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ORIGINAL ARTICLE

# Human Vγ9Vδ2 T cells show potent antitumor activity against zoledronate-sensitized OSCC cell lines

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## Summary

***Purpose:*** *Vγ9Vδ2 T cells exhibit potent antitumor effects against multiple types of tumors in preclinical models. In the present study, we examined whether human Vγ9Vδ2 T cells can be effective against oral squamous cell carcinoma (OSCC) cell lines in vitro because the interaction between OSCC and Vγ9Vδ2 T cells has not been explored previously.*

***Methods:*** *Eight OSCC cell lines were analyzed for their expression of ligands that potentially activate Vγ9Vδ2 T cells. Vγ9Vδ2 T cells were expanded in vitro from peripheral blood mononuclear cells (PBMCs) using zoledronate and IL-2. Expanded Vγ9Vδ2 T cells were tested for IFNγ production and cytotoxicity in response to zoledronate-treated OSCC cell lines. Flow cytometry was used to obtain and analyze data.*

***Results:*** *All OSCC cell lines expressed CD277. The cell lines also expressed at least one type of NKG2D ligand. Zoledronate-treated OSCC cell lines induced IFNγ expression in Vγ9Vδ2 T cells. We thus found that Vγ9Vδ2 T cells efficiently kill zoledronate-sensitized OSCC cell lines.*

***Conclusions:*** *We found that zoledronate-treated OSCC cell lines are effectively killed by Vγ9Vδ2 T cells. Our results indicate that developing Vγ9Vδ2 T cell-based immunotherapy will be promising in treating patients with OSCC.*

***Key words:*** *aminobisphosphonate, oral squamous cell carcinoma, Vγ9Vδ2 T cells, zoledronate*

## Introduction

Oral squamous cell carcinoma (OSCC) is a malignant neoplasm arising from the oral cavity and is the sixth most common cancer reported globally [1]. The most important risk factors include tobacco use, alcohol consumption, and human papilloma virus infection [2]. The major strategies for treating OSCC are surgery, radiotherapy, and chemotherapy, and of late, immunotherapy involving the use of immune checkpoint inhibitors has been shown to be effective against OSCC as well as head and neck squamous cell carcinomas [3].

To date, adoptive immunotherapy including ex vivo expanded T cells has been attractive approach to treat cancer patients [4]. However, the use of alpha beta T cells is limited to immunogenic tumors with known tumor antigens and certain HLA haplotypes. Moreover, loss of the HLA molecules is often observed in cancer cells. Gamma delta (γδ) T cells have been shown to have potent antitumor properties in an HLA-independent manner [5]. Vγ9Vδ2 T cells (also termed Vγ2Vδ2 T cells) are one of the most widely studied human γδ T cell subsets. Vγ9Vδ2 T cells, found in the peripheral blood, are exclusive to primates and play an important role in human immunity against microbes and tumors [6]. The response of Vγ9Vδ2 T cells is not restricted to

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the HLA but requires the unconventional antigen presenting molecule butyrophilin 3A1 (BTN3A1) [7-9]. The Vγ9Vδ2 T cell receptor (TCR) recognizes a conformational change in BTN3A1 induced by the association with prenyl pyrophosphates such as isopentenyl pyrophosphate (IPP) [10].

IPP is produced as an intermediate of the mevalonate pathway, and the dysregulation of this pathway is observed in transformed cells or can be induced by pharmacological inhibition by aminobisphosphonates (N-BPs). The resulting accumulation of IPP can be detected by Vγ9Vδ2 T cells through their TCR and results in their activation [11,12].

In preclinical models, Vγ9Vδ2 T cells activated by N-BPs show potent antitumor activity in vitro and in vivo [13]. Moreover, clinical trials involving Vγ9Vδ2 T cell-based immunotherapy for treating patients with lymphoma, multiple myeloma, and solid tumors have shown that it is well tolerated and effective [14-16]. However, no previous study, to the best of our knowledge, has explored whether the Vγ9Vδ2 T cell-mediated antitumor activity is also effective against OSCC cell lines.

Here, we examined the expression of BTN3A1 and NK ligands using eight OSCC cell lines and checked the activation status and cytotoxic activity of Vγ9Vδ2 T cells co-cultured with zoledronate-treated OSCC cell lines.

## Methods

*Cell culture*

All OSCC cell lines (HSC-2, HSC-3, HSC-4, SAS, Ca9-22, HO-1-N-1, KOSC-2 cl3-43, and SKN3) were obtained from the JCRB cell bank (Osaka, Japan) and were cultured in DMEM (Wako, Osaka, Japan) supplemented with 10% FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque, Kyoto, Japan).

*Preparation of ex vivo expanded Vγ9Vδ2 T cells*

Peripheral blood was obtained from healthy volunteers after obtaining written informed consent with the approval of the Research Ethics Committee of Osaka Dental University. Peripheral blood mononuclear cells (PBMCs) were isolated from the fresh blood by density gradient centrifugation using Lymphoprep with SepMate-50 (STEMCELL Technologies, Vancouver, Canada). The PBMCs were cultured at 37°C and 5% $CO_2$ in a 24-well plate at a density of $1\times10^6$ cells/mL/well in RPMI 1640 (Wako) supplemented with 10% FBS (Sigma-Aldrich, Missouri, USA), 4 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 U/mL IL-2 (Primmune, Osaka, Japan), and 5 μM zoledronic acid (Cayman Chemical, Michigan, USA). The cell density was maintained at $0.5\text{-}2\times10^6$ cells/mL by adding fresh medium supplemented with IL-2 (100 U/mL). On day 14, the expanded cells were collected and extensively washed with the medium, after which they were cryopreserved for further use. The purity of Vγ9Vδ2 T cells was >93%. On the day before commencing the experiment, the cryopreserved Vγ9Vδ2 T cells were quickly thawed and washed in growth medium, and were cultured overnight at 37°C and 5% $CO_2$.

*Flow cytometric analysis*

OSCC cell lines were labeled with the following monoclonal antibodies: CD277 (clone BT3.1) from BioLegend (California, USA); MICA/B (clone 6D4) from eBioscience (California, USA); ULBP-1 (clone #170818), ULBP-2 (clone #165903), ULBP-4 (clone #709116), and ULBP-2,5,6 (clone #165903) from R&D Systems (Minnesota, USA). After staining with anti-CD3 (clone OKT3) and anti-TCR Vδ2 (clone B6) (both from BioLegend), the Vγ9Vδ2 T cells were fixed and permeabilized using Fixation Buffer and Intracellular Staining Permeabilization Wash Buffer (BioLegend) and were then treated with anti-IFNγ antibody (clone 4S. B3, BioLegend). Data were collected using BD FACSVerse (BD, California, USA) and analyzed using FlowJo software X 10.0.7 (Tree Star, Oregon, USA).

*Vγ9Vδ2 T cell IFNγ expression after co-culturing with OSCC cells*

The OSCC cell lines were plated at a density of $1.2\times10^5$ cells per well in a 24-well plate in a growth medium supplemented with 3 μM zoledronate and in medium not containing zoledronate. After 18 h of culturing, the plate was washed with RPMI-1640, followed by co-incubation with $1.2\times10^5$ Vγ9Vδ2 T cells in RPMI-1640 for 4 h. During the last 2 h of culturing, monensin was added to enhance the intracellular staining. The cells were then collected and the intracellular IFNγ was assessed by flowcytometry.

*In vitro tumor-killing assay*

The OSCC cell lines were cultured overnight at a density of $6\times10^4$ cells per well in a 48-well plate. The next day, the cells were labeled with 1 μM CFSE (Dojindo, Kumamoto, Japan) before incubation with $2.4\times10^5$ Vγ9Vδ2 T cells in RPMI-1640 for 4 h. The cells were collected and stained with Annexin V-APC (BioLegend) and the viability of the cells was assessed by flowcytometry.

*Statistics*

Statistical analysis was performed using GraphPad Prism 6.0 for Mac OS X (GraphPad Software, California, USA). Two-tailed Student's *t*-test was used to assess the statistical significance, and a p value of 0.05 was set as significant.

## Results

*OSCC cells express BTN3A (CD277) and NKG2D ligands*

In humans, BTN3A (CD277) occurs in three isoforms -BTN3A1, BTN3A2, and BTN3A3- and BTN3A1 induces IPP-dependent Vγ9Vδ2 T cell activation [17]. It was recently found that BTN3A2 is

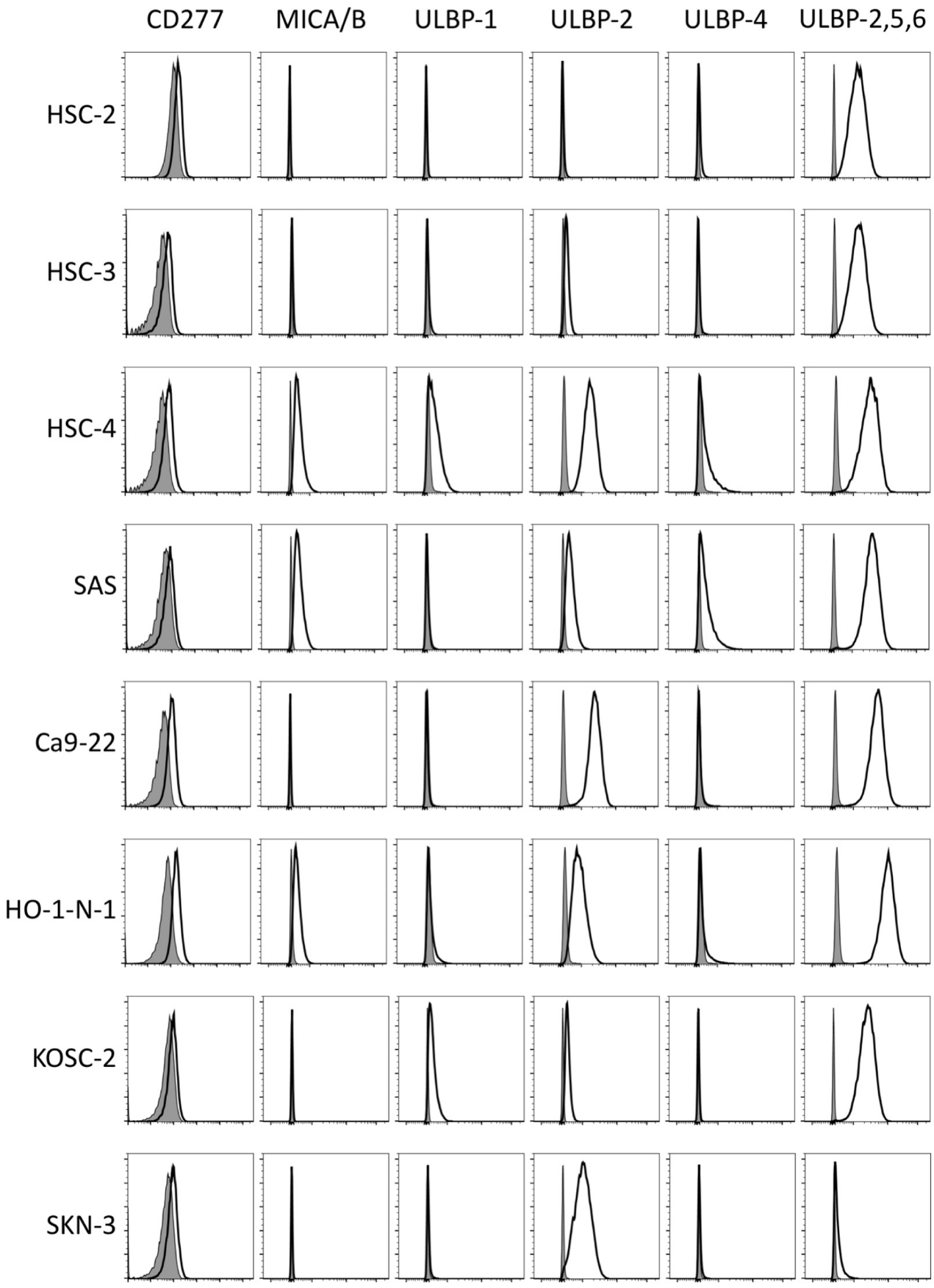



**Figure 1.** Expression of CD277 and NKG2D ligands. Representative histograms of surface expression of CD277 and NKG2D ligands in the OSCC cell lines. MICA and MICB were detected by the antibody cross-reacting with both the ligands. ULBP-2, ULBP-5, and ULBP-6 were detected by the antibody cross-reacting with all the three ligands. Tinted histograms show staining by isotype control antibody.

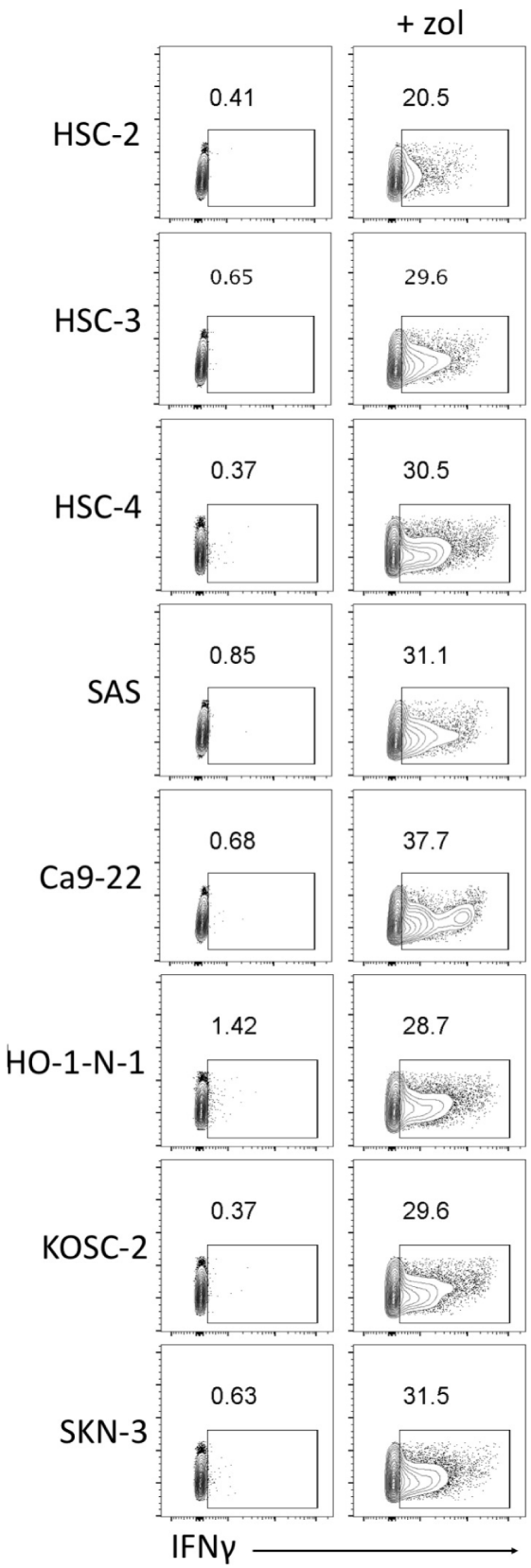



**Figure 2.** IFNγ production in Vγ9Vδ2 T cells in response to co-culturing with OSCC cell lines. Representative contour plots of IFNγ-positive cells gated on CD3+ Vδ2 TCR+ cells. Vγ9Vδ2 T cells were cultured with zoledronate (zol) untreated (left) and treated (right) OSCC cell lines for 4 hrs. IFNγ production was assessed by flow cytometry using intracellular staining.

also involved in the activation of Vγ9Vδ2 T cells [17]. We first examined the cell-surface expression of CD277 by using the 8 OSCC cell lines. As shown in Figure 1, flowcytometric analysis revealed that all the OSCC cell lines weakly expressed CD277. Because NKG2D is known to positively regulate Vγ9Vδ2 T cell activation [18], we examined the cell-surface expression of NKG2D ligands (MICA/B and ULBP1-6) in the OSCC cells (Figure 1). All the OSCC cell lines expressed at least one type of NKG2D ligand. These results confirmed that all the OSCC cells could be targeted by the Vγ9Vδ2 T cells.

*Zoledronate-treated OSCC cells induce IFNγ expression in Vγ9Vδ2 T cells*

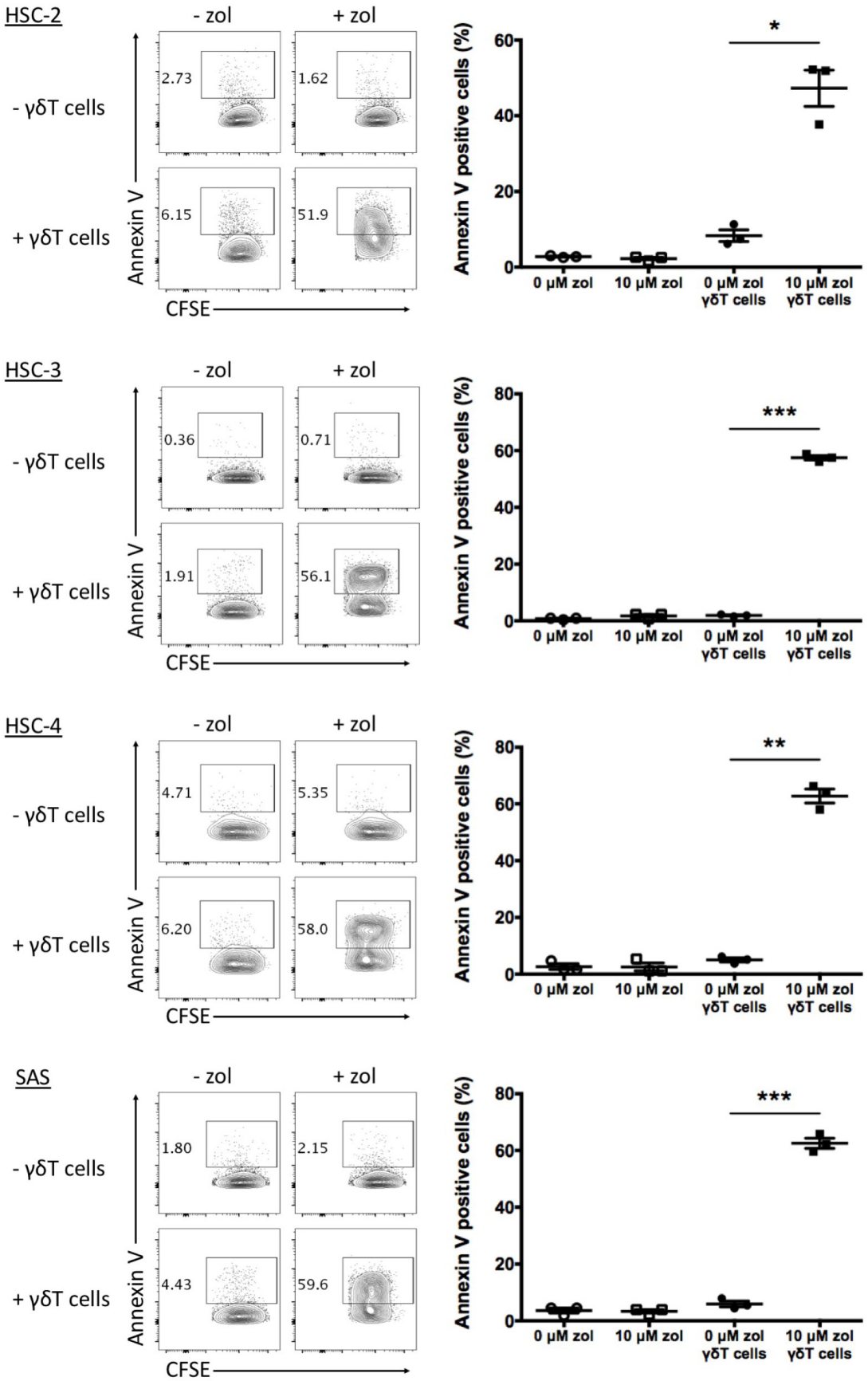
N-BPs, including zoledronate, inhibit the mevalonate pathway in eukaryotic cells and cause IPP accumulation [11]. Accumulation of high levels of IPP in turn induces conformational changes BTN3A1, which results in the activation of Vγ9Vδ2 T cells through the interaction with their TCR [10]. By using expanded Vγ9Vδ2 T cells, we evaluated the ability of OSCC cells to induce the production of IFNγ in Vγ9Vδ2 T cells. As shown in Figure 2, co-culturing Vγ9Vδ2 T cells with OSCC cells not treated with zoledronate did not produce IFNγ. In contrast, zoledronate-treated OSCC cells induced IFNγ production in Vγ9Vδ2 T cells. Therefore, we concluded that OSCC cells can respond to zoledronate and be targeted by the Vγ9Vδ2 T cells.

*Vγ9Vδ2 T cell-mediated cytotoxicity against OSCC cells is induced by zoledronate*

Because exposure to zoledronate enabled OSCC cells to activate Vγ9Vδ2 T cells, we examined whether zoledronate sensitizes OSCC cells to Vγ9Vδ2 T cell-mediated cytotoxicity. Exposure to zoledronate alone did not affect the viability of OSCC cells. OSCC cells not treated with zoledronate did not induced cytotoxic activity of Vγ9Vδ2 T cells. However, pre-treatment of OSCC cells with zoledronate dramatically increased their sensitivity to Vγ9Vδ2 T cell-mediated cytotoxicity (Figure 3). These results suggest that Vγ9Vδ2 T cells can effectively target and induce cytotoxicity in zoledronate-sensitized OSCC cells.

## Discussion

Adoptive immunotherapy with Vγ9Vδ2 T cells for treating cancer patients has proven to be safe and well tolerated [14-16]. However, increasing the effectiveness of Vγ9Vδ2 T cells is necessary to realize the treatment's full potential. Continuous exposure to N-BPs, which are generally used

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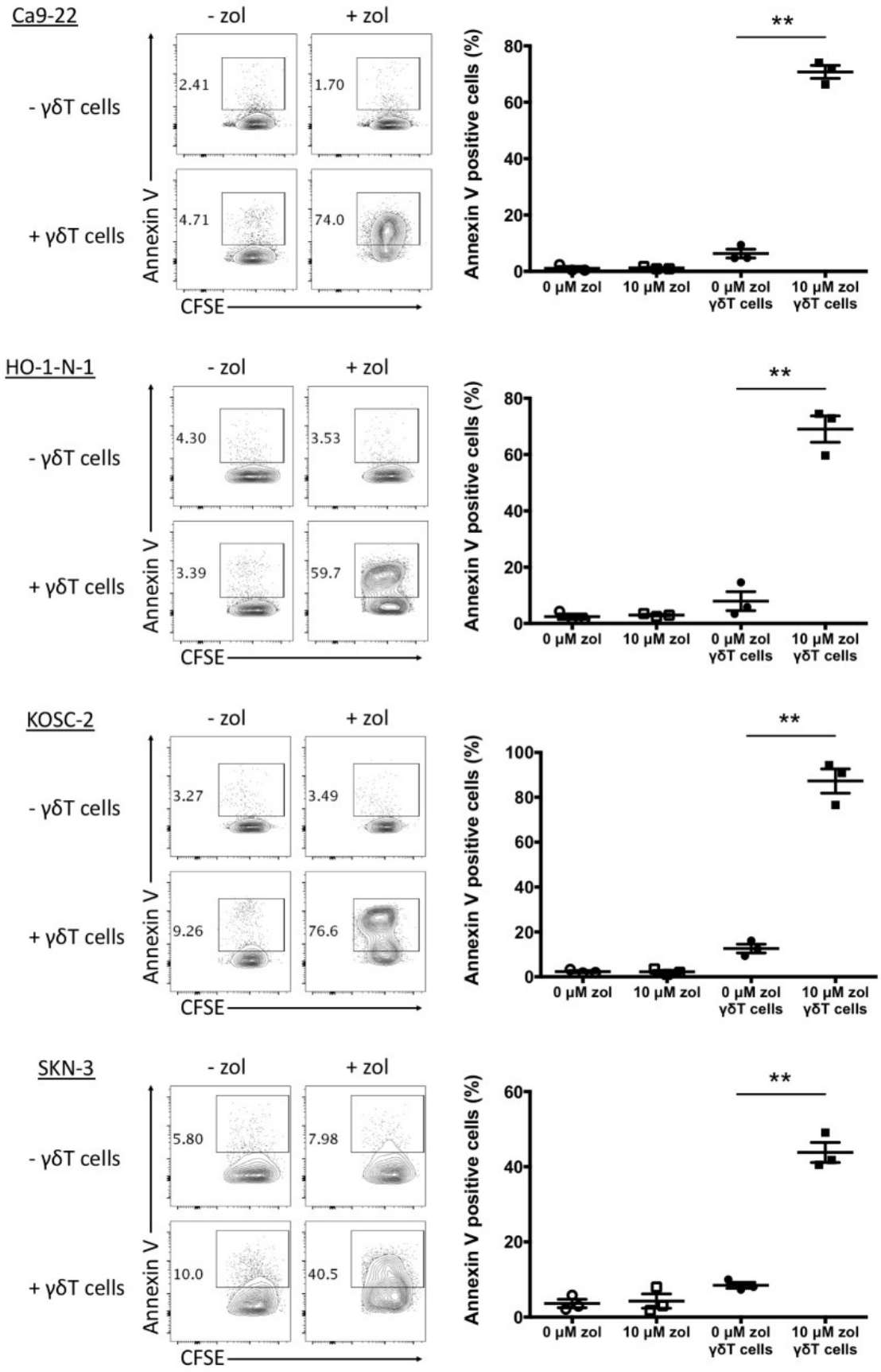


**Figure 3.** Cytotoxicity of Vγ9Vδ2 T cells against multiple OSCC cell lines. Representative contour plots (left) and percentage (right) of annexin V-positive OSCC cell lines after 4 h of incubation with Vγ9Vδ2 T cells. The data were obtained from three independent experiments, with each using Vγ9Vδ2 T cells from different donors. Bars represent the mean and SEM; *p < 0.05, **p < 0.01, ***p < 0.001; two-tailed paired Student's *t*-test.

to expand Vγ9Vδ2 T cells, is toxic to Vγ9Vδ2 T cells. In contrast, exposing Vγ9Vδ2 T cells in short durations (pulse exposure) to N-BPs maximizes their purity, quantity, and quality [19]. We have previously reported that a combined exposure to IL-12 and IL-18 increases the effector functions of Vγ9Vδ2 T cells [20]. Therefore, improving preparation protocols and activation methods of expanded Vγ9Vδ2 T cells is vital in increasing their effectiveness in immunotherapy.

Vγ9Vδ2 T cells kill a broad range of tumor cells [13]. Here, we demonstrated that all the OSCC cell lines we used can activate Vγ9Vδ2 T cells in response to N-BP treatment.

We first examined whether the OSCC cells we selected expressed the ligands that can be used for Vγ9Vδ2 T cell activation. All the OSCC cell lines expressed CD277, which is a cognate ligand for their TCR. Because Vγ9Vδ2 T cells may be activated through NKG2D, we examined the expression of its subtypes, MICA/B or ULBP1-6, on the OSCC cell surface. Although the expression of NKG2D ligands was different across the OSCC cell lines, all the OSCC cell lines expressed at least one type of NKG2D ligand. Thus, OSCC cell lines express CD277 and NKG2D ligands which can activate Vγ9Vδ2 T cells through interaction with TCR and NKG2D.

Next, we examined whether the OSCC cells could activate Vγ9Vδ2 T cells through its activating ligand. N-BP treatment induces the accumulation of IPP in multiple tumor cells, which results in the activation of Vγ9Vδ2 T cells through the interaction of CD277 and Vγ9Vδ2 TCR. Similar to other tumor cells, OSCC cells induce the production of IFNγ in Vγ9Vδ2 T cells in response to zoledronate treatment.

Finally, we examined whether the activated Vγ9Vδ2 T cells could target OSCC cells. Neither zoledronate treatment alone nor Vγ9Vδ2 T cell incubation with untreated OSCC cells result in tumor suppression. However, zoledronate-sensitized OSCC cells were efficiently killed by activated Vγ9Vδ2 T cells.

To summarize, all OSCC cell lines we tested were highly sensitive to zoledronate and were effectively killed by the Vγ9Vδ2 T cells. Our in vitro data suggest that adoptive immunotherapy including activated Vγ9Vδ2 T cells is promising in treating patients with OSCC. It is important to test the effectiveness of Vγ9Vδ2 T cells in vivo; e.g., by using a humanized mouse model.

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## Conflict of interests

The authors declare no conflict of interests.

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