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A Quantitative Deficiency in Peripheral Blood V γ 9V δ 2 Cells Is a Negative Prognostic Biomarker in Ovarian Cancer Patients

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Abstract

V γ 9V δ 2 cells are cytotoxic T cells that are able to recognize epithelial ovarian carcinoma (EOC) cells. Therefore, V γ 9V δ 2 cell-based adoptive transfer is an attractive therapy for EOC. However, the inefficient *ex vivo* expansion after specific stimulation of V γ 9V δ 2 cells from some patients and the relationships between V γ 9V δ 2 cells and clinical course of EOC are issues that remain to be clarified. Herein, peripheral blood mononuclear cells (PBMCs) from 60 EOC patients were stimulated with bromohydrin pyrophosphate (BrHPP) or zoledronate, which are specific agonists of V γ 9V δ 2 cells. The compounds differed in their efficacies to induce *ex vivo* V γ 9V δ 2 PBMC expansion, but 16/60 samples remained inefficiently expanded with both stimuli. Interestingly, the V γ 9V δ 2 cells in these low-responding PBMCs displayed before expansion (*ex vivo* PBMCs) an altered production of the pro-inflammatory cytokines IFN- γ and TNF- α , a decreased naïve fraction and a reduced frequency. No evidence of an involvement of CD4⁺CD25⁺Foxp3⁺ regulatory cells was observed. Importantly, our data also demonstrate that a V γ 9V δ 2 cell frequency of 0.35% or less in EOC PBMCs could be used to predict low responses to both BrHPP and zoledronate. Moreover, our data highlight that such a deficiency is not correlated with advanced EOC stages but is associated with more refractory states to platinum-based chemotherapy and is an independent predictor of shorter disease-free survival after treatment. These results are the first to suggest a potential contribution of V γ 9V δ 2 cells to the anti-tumor effects of chemotherapeutic agents and they strengthen interest in strategies that might increase V γ 9V δ 2 cells in cancer patients.

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Introduction

Human V γ 9V δ 2 cells are a predominant subset of peripheral blood $\gamma\delta$ T cells that express a unique TCR with V γ 9-V δ 2 regions. These cells, which usually represent 0.5–10% of the peripheral lymphoid pool, react against various tumor cells through the recognition of phosphorylated isoprenoid derivatives defined as phosphoantigens [1,2]. V γ 9V δ 2 cells can directly kill their targets and release pro-inflammatory cytokines that boost the anti-tumor effector cells of the adaptive immune system [3]. Due to these characteristics, the selective triggering of these cells could be of major interest in cancer immunotherapy [4]. Several currently available clinical-grade compounds are able to strongly activate V γ 9V δ 2 cells and, with IL-2, can induce the selective outgrowth of these cells *in vitro* and *in vivo*. These compounds are either synthetic phosphoantigens, such as bromohydrin pyrophosphate (BrHPP, PhosphostimTM), or pharmacological inhibitors of the mevalonate pathway, such as

the aminobisphosphonates (i.e., zoledronate, ZometaTM). Such compounds have been recently assessed in passive or active immunotherapeutic trials in patients with hematological malignancies or solid tumors [5–14]. These treatments have been generally well tolerated and have induced encouraging objective responses in some patients [12,15,16].

Epithelial ovarian cancer (EOC) is the fifth most frequently occurring cancer in women and causes more deaths than any other gynecologic cancer. Most EOC patients are diagnosed at an advanced stage. Currently, all patients undergo surgical procedures, and 90% of patients also receive a platinum-based chemotherapy. However, the 5-year survival rate remains below 40%. Therefore, the increasing knowledge about the role of immunosurveillance in EOC has led to the exploration of innovative therapeutic strategies that target the immune system [17]. Recently, our group demonstrated that *in vitro* phosphoantigen-expanded V γ 9V δ 2 cells from EOC patients display high cytolytic activity against fresh ovarian autologous tumor cells,

thus providing a rationale for V γ 9V δ 2 cell-based adoptive transfer in EOC patients [18]. However, the relationships between V γ 9V δ 2 cells and progression or clinical outcomes of EOC remain unexplored. Additionally, some concerns exist about the efficacy of V γ 9V δ 2 cell expansions with conventional protocols that are based on the *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) with a single dose of either BrHPP or zoledronate and culture conditions that require IL-2. These protocols are suitable for cells from healthy donors [19,20]. However, they failed to efficiently expand the V γ 9V δ 2 cells from some EOC patients [18], similar to observations in other cancers [12,14,20–22]. It remains to be seen whether these failures in some EOC patients are related to intrinsic differences in the V γ 9V δ 2 cells or are due to differences in other environmental parameters. An understanding of such differences would help to optimize future clinical trials of V γ 9V δ 2 cell-based adoptive transfer therapies in EOC.

In this study, we investigated the following in a cohort of 60 EOC patients: the parameters associated with inefficient BrHPP- and zoledronate-induced V γ 9V δ 2 cell expansions and the possibility of an association between the presence of V γ 9V δ 2 cells and the clinical course of EOC. We report that PBMCs that were inefficiently expanded with BrHPP and with zoledronate have before expansion (*ex vivo* PBMCs) reduced frequencies of V γ 9V δ 2 cells and that these cells display alterations in their phenotype and functionality. In addition, we reveal that a V γ 9V δ 2 cell frequency of 0.35% or less in *ex vivo* EOC PBMCs predicts low responses to both BrHPP- and zoledronate-based stimulation protocols and that such a cellular deficiency is related to the clinical progression and recurrence of EOC after chemotherapy-based treatment.

Results

The Expansions of V γ 9V δ 2 PBMCs in Response to BrHPP and to Zoledronate are Lower in EOC Patients than in Healthy Donors

First, we compared the expansions of PBMCs from 60 EOC patients (EOC PBMCs) and from 13 healthy female donors after a specific V γ 9V δ 2 cell stimulation with a single dose of either BrHPP or zoledronate (Zol), which were relevant to clinical trial protocols, and a culture for two weeks in presence of IL-2 (Fig. 1). The median frequency of V γ 9V δ 2 cells in expanded PBMCs (Fig. 1A) and the median number of expanded V γ 9V δ 2 cells (Fig. 1B) were significantly lower in EOC patients than in donors at 14 days post-stimulation with either BrHPP or Zol. These data confirm that the responses of V γ 9V δ 2 PBMCs to both stimulation protocols are significantly reduced in EOC patients.

BrHPP and Zoledronate Differ in their Capacities to Expand V γ 9V δ 2 PBMCs from EOC Patients

Taking into account our results from EOC patient PBMC expansions (Fig. 1) and the minimal rate of purity that was previously defined for a V γ 9V δ 2 cell-based therapy product [6,23], we selected the expanded V γ 9V δ 2 cell number of 2×10^6 cells (generated from 1×10^6 PBMCs) (see Table 1 legend for details) and the V γ 9V δ 2 cell frequency of 70% among the expanded cells as cut-off values to distinguish efficient expansions ($\geq 2 \times 10^6$ cells and $\geq 70\%$) from inefficient expansions ($< 2 \times 10^6$ cells or $< 70\%$). Four statistically distinct groups of samples were thus identified in our experimental conditions (Table 1): the responding PBMCs (R) that were efficiently expanded with both stimuli (53%); the BrHPP-low-responding PBMCs (Br-LR) that were only efficiently expanded with Zol (13%); the Zol-low-

responding PBMCs (Zol-LR) that were only efficiently expanded with BrHPP (7%); and the low-responding PBMCs (LR) that were inefficiently expanded regardless of the stimulus applied (27%). These data reveal the distinct abilities of conventional BrHPP- and zoledronate-based protocols to efficiently expand V γ 9V δ 2 PBMCs from EOC patients.

The Capacities of V γ 9V δ 2 Cells to Proliferate and to Produce the Pro-inflammatory Cytokines IFN- γ and TNF- α are Reduced in LR EOC PBMCs

To investigate the parameters associated with the low-response status of V γ 9V δ 2 PBMCs to both BrHPP- and zoledronate-based stimulation protocols, comparative analyses were restricted to the LR and R groups.

We first compared the fold increases of V γ 9V δ 2 PBMCs 14 days after BrHPP or Zol stimulation (Fig. 2A and 2B). The fold increases were significantly lower in the LR PBMCs than in the R PBMCs after treatment with either agonist (Fig. 2A). The median BrHPP-induced V γ 9V δ 2 cell fold increase was 812 [352–2333] for the R group and only 132 [74–609] for the LR group (Fig. 2A). A similar trend was observed in Zol-treated cells, with median fold increases of 712 [339–1854] for the R group and 403 [140–872] for the LR group (Fig. 2B). The production of the pro-inflammatory cytokines IFN- γ and TNF- α by V γ 9V δ 2 cells in LR and R EOC PBMCs before expansion (*ex vivo* PBMCs) was also addressed with intracellular staining. Experiments were performed by stimulating *ex vivo* PBMCs with BrHPP or PMA/Ionomycin. Due to few PBMC amounts in samples coming from patients, zoledronate was skipped from the following activation assays. The productions of TNF- α and IFN- γ were found to be significantly reduced in LR PBMCs as compared to R PBMCs (Fig. 2C and 2D). Importantly, doses of BrHPP that were saturating for R PBMCs failed to restore the IFN- γ and TNF- α responses in LR PBMCs (Fig. S1). All together, these results demonstrate an altered functional profile of V γ 9V δ 2 cells in LR EOC PBMCs.

The reduced proliferation and cytokine production of V γ 9V δ 2 cells could result from various factors including a reduced expression of T-cell receptor CD3 ζ chains, altered proportions of naive and memory subsets or an unfavorable ratio of regulatory T cells to V γ 9V δ 2 cells. These possibilities were subsequently investigated.

The Expression Level of CD3 ζ Chains is Similar between the *ex vivo* LR and R EOC PBMCs

The activation signal transduction of T lymphocytes passes through the cytoplasmic tails of the T-cell receptor CD3 ζ chains. A defect in the expression of these components on *ex vivo* V γ 9V δ 2 cells from LR PBMCs was explored. However, no significant differences were detected in the percentages and levels (MFI) of CD3 ζ chain expression between *ex vivo* LR and R V γ 9V δ 2 PBMCs (Fig. 2E). We also compared the abilities of V γ 9V δ 2 cells in LR and R EOC PBMCs to express the class I-restricted T cell-associated molecule (CRTAM) [24], which was identified recently by our group as a phenotypic marker that is strongly associated with the activation of V γ 9V δ 2 PBMCs [25]. Interestingly, after BrHPP stimulation, CRTAM was found to be expressed similarly (percentage and MFI) on V γ 9V δ 2 cells from both LR and R PBMCs (Fig. 2F and data not shown).

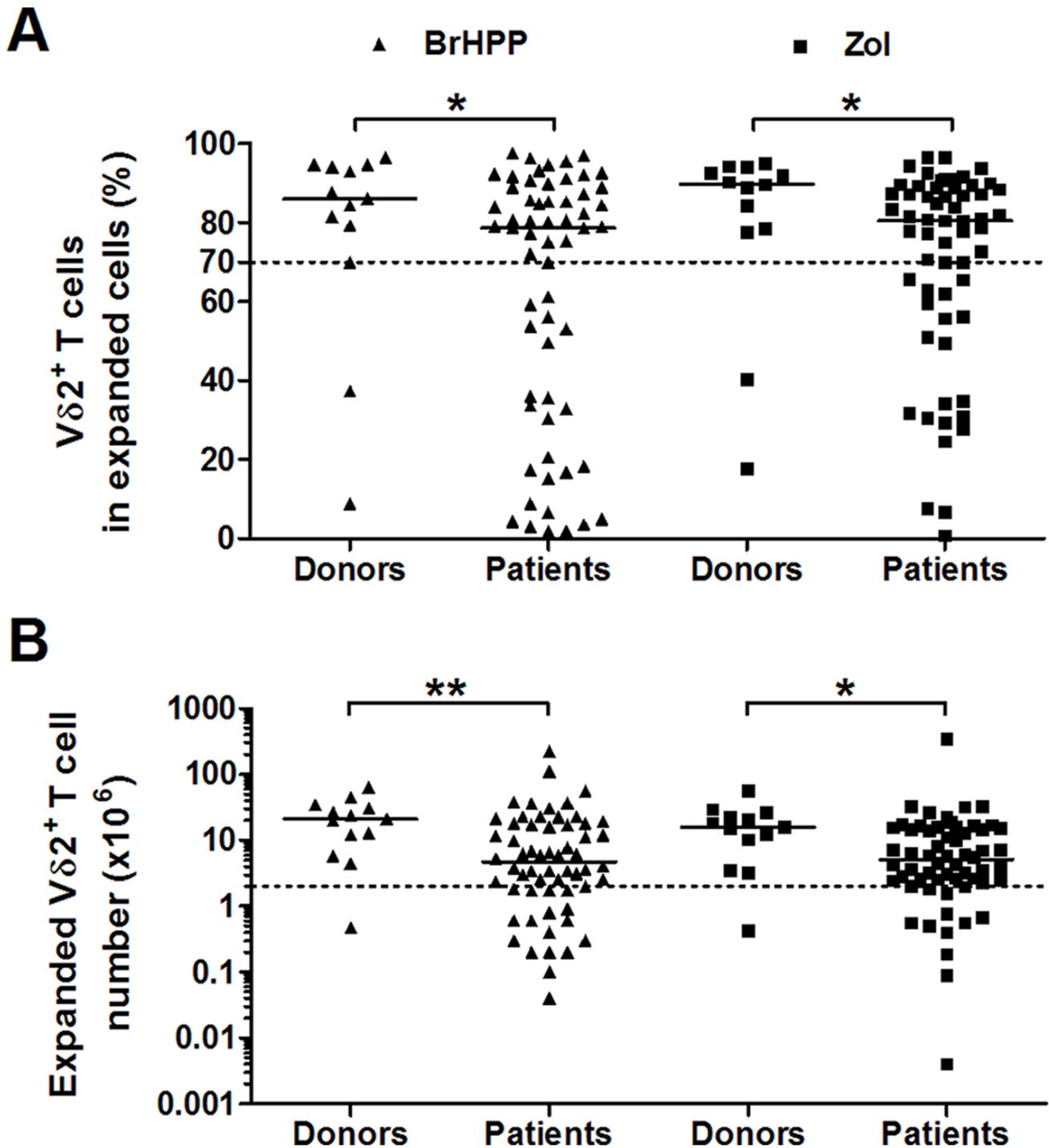


Figure 1. Proliferation of $V\gamma 9V\delta 2$ PBMCs from EOC patients and from healthy female donors following BrHPP or zoledronate stimulation. $V\delta 2^+CD3^+$ cell frequencies among expanded cells (A) and $V\delta 2^+CD3^+$ cell numbers generated from 1×10^6 PBMCs (B) were measured at 14 days after BrHPP (▲) or zoledronate (Zol) (■) stimulation. The results from $n=60$ patient PBMCs and $n=13$ donor PBMCs are shown. doi:10.1371/journal.pone.0063322.g001

The Naive $V\gamma 9V\delta 2$ Cell Fraction is Reduced in *ex vivo* LR EOC PBMCs

The naive and memory subsets of $V\gamma 9V\delta 2$ cells were analyzed in *ex vivo* LR and R EOC PBMCs according to the expression of CD27 and CD45-RA markers (Fig. 3). The median naive cell frequency among $V\gamma 9V\delta 2$ PBMCs was

significantly lower in the LR group (37%) than in the R group (53%; Fig. 3). Accordingly, the median memory cell frequency was higher in the LR group. Significant positive correlation was found between the naive cell frequencies among $V\gamma 9V\delta 2$ PBMCs and the fold increases of $V\gamma 9V\delta 2$ cells after BrHPP stimulation ($p=0.041$ according to the Spearman's correlation test). Such correlation was not found in experiments performed

Table 1. Distinct expansions of V γ 9V δ 2 cells from 60 EOC patients in response to BrHPP and to Zol.

Groups	Nb. (%)	V δ 2 ⁺ T cell frequency (%)			V δ 2 ⁺ T cell number ($\times 10^6$)		
		BrHPP	Zol	p value [#]	BrHPP	Zol	p value [#]
All	60 (100)	62+/-4	70+/-3	0.002	4.5 [1.5–17.5]	5 [2.5–15]	0.473
R	32 (53)	86+/-1	86+/-1	0.666	14 [6–22.5]	12 [4.5–17]	0.026
Zol-LR	4 (7)	77+/-2	56+/-4	<0.001	12.5 [2.5–22.5]	2 [0.5–13]	0.125
Br-LR	8 (13)	38+/-7	80+/-3	0.015	1.5 [1–3.5]	6 [3–12]	0.015
LR	16 (27)	20+/-5	35+/-5	<0.001	0.5 [0–2]	1.5 [0.5–3]	0.022

V δ 2⁺ T cell frequencies in expanded cells (V δ 2⁺CD3⁺ staining) and V δ 2⁺ T cell numbers generated from 1×10^6 PBMCs were measured 14 days after BrHPP or zoledronate (Zol) stimulation; data are presented as means +/- SEM or medians with [first quartile - third quartile]; The V δ 2⁺ T cell frequency of 70% and the V δ 2⁺ T cell number of 2×10^6 (corresponding to the mean of the first quartile values of expanded V δ 2⁺ T cell number with BrHPP and with Zol) were both used as cut-off values to distinguish efficient expansions ($\geq 70\%$ and $\geq 2 \times 10^6$ cells) from inefficient expansions ($< 70\%$ or $< 2 \times 10^6$ cells); four PBMC groups were distinguished: R, Zol-LR, Br-LR and LR; Nb. (%) denotes the number of samples and the corresponding percentage; BrHPP and Zol data were compared using [#]paired t-test or [#]wilcoxon matched pairs test; p value <0.05 indicates a significant difference.
doi:10.1371/journal.pone.0063322.t001

with Zol. Within the memory compartment, no statistically significant differences were detected between the LR and R groups in the frequencies of central, effector or terminally differentiated effector subsets among V γ 9V δ 2 PBMCs (Fig. 3). Thus, no memory V γ 9V δ 2 subsets were enriched preferentially in the *ex vivo* LR EOC PBMCs.

An Imbalance between CD4⁺CD25^{high}FoxP3^{high} Regulatory T cells and V γ 9V δ 2 Cells Exists in *ex vivo* LR EOC PBMCs but is Not Involved in the Impaired Expansion of V γ 9V δ 2 Cells

The frequencies of CD4⁺CD25^{high}FoxP3^{high} regulatory T cells (Tregs) and the ratios of Tregs to V γ 9V δ 2 cells (Treg:V γ 9V δ 2 ratio) were explored in *ex vivo* LR and R EOC PBMCs. The Treg cell frequencies were not found to be significantly different between the LR and R EOC PBMCs (Fig. 4A). However, significant decreases in the V γ 9V δ 2 cell frequencies in LR EOC PBMCs led to Treg:V γ 9V δ 2 ratios that were significantly higher in the LR group than in the R group (Fig. 4B). Importantly, no inverse correlations were observed between these ratios and the fold increases of V γ 9V δ 2 cells that were measured after stimulation with either BrHPP or Zol (according to the Spearman's correlation coefficient analyses, $p = 0.07$ and $p = 0.67$, respectively). Additionally, the depletion of Tregs in LR EOC PBMCs with anti-CD25 microbeads did not improve the expansion capacities of V γ 9V δ 2 cells after stimulation with BrHPP or Zol (Fig. 4C). Indeed, no significant differences were observed in the frequencies, numbers or fold increases of V γ 9V δ 2 cells from CD25-depleted LR PBMCs when compared to non-depleted LR PBMCs after stimulation with either BrHPP or Zol (Fig. 4C and data not shown). Of note, no depletions of V γ 9V δ 2 cells and altered cell viabilities were apparent after CD25-depletion (data not shown). Altogether, these observations suggest that the increased proportion of Tregs compared to V γ 9V δ 2 cells in LR EOC PBMCs is not involved in the impaired response of V γ 9V δ 2 cells.

A Reduced Frequency of V γ 9V δ 2 Cells in *ex vivo* EOC PBMCs $\leq 0.35\%$ Predicts an Inefficient Response to Both BrHPP and Zoledronate

An analysis of the *ex vivo* V γ 9V δ 2 cell frequencies was then performed in the four groups previously described in Table 1. The frequency of V γ 9V δ 2 cells was significantly reduced in the LR

group when compared to all other groups (Fig. 5A). The median values of V γ 9V δ 2 cell frequencies in PBMCs were 0.28% for the LR group and 1.38% for the R group (Fig. 5A). Intermediate values were observed for the Br-LR and Zol-LR groups, with median frequencies of 0.48% and 0.64%, respectively. Interestingly, no significant differences were observed between the LR, Br-LR, Zol-LR and R EOC groups in the frequencies of non-V δ 2⁺ $\gamma\delta$ T cells (Fig. 5B) or of CD3⁺ cells in PBMCs (Fig. 5C). Besides, considering the whole cohort, a significant positive correlation was found between the *ex vivo* V γ 9V δ 2 cell frequencies and the fold increases after stimulation with BrHPP but not with Zol ($p = 0.015$ according to Spearman's correlation tests). In addition, positive correlations between the *ex vivo* V γ 9V δ 2 cell frequencies and the IFN- γ -productions in response to either BrHPP or PMA/ionomycin (measured in Fig. 2C) were evidenced ($p = 0.015$ and $p < 0.001$, respectively). These data reveal a specific reduction in the frequency of the V γ 9V δ 2 subset that is strongly associated with functional anomalies in LR EOC PBMCs.

To determine whether a particular V γ 9V δ 2 cell frequency value could be used to predict inefficient responses to both BrHPP and Zol, a receiver-operator characteristic (ROC) analysis was performed. A V γ 9V δ 2 cell frequency among PBMCs of 0.35% or less was always associated with low responses to both BrHPP and Zol (100% specificity). For frequencies of 0.8% or greater, all the expansions were efficient with at least one stimuli. For frequencies between 0.35% and 0.8%, inefficient responses to both stimuli and efficient responses were observed. These results demonstrate that a V γ 9V δ 2 cell frequency of 0.35% or less among *ex vivo* PBMCs is predictive of inefficient responses to both BrHPP and Zol.

A V γ 9V δ 2 Cell Frequency of 0.35% or Less in *ex vivo* EOC PBMCs is a Negative Prognostic Marker in EOC Patients

To investigate the relationships between V γ 9V δ 2 cells and EOC disease, clinical data from patients were analyzed relative to the V γ 9V δ 2 cell frequencies in *ex vivo* PBMCs (Table 2 and Fig. 5). Among all the parameters that were recorded at the time of blood collection, an association was established only with patient age (Table 2). The median patient age was significantly higher in the group that had V γ 9V δ 2 cell frequencies in PBMCs of 0.35% or less (group $\leq 0.35\%$) than in the group with frequencies greater than 0.35% (group $> 0.35\%$). Importantly, no decreases in the blood concentrations of leukocytes and PBMCs were observed in the group $\leq 0.35\%$, which confirms the specific quantitative deficiency of V γ 9V δ 2 cells in the peripheral blood. No association

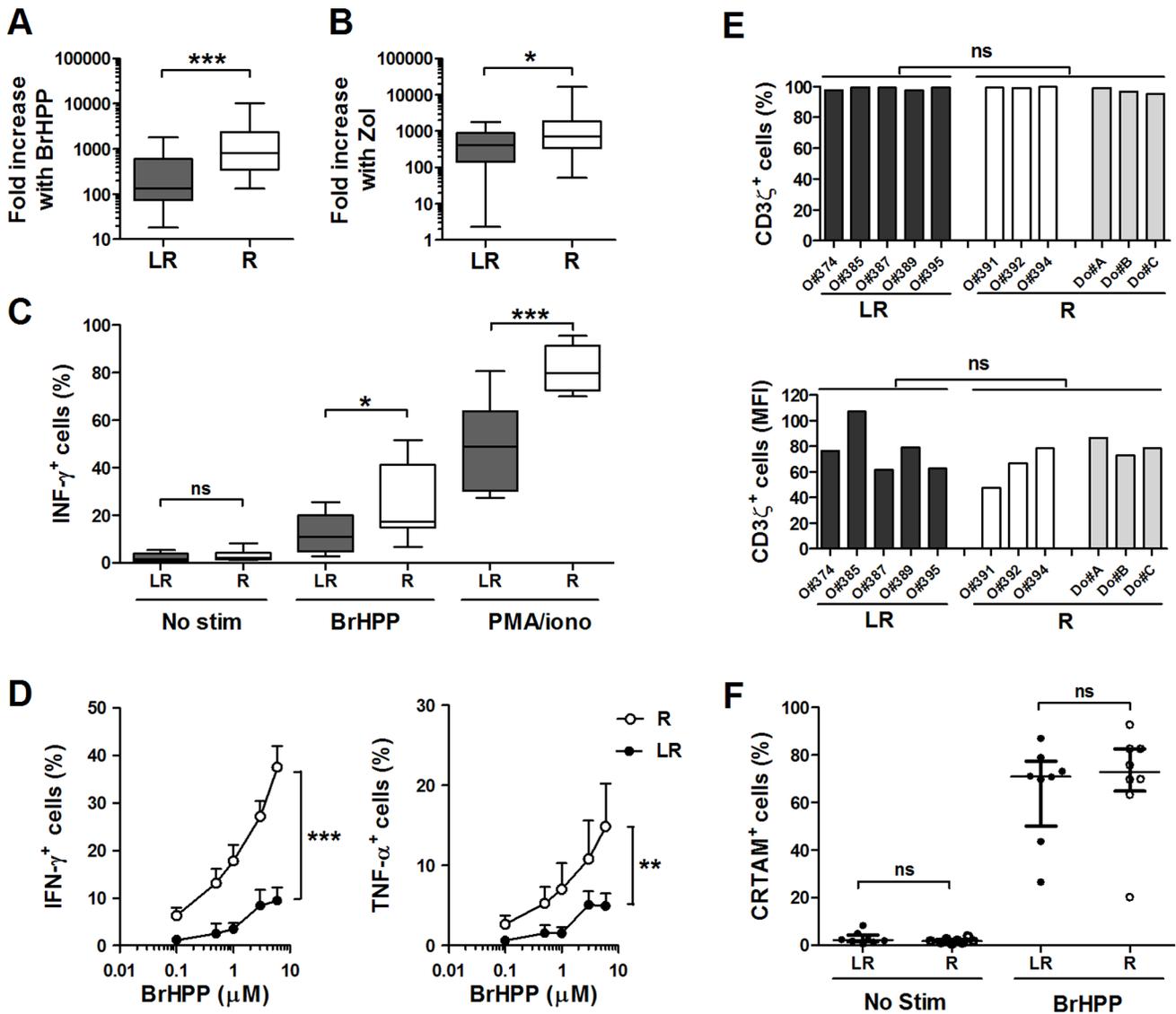


Figure 2. Reduced functionality of V γ 9V δ 2 cells in LR EOC PBMCs without defects in expression level of CD3 ζ and CRTAM molecules. **A, B**) V δ 2⁺CD3⁺ cell fold increases in LR (n=16) and R (n=32) EOC PBMCs at 14 days after BrHPP (**A**) or Zol (**B**) stimulation. **C**) Percentages of IFN- γ ⁺ cells in the V δ 2⁺CD3⁺ cells measured at 5 h after the stimulation of *ex vivo* LR (n=10) and R (n=10) PBMCs with BrHPP (3 μ M) or PMA/ionomycin (PMA/iono). **D**) Percentages of IFN- γ ⁺ cells (*left panel*) and TNF- α ⁺ cells (*right panel*) among the V δ 2⁺CD3⁺ cells measured at 5 h after stimulations of *ex vivo* LR (n=4) and R (n=4) PBMCs with increasing doses of BrHPP (0.1 to 6 μ M). **E**) Expression of the CD3 ζ chain measured on V γ 9V δ 2 PBMCs from the LR and R groups. Percentage (*upper panel*) and MFI (*lower panel*) of CD3 ζ staining in the V δ 2⁺CD3⁺ cells from LR EOC PBMCs (n=5), R EOC PBMCs (n=3) and R donor PBMCs (n=3). **F**) CRTAM expression on V γ 9V δ 2 cells measured at 20 h after stimulation of LR (n=8) and R (n=8) EOC PBMCs with BrHPP. No stim: no stimulation. doi:10.1371/journal.pone.0063322.g002

was established between this deficiency and the administration of chemotherapy prior to blood collection. Moreover, no correlation was found between the deficiency in peripheral blood V γ 9V δ 2 cells and an advanced stage of disease or higher tumor grade. With regard to the treatment of EOC patients (Table 2), no significant differences were observed between the group $\leq 0.35\%$ and the group $> 0.35\%$ in the efficacy of debulking surgery or in the type of received chemotherapy (Table 2). However, the clinical outcomes of chemotherapy-treated EOC patients were found to be distinct between the groups. Interestingly, the proportion of patients who were refractory to chemotherapy was significantly higher in the group $\leq 0.35\%$ (62.5%) than in the group $> 0.35\%$ (17.5%) (Table 2). In addition, univariate analyses of disease-free survival

with different relevant factors revealed that a V γ 9V δ 2 cell frequency of 0.35% or less in PBMCs and a non-optimal debulking are predictors of shorter disease-free survival duration (Table 3). With an average follow-up duration of 13 months for these patients, the median duration of disease-free survival was 1 month in the group $\leq 0.35\%$ versus 10 months in the group $> 0.35\%$ (Fig. 5). Importantly, the negative prognostic value of the reduced V γ 9V δ 2 cell frequency was maintained after an adjustment in a multivariate analysis (Table 3).

Discussion

Previous studies have shown that conventional *ex vivo* V γ 9V δ 2 cell expansion protocols based on the stimulation of PBMCs with

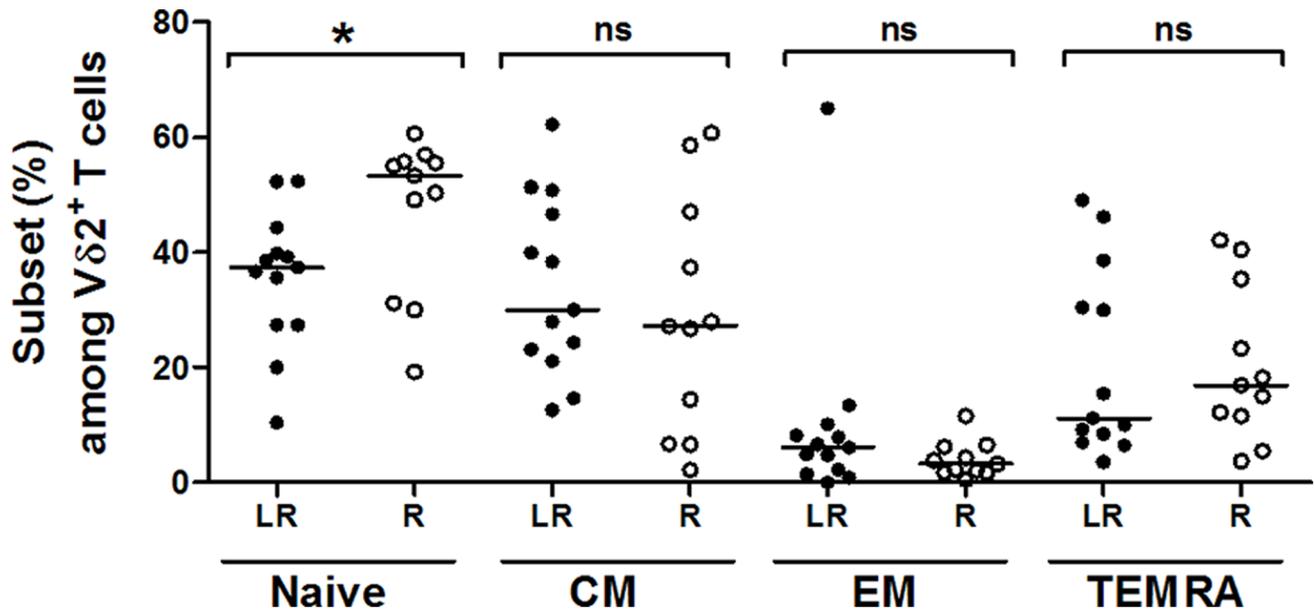


Figure 3. A reduced naive V γ 9V δ 2 subset in *ex vivo* LR EOC PBMCs. Percentages of naive (CD27⁺CD45RA⁺), central memory (CM) (CD27⁺CD45RA⁻), effector memory (EM) (CD27⁻CD45RA⁻) and terminally differentiated effector memory (TEMRA) (CD27⁻CD45RA⁺) cells among the V δ 2⁺CD3⁺ cells in *ex vivo* LR (n = 13) and R (n = 11) EOC PBMCs. doi:10.1371/journal.pone.0063322.g003

BrHPP or Zol failed in an average of 35% of cancer patients [12,20–22,26,27]. Here, we confirmed these observations in EOC. From 60 EOC PBMC samples, inefficient expansions after stimulation with either BrHPP or Zol occurred in 40% and 33% of cases, respectively. Interestingly, our comparative analyses also revealed that BrHPP and Zol differed in their capacities to expand V γ 9V δ 2 PBMCs from patients. Samples that were not efficiently expanded with BrHPP could be efficiently expanded with Zol, and the reverse was also true. These results suggest that each compound might be tested in small-scale expansion assays to allow for the choice of the best compound for each patient. Nevertheless, 27% of PBMC samples from EOC patients remained inefficiently expanded with both compounds (LR PBMCs). We showed that the V γ 9V δ 2 cells in these *ex vivo* LR PBMCs not only had reduced proliferative capacities but also displayed an altered production of pro-inflammatory cytokines IFN- γ and TNF- α compared to the V γ 9V δ 2 cells from responding (R) PBMCs. Saturating doses of BrHPP failed to restore the response of V γ 9V δ 2 cells in LR PBMCs. These results support an intrinsic functional defect of LR V γ 9V δ 2 PBMCs and suggest that the use of higher doses of phosphoantigens to improve response of V γ 9V δ 2 cells in LR EOC PBMCs would be unsuccessful. To our knowledge, this is the first *ex vivo* demonstration of altered V γ 9V δ 2 cell effector functions in EOC patients.

Many parameters could be related to this reduced functionality. The loss or reduced expression of the CD3 ζ chain on T cells from cancer patients has been implicated in impaired T cell activation [28]. However, no differences were observed in CD3 ζ chain expression on *ex vivo* V γ 9V δ 2 cells between the LR and R PBMCs in our EOC cohort, which indicates that the expression of this chain is not causative. Defects in expression of activation markers could also be associated. Here, the molecule CRTAM, recently described by our group as a phenotypic marker that is strongly related with the activation of V γ 9V δ 2 PBMCs [25], was found to be expressed similarly on V γ 9V δ 2 cells from both LR and R EOC PBMCs after BrHPP stimulation. This observation suggests that

signaling events leading to CRTAM expression are not defective in V γ 9V δ 2 from LR PBMCs. Besides, the presence of CD4⁺CD25^{high}FoxP3^{high} T regulatory cells (Tregs) could be involved in the V γ 9V δ 2 cell deficiencies in cancer patients [29]. Recent data from Kunzmann *et al.* have documented that an increase in the Treg:V γ 9V δ 2 ratio suppressed phosphoantigen-induced $\gamma\delta$ T cell proliferation and contributed to the state of apparent immunological unresponsiveness to phosphoantigens that was observed in some cancer patients [27]. Our results in EOC patients showed an increased Treg:V γ 9V δ 2 ratio in *ex vivo* LR PBMCs. However, we did not find an inverse correlation between this ratio and the fold increases of V γ 9V δ 2 cells in response to either BrHPP or Zol. Moreover, Kunzmann *et al.* have reported that the depletion of Tregs (using CD25-depletion) in low-responding PBMCs from patients with tumors others than EOC restored V γ 9V δ 2 cell expansion in response to phosphoantigens. Here, the same cell depletion in LR PBMCs from EOC patients did not improve the proliferation of V γ 9V δ 2 cells in response to phosphoantigens or aminobisphosphonates. In additional assays, we tested an oblique method of cell depletion to remove Tregs from LR EOC PBMCs while preserving CD25⁺ activated cells with the use of the CD4⁺CD25⁺CD127^{dim/-}Regulatory T Cell Isolation Kit II from Miltenyi Biotec (data not shown). Interestingly, effects of such depletion on the proliferation of V γ 9V δ 2 cells were similar to those observed with the CD25-depletion. All together, these results indicate that Tregs are not involved in the impaired proliferation of V γ 9V δ 2 cells in EOC.

Alternatively, differences in the *ex vivo* proportions of naive and memory V γ 9V δ 2 cell subsets could be associated with the reduced functionality of V γ 9V δ 2 EOC PBMCs, as it has been observed in other cancers [12,21,22]. Herein, we found that the naive subset was significantly reduced in *ex vivo* LR EOC PBMCs. This decrease might have been triggered by the repeated antigen priming of V γ 9V δ 2 cells *in vivo* during the development of the disease and with age, leading to a marked differentiation of naive cells to the memory cell compartment in some patients. The

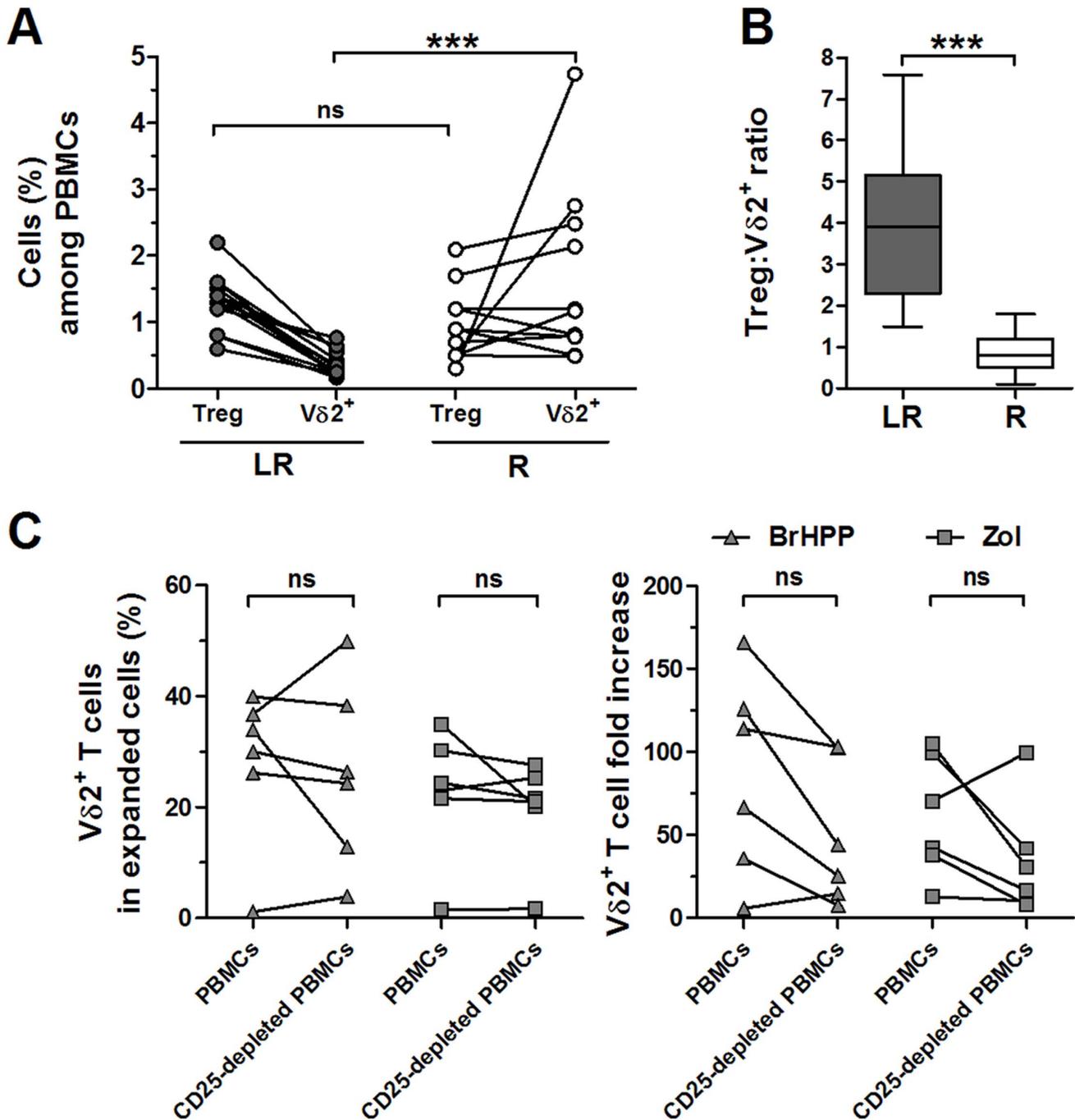


Figure 4. An imbalanced ratio of Tregs and V γ 9V δ 2 cells in *ex vivo* LR EOC PBMCs. **A** CD4⁺CD25^{high}FoxP3^{high} cell (Treg) frequencies and corresponding V δ 2⁺CD3⁺ cell frequencies in *ex vivo* LR (n = 13) and R (n = 11) PBMCs. **B** The ratios of Tregs (%) to V δ 2⁺CD3⁺ cells (%) (Treg:V δ 2⁺ ratio) among PBMCs in the LR and R groups. **C** LR PBMCs from EOC patients (n = 6) were stimulated with BrHPP (▲) or Zol (■) and IL-2 before and after the depletion of CD25⁺ cells; the proliferation of V δ 2⁺CD3⁺ cells was analyzed on day 7. V δ 2⁺CD3⁺ cell frequencies among expanded cells (*left panel*) and V δ 2⁺CD3⁺ cell fold increases (*right panel*) are shown. doi:10.1371/journal.pone.0063322.g004

V γ 9V δ 2 cell frequencies in *ex vivo* PBMCs from patients could also be related to inefficient V γ 9V δ 2 cell expansions. Data from the literature on this issue are diverging. A correlation between the baseline percentages of V γ 9V δ 2 cells in PBMCs from different cancer patients and the *ex vivo* expansion capacities has been established recently [12], while another previous study reported no association between the same parameters in a large cohort of

patients with different types of cancer [27]. Our study of EOC patients supports the most recent observation of specifically reduced V γ 9V δ 2 cell frequencies in *ex vivo* LR PBMCs. These deficiencies in V γ 9V δ 2 PBMCs, combined with decreases in proportion of the naive subset that is endowed with proliferative capacities [30], might be partly responsible for the reductions in V γ 9V δ 2 cell proliferation that were observed in some LR EOC

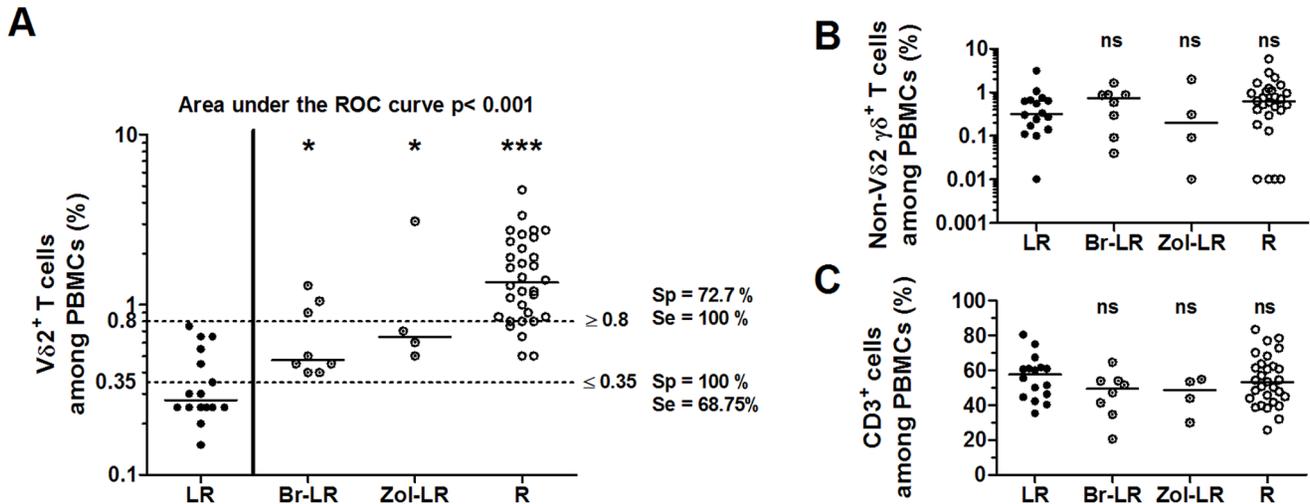


Figure 5. Specific quantitative deficiencies of V γ 9V δ 2 cells in *ex vivo* LR EOC PBMCs. Frequencies of V $\delta 2^+$ CD3 $^+$ cells (A), V $\delta 2^- \gamma\delta^+$ CD3 $^+$ cells (B) and CD3 $^+$ cells (C) among LR (n = 16), Br-LR (n = 8), Zol-LR (n = 4) and R (n = 32) EOC PBMCs are shown. Differences between LR group and each of other groups are indicated. A) A receiver-operator characteristic (ROC) analysis was performed in which LR PBMC samples were compared to the other PBMC samples. Dashed lines indicate cut-offs at 0.35% and 0.8%. Sp: Specificity. Se: sensibility. doi:10.1371/journal.pone.0063322.g005

PBMCs. Of note, the peripheral blood V γ 9V δ 2 cell frequencies in EOC patients were not affected by chemotherapy and we confirmed an age-dependent decrease of V γ 9V δ 2 cell frequencies in EOC patients, as was observed by other authors in healthy subjects [31,32]. Importantly, we demonstrated that a V γ 9V δ 2 cell frequency among *ex vivo* PBMCs from EOC patients of 0.35% or less was always associated with inefficient expansions in response to BrHPP and to Zol. These data identify the V γ 9V δ 2 cell frequency in PBMCs of 0.35% or less as a unique biomarker that might be useful for the prediction of inefficient expansions of PBMCs from EOC patients in response to both conventional BrHPP- or Zol-based $\gamma\delta$ expansion protocols.

Another important point addressed in this study was the relationships between V γ 9V δ 2 cells and the clinical outcomes of EOC patients who were treated with surgery plus platinum-based chemotherapy. Platinum derivatives have been described as immunomodulatory compounds, and candidate immune biomarkers such as $\alpha\beta$ T cells have already been implicated in the efficacy of these anticancer agents [33,34]. Two studies in human advanced ovarian carcinoma have reported that the presence of tumor-infiltrating CD3 $^+$ T cells is correlated with improved clinical outcomes of patients who were treated with surgery plus platinum-based chemotherapy [35,36]. Of these two studies, one has also reported a correlation between the presence of infiltrating $\gamma\delta$ T cells and a brief disease-free interval after treatment [36]. However, this study, which was based on a molecular assessment of TCR γ by PCR analysis, did not discriminate between V γ 9V δ 2 and other $\gamma\delta$ cells. Here, we demonstrate that EOC patients with a decreased peripheral blood V γ 9V δ 2 cell frequency of 0.35% or less, which is correlated strongly with an impaired V γ 9V δ 2 PBMC functional profile, are more likely to be refractory to platinum-based chemotherapy and display a shorter disease-free survival time after treatment. A multivariate analysis confirms that this reduced frequency is an independent predictor for disease-free survival time. Therefore, the V γ 9V δ 2 cell frequency in PBMCs could be used as a prognostic biomarker in EOC patients. Taken together, our observations support the conclusion that the outcomes for chemotherapy-treated patients are more favorable when there are no deficiencies in the numbers and functionality of

peripheral blood V γ 9V δ 2 cells. Interestingly, these data are the first to suggest that the V γ 9V δ 2 cell subset could contribute to the anti-tumor effects of conventional anticancer chemotherapeutics.

In conclusion, our results indicate that a specific V γ 9V δ 2 cell frequency value in *ex vivo* PBMCs could be used to select eligible EOC patients for conventional BrHPP- or Zol-based $\gamma\delta$ expansion protocols and to predict the clinical outcomes for chemotherapy-treated EOC patients. The data suggest that the combination of current chemotherapeutic treatments in EOC with V γ 9V δ 2 cell-based immunotherapies could have a clinical interest for patients and should be explored. Our data also reveal that EOC patients with the worst prognostic outcomes are those with inefficient V γ 9V δ 2 PBMC expansions to both conventional BrHPP- or Zol-based $\gamma\delta$ expansion protocols. Fortunately, other strategies that could increase V γ 9V δ 2 cells in these patients are conceivable. A powerful expansion protocol using aminobisphosphonates-pre-treated dendritic cells or the transfer of allogeneic V γ 9V δ 2 cells from healthy donors could represent alternative solutions [20].

Materials and Methods

Ethics Statement

This paper contains experiments using human cells. The paper satisfies PLOS ONE policies regarding human subject research. The Centre de Ressources Biologiques (CRB) from CHU of Rennes approved this study. The study was agreed by the french ministry of research and recorded under the n $^{\circ}$ DC 2008-738". Written informed consent was obtained from all participating subjects.

Cells from Donors and EOC Patients

PBMCs were isolated from peripheral blood samples from healthy female donors (Do#, n = 13 from the Etablissement Français du Sang, Villejean, Rennes, France) and patients with epithelial ovarian adenocarcinoma (EOC) (O#; n = 59 from the Gynecology Department, CHU of Rennes, France; n = 1 from Surgical Oncology, ICO-Cancer Center, Nantes; France) by the density separation method (Unisep $^{\circ}$, Novamed, Jerusalem, Israel). Blood samples from EOC patients were collected before (n = 46)

Table 2. Clinical characteristics of EOC patients according to their V δ 2⁺ T cell frequency in PBMCs.

			$\leq 0.35\%$	$> 0.35\%$	p value \ddagger
			n = 11	n = 49	
At blood collection	Tumor histological type	SP	7 (64)	36 (73.5)	0.712
		E	2 (18)	8 (16.5)	1.000
		M	2 (18)	1 (2)	0.084
		CC	0 (0)	4(8)	1.000
	Tumor grade	I	2 (20)	4 (9)	0.297
		II	5 (50)	12 (27)	0.254
		III	3 (30)	29 (64)	0.075
		nd	1	4	
	FIGO stage	I and II	2 (18)	9 (18.5)	1.000
		III	7 (64)	27 (55)	0.742
		IV	0 (0)	9 (18.5)	0.188
		recurrence	2 (18)	4 (8)	0.302
		Ca125 (UI/ml)	median [–]	203 [52–1042]	451 [51–1850]
Leucocytes (GIGA/L)		median [–]	9.1 [5.6–10.4]	8.0 [6.3–10.3]	1.000
PBMCs (GIGA/L)		median [–]	1.4 [0.9–2.4]	1.5 [0.9–1.9]	0.573
Blood puncture after chemo			4 (36)	9 (18)	0.231
Age (years)		median [–]	76 [70–78]	62 [54–71]	0.003
Treatment	Optimal debulking	yes	6 (60)	25 (62.5)	1.000
		no	4 (40)	15 (37.5)	
		nd	1	9	
	Platinum-based chemotherapy received		9 (82)	43 (88)	0.630
		Carboplatin	2 (22)	5 (12)	0.590
		Carboplatin- taxol	6(67)	38 (88)	0.130
		Gemcitabine-oxaliplatin	1 (11)	0 (0)	0.173
	Refractory to chemotherapy	no	3 (37.5)	33 (82.5)	0.016
		yes	5 (62.5)	7 (17.5)	
		nd	1	3	

Patients with V δ 2⁺ T cell frequencies in PBMCs of 0.35% or less ($\leq 0.35\%$) (n = 11) or greater than 0.35% ($> 0.35\%$) (n = 49) at the time of blood collection. Tumor histological type (serous-papillary (SP), endometrioid (E), mucinous (M), clear cell (CC)), tumor grade, FIGO stage, Ca125 concentration, leucocyte concentration, PBMC concentration and patient age at time of blood puncture are indicated; PBMC samples collected after chemotherapy are noticed; the optimal debulking of tumor after surgery (no residual tumor nodules), the number of patients who received chemotherapy, the type of chemotherapy and the numbers of patients who were refractory to chemotherapy are indicated; data are presented as medians [first quartile – third quartile] for continuous variable and as numbers (with corresponding percentages) for qualitative variables; nd denotes case not determined;

\ddagger continuous variables were compared using the Mann-Whitney test and categorical variables using the Fisher's exact test; p value <0.05 indicates a significant difference.

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and after (n = 14) chemotherapeutic treatment. The mean age of the patients was 63.9 years (\pm SEM 1.5). Serous-papillary, endometrioid, mucinous and clear cell adenocarcinoma histological types were diagnosed anatomopathologically in 43, 10, 3 and 4 cases, respectively. Grades I, II, and III were noted in 11%, 31% and 58% of cases, respectively. The EOC staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification system [37]. The majority of the patients had advanced disease; 56.7% and 15% had FIGO stage disease III and IV, respectively, and 10% were in relapse. All patients underwent a surgical procedure. Platinum derivative-based chemotherapy was also performed for 52/60 patients. Carboplatin, carboplatin-taxol and gemcitabine-oxaliplatin agents were used in 7, 44 and 1 cases, respectively. Optimal debulking with surgery was defined by the absence of residual individual tumor nodules after surgery. A state refractory to chemotherapy was defined by increases in tumor size and/or CA-125 values at

the end of the chemotherapy treatment. Refractory states were reported in 25% of the cases. The duration of disease-free survival was measured as the time between the end of treatment (surgery plus chemotherapy) and the first disease recurrence or progression. The duration of overall survival was calculated as the time between the end of treatment and death. Data were censored at the final follow-up for patients without disease recurrence, progression or death. The follow-up duration was defined as the time between the end of treatment and either death or the final follow-up.

Antibodies and Reagents

Monoclonal antibodies (mAbs) against the following antigens were used for staining: V δ 2 (IMMU389), pan- $\gamma\delta$ (IMMU510), CD45RA (ALB11), CD3 (CD3 ϵ ; UCHT1), CD3 ζ (2H2D9), CD4 (13B8.2), CD14 (RMO52) and CD25 (B1.49.9) were obtained

Table 3. Regression analyses of disease-free survival in surgery plus chemotherapy-treated patients.

Variables	Univariate analysis				Multivariate analysis ^Φ			
	Nb	HR	95% CI	p value	Nb	HR	95% CI	p value
Vδ2⁺ T cells in PBMC (%)								
≤0.35	9	2.30	1.03 to 5.13	0.042	8	2.99	1.22 to 7.29	0.016
>0.35	43				43			
Histologic type								
CC	4	1.71	0.52 to 5.59	0.378	NI			
SP, E or M	48				NI			
Tumour grade^a								
1	4	0.51	0.11 to 2.19	0.367	NI			
2	15	1.28	0.62 to 2.64	0.496	NI			
3	29				NI			
FIGO stage								
I or II	6	0.00	0 to 39.5E+168	0.947	NI			
III	32	1.15	0.39 to 3.30	0.800	NI			
IV	9	1.50	0.44 to 5.11	0.513	NI			
Relapse	5				NI			
Optimal debulking^b								
no (>1 cm)	26	2.50	1.22 to 5.11	0.012	26	2.93	1.39 to 6.17	0.005
yes (≤1 cm)	25				25			
Platinum-based chemotherapy								
without taxol	8	1.50	0.62 to 3.63	0.366	NI			
with taxol	44				NI			
Age (years)								
≥70	21	1.20	0.61 to 2.35	0.596	NI			
<70	31				NI			

Results obtained using the Cox proportional hazard regression model; Nb denotes number of patients; HR denotes Hazard Ratio; CI denotes confidence interval; Tumor histological type (serous-papillary (SP), endometrioid (E), mucinous (M), clear cell (CC));

^adata available in 48 patients;

^bdata available in 51 patients;

^Φmultivariate analysis with data available in 51 patients: variables found to have a p value <0.1 in the univariate analysis were included in the model; NI denotes not included; p value <0.05 indicates a significant difference.

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from Beckman Coulter (Villepinte, France); CD27 (o323) and Foxp3 (236A/E7) were obtained from eBioscience (Paris, France); CRTAM (210213) was obtained from R&D systems (Lille, France); and IFN- γ (B27) and TNF- α (Mab11) were obtained from BD Biosciences (Le Pont de Claix, France). Isotype-matched mAbs (Beckman Coulter, eBioscience, R&D systems or BD Biosciences) were used as staining controls. Synthetic BrHPP (PhosphostimTM) was kindly provided by Innate Pharma (Marseille, France). Synthetic Zoledronate (Zometa[®]) was obtained from Novartis (Rueil-Malmaison, France). Recombinant human IL-2 (Proleukin[®]) was obtained from Chiron Therapeutics (Suresnes, France). Monensin, saponin, PMA and ionomycin were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France).

V γ 9V δ 2 Cell Expansion Assay

Ex vivo PBMCs were resuspended at 1.2×10^6 cells/ml in RPMI 1640 medium (Eurobio, Les Ullis, France) that was supplemented with 10% fetal calf serum (FCS) (Gibco Invitrogen Life Technologies, Cergy Pontoise, France), 2 mM L-glutamine, 100 μ g/mL streptomycin and 100 U/mL penicillin (hereafter referred to as RPMI-FCS) and were specifically activated with 3 μ M BrHPP or 1 μ M zoledronate plus 400 UI/ml recombinant

IL-2. Cultures were maintained at 37°C for two weeks at 0.5×10^6 cells/mL in RPMI-FCS plus 400 UI/ml IL-2. The concentrations of BrHPP, zoledronate and IL-2 that were used in this study were defined in accordance to clinical protocols [6,12] and dose-scaling studies that ranged from 0.3 to 30 μ M for BrHPP, from 1 to 10 μ M for zoledronate and from 400 UI/ml to 3200 UI/ml for IL-2 (laboratory data). V γ 9V δ 2 cell specific expansion was measured by calculating the frequencies of V δ 2⁺CD3⁺ cells and the absolute V δ 2⁺CD3⁺ cell numbers at day 0 among PBMCs and at day 14 among expanded cells. The fold increases of V γ 9V δ 2 cells at day 14 were calculated according to the following formula: fold increase = (absolute V δ 2⁺CD3⁺ cell number at day 14) / (absolute V δ 2⁺CD3⁺ cell number at day 0).

Depletion of CD25⁺ Cells and V γ 9V δ 2 Cell Proliferation Assay

Ex vivo PBMCs were depleted of CD25⁺ cells by a magnetic cell separation with CD25 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The efficacy of CD25 depletion in PBMCs and the frequency of V δ 2⁺CD3⁺ cells in depleted-PBMCs were evaluated by flow cytometric analysis. Next, CD25⁺-depleted PBMCs and non-

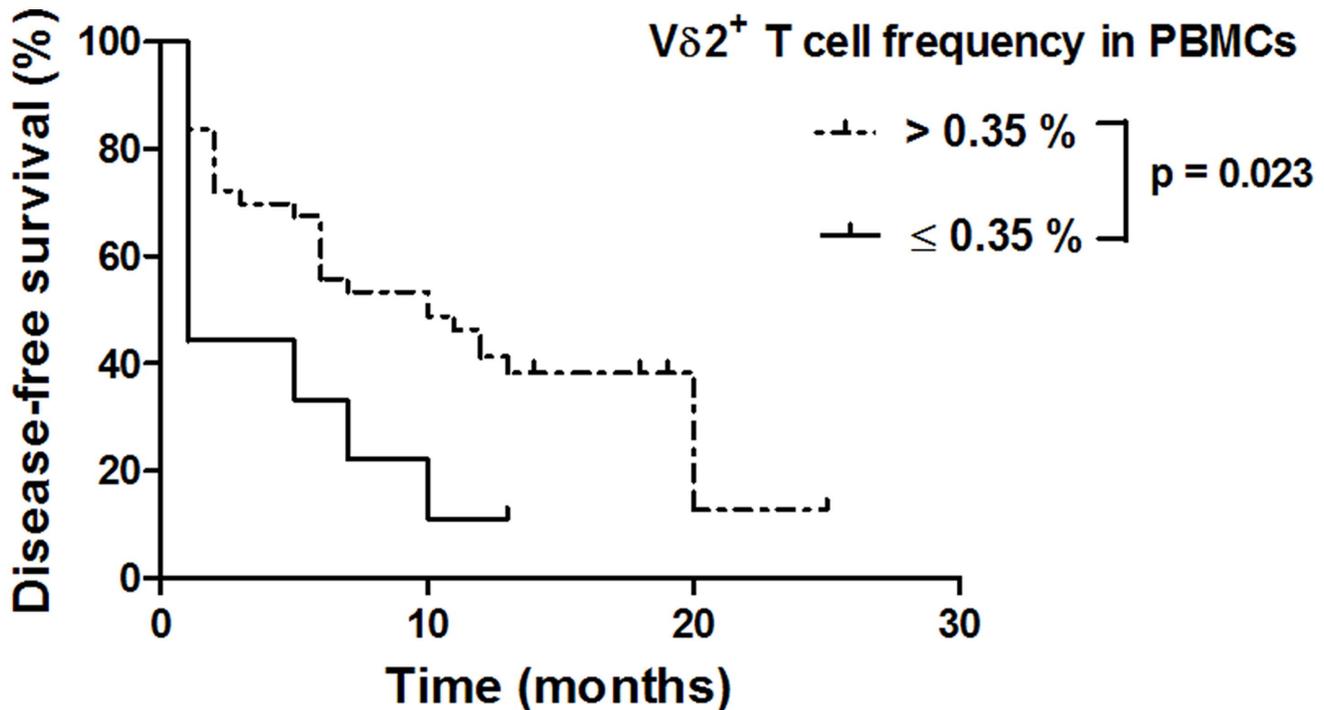


Figure 6. Disease-free survival of chemotherapy-treated EOC patients according to V γ 9V δ 2 cell frequencies among *ex vivo* PBMCs. Disease-free survival Kaplan-Meier curves of patients with V γ 9V δ 2 cell frequencies in PBMCs of 0.35% or less ($\leq 0.35\%$) ($n=9$) or greater than 0.35% ($>0.35\%$) ($n=43$) at the time of blood collection. p value <0.05 indicates a significant difference. doi:10.1371/journal.pone.0063322.g006

depleted PBMCs from the same patients were resuspended at 1.2×10^6 cells/ml in RPMI-FCS with 400 UI/ml IL-2 (0.6×10^6 cells per well of a 48-well plate). The cells were stimulated by the addition of 3 μ M BrHPP or 1 μ M zoledronate and were incubated at 37°C for 7 days. At day 4, 0.25 ml of RPMI-FCS with IL-2 (400 UI/ml) was added to each well. The frequencies, numbers and fold increases of V γ 9V δ 2 cells at day 7 were measured as described previously.

IFN- γ and TNF- α Responses and CRTAM Expression Assay

Ex vivo PBMCs were activated at 37°C in RPMI-FCS (1.2×10^6 cells/ml) by the addition of BrHPP (0.1 to 30 μ M) or PMA (20 ng/ml) plus ionomycin (0.5 μ M). To measure IFN- γ and TNF- α responses, intracellular cytokine accumulation was induced by the addition of 3 μ M monensin after 1 h of activation. The cells were collected 4 h later, stained for V δ 2 TCR chain and CD3 expression, and fixed with 1% formal. Fixation was followed by permeabilization with 0.5% saponin for 20 min and incubation with IFN- γ -specific or TNF- α specific mAbs for 30 min. To measure CRTAM expression, cells were collected 20 h after activation, stained for V δ 2 TCR chain, CD3 and CRTAM expression and fixed with 1% formal.

Flow Cytometry and Statistical Analysis

Stained cells were analyzed by flow cytometry with a FACSCalibur system (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA). Statistical analyses were performed with GraphPad Prism 5.0 (La Jolla, CA, USA) and Sigma plot 12.0 softwares (San Jose, CA, USA). The column scatter plots all show lines drawn at the medians. Comparison tests between groups were performed using Mann-Whitney U tests in Fig. 1, Fig. 2 (A,

B, C, E and F), Fig. 3, Fig. 4 (A and B) and Fig. 5; a two-way ANOVA in Fig. 2D; a wilcoxon matched pairs test in Fig. 4C; and a log-rank (Mantel-Cox) test in Fig. 6. *, ** and *** indicate statistically significant differences for which $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. ns indicates that the difference was not significant.

Supporting Information

Figure S1 Reduced IFN- γ and TNF- α responses of V γ 9V δ 2 cells in LR EOC PBMCs. Percentages of IFN- γ ⁺ cells and TNF- α ⁺ cells among the V δ 2⁺CD3⁺ cells measured at 5 h after stimulation of *ex vivo* LR and R EOC PBMCs with increasing doses of BrHPP ($n=3$). Comparison tests between groups were performed using a two-way ANOVA. *** indicates statistically significant differences for which $p < 0.001$. (TIF)

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Author Contributions

Conceived and designed the experiments: VC FC. Performed the experiments: AT BD PD. Analyzed the data: AT VL. Contributed reagents/materials/analysis tools: VL SH IJ JL. Wrote the paper: AT VL VC FC.

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