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Zoledronic Acid-Induced Expansion of $\gamma\delta$ T Cells from Early-Stage Breast Cancer Patients: Effect of IL-18 on Helper NK Cells

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Abstract

Human $\gamma\delta$ T cells display potent cytotoxicity against various tumor cells pretreated with zoledronic acid (Zol). Zol has shown benefits when added to adjuvant endocrine therapy for patients with early-stage breast cancer or to standard chemotherapy for patients with multiple myeloma. Although $\gamma\delta$ T cells may contribute to this additive effect, the responsiveness of $\gamma\delta$ T cells from early-stage breast cancer patients has not been fully investigated. In this study, we determined the number, frequency, and responsiveness of V γ 2V δ 2 T cells from early- and late-stage breast cancer patients and examined the effect of IL-18 on their ex vivo expansion. The responsiveness of V γ 2V δ 2 T cells from patients with low frequencies of V γ 2V δ 2 T cells was significantly diminished. IL-18, however, enhanced ex vivo proliferative responses of V γ 2V δ 2 T cells and helper NK cells from patients with either low or high frequencies of V γ 2V δ 2 T cells. Treatment of breast cancer patients with Zol alone decreased the number of V γ 2V δ 2 T cells and reduced their ex vivo responsiveness. These results demonstrate that Zol can elicit immunological responses by $\gamma\delta$ T cells from early-stage breast cancer patients but that frequent in vivo treatment reduces V γ 2V δ 2 T cell numbers and their responsiveness to stimulation.

Keywords

$\gamma\delta$ T cells; helper NK cells; breast cancer; phosphoantigen; nitrogen-containing bisphosphonate; IL-18

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Introduction

V γ 2V δ 2 T cells (also termed V γ 9V δ 2 T cells) recognize phosphoantigens derived from isoprenoid pathways, including isopentenyl diphosphate (IPP) and (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP), in an MHC class I- and MHC class II-independent manner [1–9]. V γ 2V δ 2 T cells target and lyse tumor cells that have been treated with nitrogen-containing bisphosphonates (N-BPs), such as pamidronate, zoledronic acid (Zol), and risedronate, in a $\gamma\delta$ TCR-dependent manner [10–12]. N-BPs have been used as therapeutic agents for osteoporosis and hypercalcemia of malignancy. The primary target for N-BPs is farnesyl diphosphate synthase (FPPS) whose inhibition leads to the accumulation of its upstream metabolite, IPP [13, 14]. Although the precise molecular mechanism underlying V γ 2V δ 2 TCR recognition of phosphoantigens presented by tumor cells remains unclear, stimulation of $\gamma\delta$ T cells has received much attention as a potential therapeutic intervention for infections and malignancies [15, 16].

Zoledronic acid has recently been tested in clinical trials as an additive therapeutic for early-stage breast cancer and multiple myeloma. In early-stage breast cancer, the addition of Zol to standard therapy improved disease-free survival in estrogen receptor-positive patients in a low-estrogen environment [17–19]. Zol also had treatment benefits in patients with newly diagnosed multiple myeloma—improving overall survival independently of the prevention of skeletal-related events [20]. Taken together, these studies clearly demonstrate that Zol has a beneficial effect on cancer patients. However, the mechanism by which Zol mediates these effects is still unclear since the effects appear to extend beyond the prevention of skeletal-related events [20, 21]. This has led to the proposal that V γ 2V δ 2 T cells contribute to the observed beneficial effect of Zol in early-stage breast cancer patients [22]. Moreover, adoptive V γ 2V δ 2 T cell therapy has shown some promise in the treatment of breast cancer with a complete remission achieved in one patient with concurrent hormonal therapy that had previously failed hormonal, radiation, and chemotherapy treatments [23]. To understand the role of V γ 2V δ 2 T cells in early-stage breast cancer patients treated with Zol and to develop $\gamma\delta$ T cell treatments for these patients, we have investigated the characteristics of V γ 2V δ 2 T cells from these patients and have determined the ability of V γ 2V δ 2 T cells to expand ex vivo and the effect of IL-18 on this expansion.

Materials and methods

Patients

Breast cancer patients (n = 80) were enrolled after institutional review board approval and with written informed consent. Patient characteristics are summarized in Online Resource 1. Early-stage (stage 0, I, or II) breast cancer patients constituted 62/80 (77.5%) of the patients whereas late-stage (stage III, IV) patients constituted 18/80 (22.5%).

Reagents

Zol was purchased from Novartis Pharmaceuticals Corp. (Basel, Switzerland) and converted to its sodium salt using a Na⁺ form of Dowex 50W $\times$ 8 (Muromachi Kogyo Kaisha Ltd., Chuo-ku, Tokyo, Japan). Recombinant human IL-18 and IL-2 were kindly provided by GlaxoSmithKline plc (Research Triangle Park, NC) and Shionogi Pharmaceutical Co., Ltd. (Chuo-ku, Osaka, Japan), respectively.

Stimulation of V γ 2V δ 2 T cells

PBMC were purified as described in Online Resource 2. The cells were stimulated with Zol/IL-2 or Zol/IL-2/IL-18 for 2 to 10 days as described in Online Resource 3.

Flow cytometry and mAbs

Flow cytometric analysis was performed using a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ). The following Abs were used; fluorescein isothiocyanate (FITC)-conjugated anti-V δ 1 and anti-V δ 2 mAbs (Beckman Coulter Inc., Fullerton, CA), phycoerythrin (PE)-conjugated anti-CD3 mAb (BD Biosciences, San Diego, CA), and isotype control Abs (Biolegend, San Diego, CA), and FcR blocking reagent (human, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The gating strategy is depicted in Online Resource 2.

Cytokine production

PBMC were stimulated with 5 μ M of Zol and 100 U/ml of IL-2 in the presence or absence of 100 ng/ml of IL-18. After incubation at 37 °C with 5% CO₂ for 2 days, the culture supernatants were frozen at -80 °C overnight. The samples were then thawed and IFN- γ and TNF- α cytokine levels determined by ELISA (Peprotech, Rocky Hill, NJ).

Effect of repeated in vivo injections of Zol

A breast cancer patient (stage II) was given 4 mg of Zol twice, 1 month apart. Peripheral blood samples were obtained just before the first infection, 1 month after the first injection, and 1 month after the second injection. PBMC were purified and cultured for 14 days as described in Online Resource 3. The cells before and after culture were stained and analyzed using a FACSCalibur flow cytometer.

Statistical analysis

Correlations between V δ 2⁺ T cell frequencies on day 0 and cell numbers or V δ 2⁺ T cell frequencies on day 10, or between the V δ 2⁺ T cell frequencies on day 0 and cytokine production on day 2 were examined using Spearman's rank correlation coefficient (ρ). Paired *t* test was used to assess the effect of IL-18 on the expansion of V δ 2⁺ T cells and helper NK cells and cytokine productions. A *p* value of less than 0.05 was considered statistically significant.

Results

The frequency of V δ 2⁺ T cells in breast cancer patients is inversely correlated with age and disease stage

To characterize $\gamma\delta$ T cells in breast cancer patients, we first determined the frequencies of V δ 1⁺ and V δ 2⁺ $\gamma\delta$ T cells among CD3⁺ T cells in peripheral blood mononuclear cells (PBMC) from 80 patients with breast cancer comprised of 62 early-stage (stage 0-II) patients and 18 late-stage (stage III, IV) patients. Overall, the frequency of V δ 2⁺ T cells ($5.2 \pm 6.8\%$) was significantly higher than that of V δ 1⁺ T cells ($1.5 \pm 1.7\%$) in both early- and late-stage patients (Fig. 1 and Online Resource 2). Whereas 57.5% of patients had V δ 1⁺ T cell frequencies of less than 1%, only 21.2% showed V δ 2⁺ T cell frequencies of less than 1% (Fig. 1). As summarized in Online Resource 1 and Table 1, the frequency of V δ 2⁺ T cells inversely correlated with the age ($p = 0.044$) and disease stage ($p = 0.001$). When stratified by disease stages, there was no significant difference in V δ 1⁺ T cell frequencies between early- and late-stage patients (early: $1.4 \pm 1.4\%$; late: $1.7 \pm 2.5\%$). In contrast, higher V δ 2⁺ T cell frequencies were observed in early-stage patients compared with late-stage patients (early: $5.9 \pm 7.4\%$; late: $2.6 \pm 2.8\%$). Although the proportion of patients whose V δ 2⁺ T cell frequencies were less than 1% was essentially the same between early- and late-stage patients (early: 21.0%; late: 22.2%), a markedly higher proportion of early-stage patients had V δ 2⁺ T cell frequencies of greater than 5.2% compared with late-stage patients (early: 35.5%; late: 5.6%). This difference in V δ 2⁺ T cell frequency between early-

and late-stage patients suggests that V γ 2V δ 2 T cells might be immunologically perturbed based on the severity of disease. Besides age and cancer stage, there was no correlation with any other patient background characteristic, with the expression of either the human epidermal growth factor receptor 2 (HER2) or the estrogen receptor (ER), or with the cancer subtype.

Ex vivo expansion of V γ 2V δ 2 T cells from early-stage breast cancer patients stimulated by Zol/IL-2

Adoptive immunotherapy with V γ 2V δ 2 T cells requires their ex vivo expansion. To assess the feasibility of adoptive immunotherapy with V γ 2V δ 2 T cells in early-stage breast cancer, PBMC from patients were stimulated with Zol in the presence of IL-2 and the expansion of V δ 2⁺ T cells determined by flow cytometry 10 days later. Dot plots of four representative breast cancer patients are shown (Fig. 2 and Online Resource 2). The proliferation of V δ 2⁺ T cells to Zol/IL-2 correlated with the initial V δ 2⁺ T cell frequency. Patients with 1% or greater V γ 2V δ 2 T cells at day 0 expanded to near 100% of the T cells by day 10. Both the number ($\rho = 0.711$; $p < 0.01$, Fig. 3a, closed circles) and the frequency ($\rho = 0.928$; $p < 0.01$, Fig. 3b, closed circles) of V γ 2V δ 2 T cells strongly correlated with the initial V δ 2⁺ T cell frequency. Patients with initial V δ 2⁺ T cell frequencies of 1% or less expanded poorly.

We also analyzed the correlation between the initial V δ 2⁺ T cell frequency and the expansion rate of V δ 2⁺ T cells. When stratified by the initial V δ 2⁺ T cell frequency, the average expansion rates $\pm$ SD were 722 ± 284 -fold, $1,610 \pm 588$ -fold, and $3,447 \pm 1,823$ -fold for patients whose initial frequency was <0.5 , 0.5 – 1 , and 1 – 10% , respectively. This demonstrated that the responsiveness of V δ 2⁺ T cells from patients with low initial frequency was functionally impaired. In addition, the V δ 2⁺ T cells/CD3 ratios after expansion by Zol for 10 days were 38.2 ± 35.3 , 83.7 ± 7.5 , and $96.9 \pm 3.1\%$, respectively. Whereas the functional impairment was prominent when the initial frequency was less than 0.5% , the relatively large SD, 35.3% , indicated variability in responsiveness among patients with low initial frequency of V δ 2⁺ T cells.

Similarly, the production of IFN- γ ($\rho = 0.566$; $p < 0.01$) and TNF- α ($\rho = 0.617$; $p < 0.01$) also correlated with the initial frequency of V δ 2⁺ T cells (Figs. 3c, d, respectively). The correlation between the initial V δ 2⁺ T cell frequency and the responsiveness of V δ 2⁺ T cells to Zol/IL-2 stimulation was also observed in late-stage breast cancer patients (Fig. 3, open circles).

Effect of IL-18 on the ex vivo expansion of V γ 2V δ 2 T cells from early-stage breast cancer patients

Significant numbers of breast cancer patients had initial V δ 2⁺ T cell frequency of 1% or less (Fig. 1b). Based on our findings, for some patients, Zol treatment might not stimulate in vivo V γ 2V δ 2 T cell expansions and ex vivo expansion of V γ 2V δ 2 T cells for adoptive immunotherapy would be poor. We previously reported that IL-18 promoted expansion of $\gamma\delta$ T cells from healthy individuals [24]. Therefore, the effect of IL-18 on the expansion of V γ 2V δ 2 T cells from breast cancer patients was investigated. Addition of IL-18 to Zol/IL-2 treatment significantly increased the expansion of V δ 2⁺ T cells by 2.7-fold ($p = 0.0053$) (Fig. 4a). For example, for three representative breast cancer patients, the addition of IL-18 increased the total number of V δ 2⁺ T cells 2.4–4.1-fold (BC08: 1.1×10^8 cells to 3.5×10^8 cells, BC09: 5.8×10^8 cells to 14.2×10^8 cells, and BC10: 2.5×10^8 cells to 10.4×10^8 cells) (Online Resource 3). The proportion and the total number of CD3⁺ cells were also significantly up-regulated by the addition of IL-18. For the three representative breast cancer patients (Online Resource 3), IL-18 addition increased CD3⁺ cells 7.7–10.3-fold (BC08: 11.6% to 38.8%; 2.3×10^7 cells to 22.9×10^7 cells, BC09: 1.8% to 7.1%; 1.1×10^8 cells to

11.3×10^8 cells, BC10: 1.4% to 3.0%; 2.7×10^8 cells to 20.8×10^8 cells). Thus, CD3⁺ cells increased by an average of 10.9-fold ($p = 0.0020$) (Fig. 4b). The addition of IL-18 to PBMC stimulated with Zol/IL-2 also increased the production of INF- γ by 6.3-fold ($p < 0.0001$) and TNF- α by 4.7-fold ($p < 0.0001$) (Fig. 4c, d, respectively). This enhancement by IL-18 of proliferative responses and cytokine secretion was observed in all patients regardless of their initial V δ 2⁺ T cell frequency, demonstrating that V γ 2V δ 2 T cells could be expanded by Zol/IL-2/IL-18 even in cancer patients with lower V δ 2⁺ T cell frequencies.

Expansion of “helper” NK cells from early-stage breast cancer patients after Zol/IL-2/IL-18 stimulation and functional analyses of expanded V δ 2⁺ T cells

After stimulation of PBMC with Zol/IL-2 in the presence or absence of IL-18 for 10 days, the majority of the cells expressed V δ 2 TCR and the remaining cells consisted of CD3⁺ cells and a few CD3⁺ $\alpha\beta$ T cells (Online Resource 3, right panels). Because NK cells with “helper” properties have been reported to develop under the influence of IL-2 and IL-18 [25], we examined cell surface markers expressed by CD3⁺ cells. Consistent with the identification of the CD3⁺ cells as NK cells, almost all of these cells expressed NK surface markers including CD56, NKG2D, and CD16 but not monocytic markers such as CD14 (Online Resource 4). We also examined expression of APC-related molecules, such as CD86, CD80, HLA-DR, and HLA-DQ—all were strongly expressed, whereas the expression of NKP44, a positive NK receptor, was down-regulated, suggesting that “helper” NK cells were expanded along with V δ 2⁺ T cells.

Frequent Zol administration as used in the AZURE trial leads to decreasing blood V γ 2V δ 2 T cell levels and decreased ability for their ex vivo expansion

Some recent clinical trials on early breast cancer patients demonstrated additional clinical benefits when Zol was added to standard cancer therapy [26]. Whereas two trials (the Austrian Breast and Colorectal Cancer Study Group trial-12 (ABCSG-12) [17, 18] and the Zometa-Femara Adjuvant Synergy Trials (ZO-FAST) [19]) showed clinical benefits for most patients, another (the Adjuvant Zoledronic Acid to Reduce Recurrence (AZURE) trial [27]) showed benefits only with postmenopausal women. Besides the increased frequency of chemotherapy [27], one other important difference between the trials was that the AZURE trial gave Zol every 3 weeks initially for 6 doses and quite frequently thereafter whereas the ABCSG-12 and ZO-FAST trials gave Zol only every 6 months. Therefore, we examined the effect of in vivo Zol administration on V δ 2⁺ T cells in breast cancer patients when given at 4 week intervals. The V δ 2⁺ T cell frequency in the peripheral blood of one stage II patient decreased after repeated Zol injection spaced at one month interval from 0.79% prior to therapy to 0.55% after the first dose, and 0.46% after the second dose (Fig. 5). The efficiency of ex vivo V δ 2⁺ T cell expansion also progressively decreased, with V δ 2/CD3 ratios of 94.2, 88.7, and 64.0%, and numbers of V δ 2⁺ cells of 4.00×10^8 , 2.68×10^8 , and 0.08×10^8 , respectively. Similar decreases in peripheral blood V γ 2V δ 2 T cells were noted in four other patients with V δ 2⁺ T cell frequencies decreasing from 3.7% to 3.0% (stage IV), 1.8% to 1.4% (stage IV), 0.6% to 0.3% (stage IV), and 0.6% to 0.3% (stage I). Overall, V δ 2⁺ T cell frequency in the blood decreased an average of 34.4% for the five patients one month after one dose of Zol. These pilot studies suggest that frequent Zol treatments of breast cancer patients leads to decreasing blood V γ 2V δ 2 T cell levels and decreased ability for ex vivo expansion.

Discussion

Recent clinical trials demonstrate that Zol provides clinical benefits in the treatment of patients with early-stage breast cancer and multiple myeloma, although the contribution of $\gamma\delta$ T cells to the beneficial effects is unclear [26]. In the present study, we have examined

the number, frequency, and responsiveness of peripheral blood $\gamma\delta$ T cells from patients with breast cancer. The average $V\delta 2^+$ T cell frequency was significantly higher in early-stage patients compared with late-stage patients (5.9 vs 2.6%) and slightly higher than healthy donors ($4.3 \pm 2.2\%$). In fact, many breast cancer patients had elevated $V\delta 2^+$ T cell frequencies with 35.5% of patients having greater than 5.2% $V\delta 2^+$ T cells (22/62) and 19.4% (12/62) greater than 10%. When stratified by patients' background, the frequency of $V\delta 2^+$ T cells correlated negatively with age and disease stage. These findings further support the hypothesis that $V\gamma 2V\delta 2$ T cells are immunologically perturbed by the development of mammary carcinomas.

The expansion of $V\delta 2^+$ T cells was impaired in some breast cancer patients, consistent with a recent report [23]. To study this hyporesponsiveness, we stimulated PBMC from donors with varying frequencies of $V\delta 2^+$ TCR with Zol/IL-2 and compared the relationship between the responsiveness of the $V\delta 2^+$ cells with the initial $V\delta 2^+$ T cell frequencies. The frequency of $V\delta 2^+$ T cells before expansion strongly correlated with both the number and the percentage of $V\delta 2^+$ T cells after expansion. This correlation was most evident when the initial frequency of $V\delta 2^+$ T cells was less than 1%. Because the proportion of patients with a $V\delta 2^+$ T cell frequency of less than 1% is significantly higher in breast cancer patients than in healthy individuals, it is thus more difficult to expand $V\delta 2^+$ T cells from patients. IFN- γ and TNF- α cytokine production also correlated with the initial frequency of $V\delta 2^+$ T cells although somewhat less strongly. This may be because the inhibition of FPPS by Zol also affects other cells in PBMC, such as monocytes and NK cells, and can cause apoptosis that may stimulate cytokine secretion by innate cells [28].

We previously reported that IL-18 enhanced proliferative responses of $V\delta 2^+$ T cells from healthy donors in response to phosphoantigens [24]. In this study, we examined whether IL-18 could compensate for the low $V\delta 2^+$ T cell frequencies observed in many breast cancer patients. IL-18 increased the numbers of $V\delta 2^+$ T cells after expansion by Zol/IL-2 and augmented cytokine secretion regardless of the initial frequency $V\delta 2^+$ T cells, thus demonstrating that IL-18 would be useful in expanding $V\delta 2^+$ T cells from breast cancer patients with low numbers of blood $V\delta 2^+$ T cells.

In healthy individuals, $CD11c^{int}CD56^+$ cells are concomitantly increased in cultures of PBMC stimulated with Zol/IL-2/IL18 and are likely involved in the enhancement of $V\delta 2^+$ T cell proliferation [24]. In fact, the depletion of $CD56^+$ cells from PBMC resulted in only poor proliferation of $V\delta 2^+$ T cells [24]. The present study demonstrates that in breast cancer patients this subset can expand to a greater degree than $V\delta 2^+$ T cells. These cells exhibited a "helper" APC phenotype because they expressed the APC-related molecules, HLA-DQ, HLA-DR, CD86, and CD80. This is consistent with previous reports that NK cells may acquire a "helper" phenotype upon exposure to IL-18 [25, 29]. We therefore have termed this subset "helper" NK cells. Although "helper" NK cells appear to preferentially promote expansion of $V\delta 2^+$ T cells under the condition in this study, the precise mechanism underlying the helper function remains to be determined. Because large aggregates of cells are observed in cultures in the presence of Zol/IL-2/IL-18 [24] and because the disruption of adhesion molecule-mediated cell-to-cell contact by anti-LFA-1 mAb greatly reduced the proliferative responses of $V\delta 2^+$ T cells [9], promotion of cell-to-cell contact may play a pivotal role in the marked expansion observed. Interestingly, the $CD11c^{int}CD56^+$ cells tend to lose the expression of CD11c as culture proceeds, and the resulting $CD11c^{-/low}CD56^+$ cells no longer support the proliferation of $V\delta 2^+$ T cells [24]. These findings suggest that IL-18/IL-2 might primarily promote the differentiation and expansion of "helper" NK cells, which in turn support the expansion of $V\delta 2^+$ T cells through the adhesion molecule-mediated interaction. Studies to identify the precursor of this NK subset and its differentiation pathway are ongoing.

While the effect of IL-18 on Vδ2⁺ T cells is not as marked as that on “helper” NK cells, Vδ2⁺ T cells also tend to develop a stronger “helper” APC phenotype upon stimulation with phosphoantigens in the presence of IL-18 given that there is further upregulation of the expression of APC-related molecules. Because both cell subsets can be considered to be types of innate immune cells and play a crucial role in provoking adaptive immune responses, it is reasonable to assume that “helper” NK cells and Vδ2⁺ T cells present classical peptide antigens to adaptive αβ T cells, through which effective, highly specific immune reactions take place to eliminate infected cells and malignant tumors [30]. Whereas we focused on the effect of “helper” NK cells on Vδ2⁺ T cells in this study, a recent report also suggests that Vδ2⁺ T cells induced robust NK-cell mediated cytotoxicity through CD137 engagement indicating a cross-talk between NK and Vδ2⁺ T cells [31].

Endogenous production of IL-18 also played a role in Zol/IL-2 responses. Both cell aggregation and cytokine production were impaired by blocking IL-18 signaling, clearly supporting a crucial role of IL-18 in the expansion of Vδ2⁺ T cells [24]. Inhibition of the mevalonate pathway by Zol inhibition of FPPS results in the depletion of downstream isoprenoid metabolites, including geranylgeraniol, that in turn is likely to lead to the activation of caspase-1 [32]. Because IL-18 is processed by caspase-1 to become a biologically active species, the exposure of cells to Zol may enhance the production of active IL-18. Although it is difficult to define the physiological function of IL-18, this study shows that the cytokine may play an important role in cytotoxic activity elicited by Vδ2⁺ cells.

The presence of IL-2 and other T cell growth factors, such as IL-15, is essential for the long-term proliferative response of Vδ2⁺ T cells, as opposed to short-term responses where Vδ2⁺ T cells themselves transiently produce IL-2 [33]. We therefore examined the in vivo effect of Zol on peripheral blood Vδ2⁺ T cells in breast cancer patients who underwent standard Zol therapy monthly without exogenous IL-2. The number of peripheral blood Vδ2⁺ T cells decreased after repetitive Zol administration as did their ex vivo expansion, clearly demonstrating that Vδ2⁺ T cells are negatively affected by frequent Zol treatment [34–36]. A previous preclinical study showed that the infusion of low-dose IL-2 together with Zol successfully expanded Vγ2Vδ2 T cells in patients with prostate cancer and breast cancer [37, 38]. Thus, IL-2 or another T cell growth factor is necessary when Zol infusion is used to augment Vδ2⁺ T cells for cancer immunotherapy. Otherwise, Zol infusion will lead to the exhaustion of Vδ2⁺ T cells and this subset will dwindle to a marginal level where the efficient expansion of Vδ2⁺ T cells is unfeasible. Consistent with these findings, it has been well documented that patients suffer from fever and flu-like symptoms due to the activation of Vδ2⁺ T cells after the initial infusion of N-BP but that the subsequent infusion of N-BP 1–2 months later does not cause such symptoms [39].

This loss of Vγ2Vδ2 T cell function may partially explain the differences observed between the various clinical trials of zoledronate in early-stage breast cancer patients. Whereas the AZURE trial gave Zol every 3–4 weeks for 6 doses, every 3 months for 8 doses, and then every 6 months [27], the ABCSG-12 [18] and ZO-FAST [19] trials gave Zol much less frequently—only every 6 months. These long intervals between Zol infusion in the ABCSG-12 and ZO-FAST trials may allow partial recovery of Vγ2Vδ2 T cell immune function. Consistent with this possibility, we found that administration of Zol twice in two months in rhesus monkeys caused decreased Vγ2Vδ2 T cell responsiveness but that partial recovery of Vγ2Vδ2 T cell responsiveness was observed in 3 out of 4 monkeys after a rest period of 9 months (C. Jin and C. T. Morita, personal observation). Thus, less frequent Zol infusion may actually be better than intensive Zol therapy at promoting the immunotherapeutic effects of Vγ2Vδ2 T cell in early-stage breast cancer patients. Less-

intensive therapy would also serve to decrease the frequency of osteonecrosis of the jaw, a major side effect of intensive Zol therapy.

Besides direct in vivo administration of Zol, adoptive immunotherapy with V γ 2V δ 2 T cells expanded ex vivo with either Zol or 2-methy-3-butenyl diphosphate has shown promise in clinical trials. Complete remissions have been observed after adoptive immunotherapy with V γ 2V δ 2 T cells in an early-stage breast cancer patient also treated with hormonal suppression therapy [23] and a renal cancer patient with metastatic disease [40]. Our results clearly demonstrate that adoptive immunotherapy with V γ 2V δ 2 T cells would be feasible for the majority of both early- and late-stage breast cancer patients given that most patients had significant ex vivo expansion of V γ 2V δ 2 T cells. Moreover, for the ~20% of patients that have V γ 2V δ 2 T cells less than 1% which is correlated with poor ex vivo expansion, IL-18 can be shown to augment V γ 2V δ 2 expansion which may allow immunotherapy even in these patients. The V γ 2V δ 2 T cells generated by ex vivo expansion have been shown to traffic to tumors in patients as early as 1 h after administration [23].

In summary, this study provides support for an immunotherapeutic role for V γ 2V δ 2 T cells in early-stage breast cancer patients. Many breast cancer patients had elevated V δ 2⁺ T cell frequencies and the majority exhibited large ex vivo expansions of V γ 2V δ 2 T cells that could be augmented further by IL-18. However, the frequent in vivo administration of Zol was detrimental to V γ 2V δ 2 T cells strongly suggesting that less frequent treatment may be preferable to preserve V γ 2V δ 2 T cell function and to avoid Zol side effects. This study shows that adoptive immunotherapy with V γ 2V δ 2 T cells is feasible for the majority of early- and late-stage breast cancer patients. Successful treatment of a breast cancer patient with V γ 2V δ 2 T cells provides proof of concept that V γ 2V δ 2 T cells can be used for immunotherapy. The challenge will be to optimize V γ 2V δ 2 immunotherapy and to select the patients most likely to respond.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABCSG-12	Austrian Breast and Colorectal Cancer Study Group trial-12
AZURE	Adjuvant Zoledronic Acid to Reduce Recurrence
ER	estrogen receptor
FPPS	farnesyl diphosphate synthase

HER2	human epidermal growth factor receptor 2
HMBPP	(<i>E</i>)-4-hydroxy-3-methylbut-2-enyl diphosphate
IPP	isopentenyl diphosphate
N-BP	nitrogen-containing bisphosphonate
ZO-FAST	Zometa-Femara Adjuvant Synergy Trials
Zol	zoledronic acid

References

1. Morita CT, Jin C, Sarikonda G, Wang H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human V γ 2V δ 2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol Rev*. 2007; 215:59–76.10.1111/j.1600-065X.2006.00479.x [PubMed: 17291279]
2. Bonneville M, O'Brien RL, Born WK. $\gamma\delta$ T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol*. 2010; 10:467–478.10.1038/nri2781 [PubMed: 20539306]
3. Hayday AC. $\gamma\delta$ T cells and the lymphoid stress-surveillance response. *Immunity*. 2009; 31:184–196.10.1016/j.immuni.2009.08.006 [PubMed: 19699170]
4. Kabelitz D, Wesch D, He W. Perspectives of $\gamma\delta$ T cells in tumor immunology. *Cancer Res*. 2007; 67:5–8.10.1158/0008-5472.CAN-06-3069 [PubMed: 17210676]
5. Constant P, Davodeau F, Peyrat M-A, Poquet Y, Puzo G, Bonneville M, Fournié J-J. Stimulation of human $\gamma\delta$ T cells by nonpeptidic mycobacterial ligands. *Science*. 1994; 264:267–270.10.1126/science.8146660 [PubMed: 8146660]
6. Tanaka Y, Sano S, Nieves E, De Libero G, Roca D, Modlin RL, Brenner MB, Bloom BR, Morita CT. Nonpeptide ligands for human $\gamma\delta$ T cells. *Proc Natl Acad Sci USA*. 1994; 91:8175–8179.10.1073/pnas.91.17.8175 [PubMed: 8058775]
7. Hintz M, Reichenberg A, Altincicek B, Bahr U, Gschwind RM, Kollas A-K, Beck E, Wiesner J, Eberl M, Jomaa H. Identification of (*E*)-4-hydroxy-3-methyl- but-2-enyl pyrophosphate as a major activator for human $\gamma\delta$ T cells in *Escherichia coli*. *FEBS Lett*. 2001; 509:317–322.10.1016/S0014-5793(01)03191-X [PubMed: 11741609]
8. Rohdich F, Hecht S, Gartner K, Adam P, Krieger C, Amslinger S, Arigoni D, Bacher A, Eisenreich W. Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein. *Proc Natl Acad Sci USA*. 2002; 99:1158–1163.10.1073/pnas.032658999 [PubMed: 11818558]
9. Morita CT, Beckman EM, Bukowski JF, Tanaka Y, Band H, Bloom BR, Golan DE, Brenner MB. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human $\gamma\delta$ T cells. *Immunity*. 1995; 3:495–507.10.1016/1074-7613(95)90178-7 [PubMed: 7584140]
10. Kunzmann V, Bauer E, Wilhelm M. γ/δ T-cell stimulation by pamidronate. *N Engl J Med*. 1999; 340:737–738.10.1056/NEJM199903043400914 [PubMed: 10068336]
11. Kato Y, Tanaka Y, Miyagawa F, Yamashita S, Minato N. Targeting of tumor cells for human $\gamma\delta$ T cells by nonpeptide antigens. *J Immunol*. 2001; 167:5092–5098. [PubMed: 11673519]
12. Bukowski JF, Morita CT, Tanaka Y, Bloom BR, Brenner MB, Band H. V γ 2V δ 2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. *J Immunol*. 1995; 154:998–1006. [PubMed: 7529807]
13. Gober H-J, Kistowska M, Angman L, Jenö P, Mori L, De Libero G. Human T cell receptor $\gamma\delta$ cells recognize endogenous mevalonate metabolites in tumor cells. *J Exp Med*. 2003; 197:163–168.10.1084/jem.20021500 [PubMed: 12538656]
14. Wang H, Sarikonda G, Puan K-J, Tanaka Y, Feng J, Giner J-L, Cao R, Mönkkönen J, Oldfield E, Morita CT. Indirect stimulation of human V γ 2V δ 2 T cells through alterations in isoprenoid metabolism. *J Immunol*. 2011; 187:5099–5113.10.4049/jimmunol.1002697 [PubMed: 22013129]

15. Tu W, Zheng J, Liu Y, Sia SF, Liu M, Qin G, Ng IH, Xiang Z, Lam KT, Peiris JS, Lau YL. The aminobisphosphonate pamidronate controls influenza pathogenesis by expanding a $\gamma\delta$ T cell population in humanized mice. *J Exp Med*. 2011; 208:1511–1522.10.1084/jem.20110226 [PubMed: 21708931]
16. Castella B, Riganti C, Fiore F, Pantaleoni F, Canepari ME, Peola S, Foglietta M, Palumbo A, Bosia A, Coscia M, Boccadoro M, Massaia M. Immune modulation by zoledronic acid in human myeloma: an advantageous cross-talk between V γ 9V δ 2 T cells, $\alpha\beta$ CD8⁺ T cells, regulatory T cells, and dendritic cells. *J Immunol*. 2011; 187:1578–1590.10.4049/jimmunol.1002514 [PubMed: 21753152]
17. Gnani M, Mlineritsch B, Schippinger W, Luschin-Ebengreuth G, Pöstlberger S, Menzel C, Jakesz R, Seifert M, Hubalek M, Bjelic-Radisic V, Samonigg H, Tausch C, Eidtmann H, Steger G, Kwasny W, Dubsky P, Fridrik M, Fitzal F, Stierer M, Rücklinger E, Greil R, Marth C. Endocrine therapy plus zoledronic acid in premenopausal breast cancer. *N Engl J Med*. 2009; 360:679–691.10.1056/NEJMoa0806285 [PubMed: 19213681]
18. Gnani M, Mlineritsch B, Stoeger H, Luschin-Ebengreuth G, Heck D, Menzel C, Jakesz R, Seifert M, Hubalek M, Pristauz G, Bauernhofer T, Eidtmann H, Eiermann W, Steger G, Kwasny W, Dubsky P, Hochreiner G, Forsthuber E-P, Fesl C, Greil R. Adjuvant endocrine therapy plus zoledronic acid in premenopausal women with early-stage breast cancer: 62-month follow-up from the ABCSG-12 randomised trial. *Lancet Oncol*. 2011; 12:631–641.10.1016/S1470-2045(11)70122-X [PubMed: 21641868]
19. Eidtmann H, de Boer R, Bundred N, Llombart-Cussac A, Davidson N, Neven P, von Minckwitz G, Miller J, Schenk N, Coleman R. Efficacy of zoledronic acid in postmenopausal women with early breast cancer receiving adjuvant letrozole: 36-month results of the ZO-FAST Study. *Ann Oncol*. 2010; 21:2188–2194.10.1093/annonc/mdq217 [PubMed: 20444845]
20. Morgan GJ, Davies FE, Gregory WM, Cocks K, Bell SE, Szubert AJ, Navarro-Coy N, Drayson MT, Owen RG, Feyler S, Ashcroft AJ, Ross F, Byrne J, Roddie H, Rudin C, Cook G, Jackson GH, Child JA. First-line treatment with zoledronic acid as compared with clodronic acid in multiple myeloma (MRC Myeloma IX): a randomised controlled trial. *Lancet*. 2010; 376:1989–1999.10.1016/S0140-6736(10)62051-X [PubMed: 21131037]
21. Aft R. Bisphosphonates in breast cancer: antitumor effects. *Clin Adv Hematol Oncol*. 2011; 9:292–299. [PubMed: 21558988]
22. Kunzmann V, Wilhelm M. Adjuvant zoledronic acid for breast cancer: mechanism of action? *Lancet Oncol*. 2011; 12:991–992.10.1016/S1470-2045(11)70252-2 [PubMed: 21968221]
23. Nicol AJ, Tokuyama H, Mattarollo SR, Hagi T, Suzuki K, Yokokawa K, Nieda M. Clinical evaluation of autologous *gamma delta* T cell-based immunotherapy for metastatic solid tumours. *Br J Cancer*. 2011; 105:778–786.10.1038/bjc.2011.293 [PubMed: 21847128]
24. Tsuda J, Li W, Yamanishi H, Yamamoto H, Okuda A, Kubo S, Ma Z, Terada N, Tanaka Y, Okamura H. Involvement of CD56^{bright}CD11c⁺ cells in IL-18-mediated expansion of human $\gamma\delta$ T cells. *J Immunol*. 2011; 186:2003–2012.10.4049/jimmunol.1001919 [PubMed: 21239711]
25. Mailliard RB, Alber SM, Shen H, Watkins SC, Kirkwood JM, Herberman RB, Kalinski P. IL-18-induced CD83⁺CCR7⁺ NK helper cells. *J Exp Med*. 2005; 202:941–953.10.1084/jem.20050128 [PubMed: 16203865]
26. Hamilton E, Clay TM, Blackwell KL. New perspectives on zoledronic acid in breast cancer: potential augmentation of anticancer immune response. *Cancer Invest*. 2011; 29:533–541.10.3109/07357907.2011.605413 [PubMed: 21843051]
27. Coleman RE, Marshall H, Cameron D, Dodwell D, Burkinshaw R, Keane M, Gil M, Houston SJ, Grieve RJ, Barrett-Lee PJ, Ritchie D, Pugh J, Gaunt C, Rea U, Peterson J, Davies C, Hiley V, Gregory W, Bell R. Breast-cancer adjuvant therapy with zoledronic acid. *N Engl J Med*. 2011; 365:1396–1405.10.1056/NEJMoa1105195 [PubMed: 21995387]
28. Nussbaumer O, Gruenbacher G, Gander H, Thurnher M. DC-like cell-dependent activation of human natural killer cells by the bisphosphonate zoledronic acid is regulated by $\gamma\delta$ T lymphocytes. *Blood*. 2011; 118:2743–2751.10.1182/blood-2011-01-328526 [PubMed: 21673342]
29. Perez-Martinez A, Iyengar R, Gan K, Chotsampancharoen T, Rooney B, Holladay M, Ramirez M, Leung W. Blood dendritic cells suppress NK cell function and increase the risk of leukemia

- relapse after hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2011; 17:598–607.10.1016/j.bbmt.2010.10.019 [PubMed: 20977942]
30. Moser B, Eberl M. $\gamma\delta$ T cells: novel initiators of adaptive immunity. *Immunol Rev*. 2007; 215:89–102.10.1111/j.1600-065X.2006.00472.x [PubMed: 17291281]
 31. Maniar A, Zhang X, Lin W, Gastman BR, Pauza CD, Strome SE, Chapoval AI. Human $\gamma\delta$ T lymphocytes induce robust NK cell-mediated antitumor cytotoxicity through CD137 engagement. *Blood*. 2010; 116:1726–1733.10.1182/blood-2009-07-234211 [PubMed: 20519625]
 32. Montero MT, Matilla J, Gómez-Mampaso E, Lasunción MA. Geranylgeraniol regulates negatively caspase-1 autoprocessing: implication in the Th1 response against *Mycobacterium tuberculosis*. *J Immunol*. 2004; 173:4936–4944. [PubMed: 15470035]
 33. Iwasaki M, Tanaka Y, Kobayashi H, Murata-Hirai K, Miyabe H, Sugie T, Toi M, Minato N. Expression and function of PD-1 in human $\gamma\delta$ T cells that recognize phosphoantigens. *Eur J Immunol*. 2011; 41:345–355.10.1002/eji.201040959 [PubMed: 21268005]
 34. Kobayashi H, Tanaka Y, Yagi J, Minato N, Tanabe K. Phase I/II study of adoptive transfer of $\gamma\delta$ T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma. *Cancer Immunol Immunother*. 2011; 60:1075–1084.10.1007/s00262-011-1021-7 [PubMed: 21519826]
 35. Lang JM, Kaikobad MR, Wallace M, Staab MJ, Horvath DL, Wilding G, Liu G, Eickhoff JC, McNeel DG, Malkovsky M. Pilot trial of interleukin-2 and zoledronic acid to augment $\gamma\delta$ T cells as treatment for patients with refractory renal cell carcinoma. *Cancer Immunol Immunother*. 2011; 60:1447–1460.10.1007/s00262-011-1049-8 [PubMed: 21647691]
 36. Dieli F, Gebbia N, Poccia F, Caccamo N, Montesano C, Fulfaro F, Arcara C, Valerio MR, Meraviglia S, Di Sano C, Sireci G, Salerno A. Induction of $\gamma\delta$ T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients *in vivo*. *Blood*. 2003; 102:2310–2311.10.1182/blood-2003-05-1655 [PubMed: 12959943]
 37. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, Roberts A, Buccheri S, D'Asaro M, Gebbia N, Salerno A, Eberl M, Hayday AC. Targeting human $\gamma\delta$ T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res*. 2007; 67:7450–7457.10.1158/0008-5472.CAN-07-0199 [PubMed: 17671215]
 38. Meraviglia S, Eberl M, Vermijlen D, Todaro M, Buccheri S, Cicero G, La Mendola C, Guggino G, D'Asaro M, Orlando V, Scarpa F, Roberts A, Caccamo N, Stassi G, Dieli F, Hayday AC. *In vivo* manipulation of V γ 9V δ 2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients. *Clin Exp Immunol*. 2010; 161:290–297.10.1111/j.1365-2249.2010.04167.x [PubMed: 20491785]
 39. Siris ES. Extensive personal experience: Paget's disease of bone. *J Clin Endocrinol Metab*. 1995; 80:335–338.10.1210/jc.80.2.335 [PubMed: 7852484]
 40. Kobayashi H, Tanaka Y, Shimmura H, Minato N, Tanabe K. Complete remission of lung metastasis following adoptive immunotherapy using activated autologous $\gamma\delta$ T-cells in a patient with renal cell carcinoma. *Anticancer Res*. 2010; 30:575–579. [PubMed: 20332473]

Fig. 1. Frequencies of peripheral blood V δ 1⁺ and V δ 2⁺ T cells in early- and late-stage breast cancer patients

a *Dot plots* of PBMC from breast cancer patients stained for expression of CD3 and V δ 1 or V δ 2. PBMC derived from 80 patients were analyzed by two-color flow cytometric analysis. Values shown are the percent of CD3⁺ T cells. *Dot plots* of three representative patients are shown (BC01: stage II; BC02: stage II; BC03: stage I). **b** Frequency of V δ 1⁺ and V δ 2⁺ T cells in early- (stage 0-II) and late-stage (stage III, IV) breast cancer patients. The percentages of V δ 1⁺ and V δ 2⁺ T cells were calculated based on the *dot plots* and are depicted in the *scatter plots*.

Fig. 2. Zol/IL-2-induced expansion of V γ 2V δ 2 T cells from PBMC of breast cancer patients PBMC derived from 18 breast cancer patients were analyzed for expression of CD3 and V δ 2. The cells were cultured in the presence of Zol and IL-2 for 10 days and analyzed by two-color flow cytometry. *Dot plots* of four representative patients (BC04: stage II; BC05: stage II; BC06: stage I; BC07: stage II) are shown. Values shown are the percent of CD3⁺ T cells.

Fig. 3. Correlation between the initial frequency of $V\delta 2^+$ T cells and the responsiveness of $V\delta 2^+$ T cells to Zol/IL-2 stimulation

a Correlation between the initial frequencies of $V\delta 2^+$ T cells and their numbers after Zol/IL-2 stimulation. **b** Correlation between the initial proportions of $V\delta 2^+$ T cells and their proportions after Zol/IL-2 stimulation. **c, d** Correlation between the initial frequency of $V\delta 2^+$ T cells and their production of IFN- γ (**c**) and TNF- α (**d**) after Zol/IL-2 stimulation. PBMC from 18 patients with breast cancer were stimulated with Zol/IL-2 for 10 days, the number of $V\delta 2^+$ T cells determined by means of dye exclusion and flow cytometry, and the data shown as a *scatter plot*. For production of IFN- γ and TNF- α , PMBC from 34 (**c**) or 36 (**d**) patients were stimulated with Zol/IL-2 for two days, the culture supernatants harvested, and IFN- γ or TNF- α levels determined by ELISA. The cytokine levels are plotted against the initial frequency of $V\delta 2^+$ T cells. Data obtained from early- and late-stage breast cancer patients were plotted as *closed* and *open circles*, respectively.

Fig. 4. IL-18 augments expansion of $V\delta 2^+$ T cells and CD3⁻ “helper” NK cells and the effector functions of $V\delta 2^+$ T cells

a, b Effect of IL-18 on the expansion of $V\delta 2^+$ T cells (**a**) and CD3⁻ “helper” NK cells (**b**). PBMC derived from 18 breast cancer patients were stimulated with Zol/IL-2 or Zol/IL-2/IL-18 for 10 days, and the numbers of $V\delta 2^+$ T cells and CD3⁻ cells determined by vital dye exclusion and flow cytometry. *Each line* connects the cell numbers after expansion by Zol/IL-2 with the cell numbers after expansion by Zol/IL-2/IL-18 from the same patient. **c, d** Effect of IL-18 on the production of IFN- γ (**c**) and TNF- α (**d**) by PBMC after stimulation with Zol/IL-2 or Zol/IL-2/IL-18. PBMC from 19 (**c**) or 20 (**d**) breast cancer patients were stimulated with Zol/IL-2 or Zol/IL-2/IL-18 for 2 days, the culture supernatants harvested, and IFN- γ and TNF- α levels determined by ELISA. *Each line* connects cytokines produced in response to Zol/IL-2 or Zol/IL-2/IL-18 by PBMC from the same patient. Data obtained from early- and late-stage breast cancer patients were plotted as *closed* and *open circles*, respectively.

Fig. 5. Effect of repeated in vivo injections of Zol on the Vγ2Vδ2 T cells of an early-stage breast cancer patient

a Dot plots showing ex vivo expansion of Vγ2Vδ2 T cells by Zol/IL-2/IL-18 before or after one or two Zol injections. **b** Effect of Zol administration to a breast cancer patient on the frequency of blood Vδ2⁺ T cells. The initial Vδ2⁺ T cell frequency before Zol injection (0), 1 month after the first injection (1), or 1 month after the second injection (2). **c** Ex vivo expansion of Vδ2⁺ T cells by Zol/IL-2/IL-18 from PBMC derived from a patient before receiving Zol or after receiving Zol once or twice. The effect of repetitive Zol injection on Vδ2⁺ T cells as % of CD3 (*left panel*) or as cell numbers (*right panel*) before treatment (*closed circles*), 1 month after the first injection (*closed triangles*), or 1 month after the second injection (*closed squares*).

Table 1

Relationship between patients' characteristics and V62/CD3 ratio.

N	80	<5.2%	5.2%	Total	p
Age	<60	23	15	38	0.044
	60	34	8	42	
Stage	0, I, II	40	22	62	0.001
	III, IV	17	1	18	
Grade	1, 2	36	18	54	0.194
	3	14	5	19	
	Unknown			7	
Node	+	20	5	25	0.113
	–	29	18	47	
	Unknown			8	
ER	Positive	39	15	54	0.384
	Negative	13	8	21	
	Unknown			5	
HER2	Positive	11	3	14	0.406
	Negative	41	20	61	
	Unknown			5	
Ki67	<14%	20	14	34	0.097
	14%	30	9	39	
	Unknown			7	
Subtype	Luminal A	17	11	28	0.494*
	Luminal B	12	4	16	
	Luminal–Her2	8	0	8	
	HER2	3	3	6	
	Triple negative	10	5	15	
	Unknown			7	

* Luminal A plus B vs. other subtypes. Correlations between the V62/CD3 ratio and clinicopathological parameters were assessed using χ^2 test. A *p* value of less than 0.05 was considered to indicate statistically significant.