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Sensitization of ovarian carcinoma cells with zoledronate restores the cytotoxic capacity of Vγ9Vδ2 T cells impaired by the prostaglandin E2 immunosuppressive factor: implications for immunotherapy

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Epithelial ovarian cancer (EOC) usually spreads into the peritoneal cavity, thereby providing an opportunity for intraperitoneal adoptive immunotherapy with Vγ9Vδ2 T lymphocytes, a T cell subpopulation endowed with high lytic properties against tumor cells. However, previous studies have reported that Vγ9Vδ2 T cells fail to expand from peripheral blood mononuclear cells in one-third of patients with cancer. Here, from a cohort of 37 patients with EOC, a multiple correspondence analysis identified three populations, one of which was not suitable for Vγ9Vδ2 T-cell adoptive therapy. Interestingly, the ineligible patients were identified based on the frequency of Vγ9Vδ2 T cells in their peripheral blood and the patients' age. The average time to tumor recurrence was also found to be significantly different between the three populations, suggesting that the innate immune response is involved in EOC prognosis. A dramatic decrease in the lytic properties of Vγ9Vδ2 T cells occurred following incubation with ascitic supernatant and was found to be associated with reduced perforin/granzyme degranulation. Prostaglandin E2, but not IL-6, IL-10, VEGF or TGF-β, showed immunosuppressive effects in Vγ9Vδ2 T cells. Interestingly, our results emphasize that pretreating ovarian tumor cells with zoledronate partially reverses the immunosuppressive effects of ovarian cancer-associated ascites and restores a high level of lytic activity. These data sustain that optimal Vγ9Vδ2 T-cell adoptive immunotherapy previously requires counteracting the tumor immunosuppressive microenvironment. Altogether, our findings provide a rationale for clinically evaluating Vγ9Vδ2 T-cell adoptive immunotherapy with intraperitoneal carcinomatosis presensitization by zoledronate in patients with EOC.

Key words: Vγ9Vδ2 T cells, immunotherapy, ovarian carcinoma, ascites, prostaglandin E2

Abbreviations: nd: not determined; PBMC: peripheral blood mononuclear cells; D: day; BrHPP: bromohydrine pyrophosphate; Zol: zoledronate.

Additional Supporting Information may be found in the online version of this article.

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Epithelial ovarian cancer (EOC) is the fifth most frequent cancer among women and the fourth most common cause of cancer-related deaths among women.[1] More than 70% of patients are diagnosed when the cancer has spread beyond the ovaries, and these patients have low median survival rates.[2] The prognosis is poor, with a 5-year survival rate of only 30%, and the rate is less than 10% for patients with bulky residual disease remaining after surgery and chemotherapy, which emphasizes the need for innovative treatments.[3] The inflammatory microenvironment of ovarian carcinomas prevents the maturation of myeloid cells, favors regulatory T-cell development and restrains the cytotoxic activity of effector T lymphocytes, leading to the escape of the tumor from the immune system.[4] Thus, research is ongoing to develop innovative approaches aimed at stimulating the immune system.[5] The preferential spread pattern of EOC in the peritoneal cavity offers an excellent opportunity for the regional administration of adoptive T-cell therapy.[2] Some pilot trials have shown the feasibility of and promising

benefits from intraperitoneal adoptive immunotherapy with conventional αβ T lymphocytes.[6] T cells bearing the αβ T cell receptor (TCR) recognize the antigenic peptides presented by Major Histocompatibility Complex (MHC) molecules. Thus, αβ T-cell-based immunotherapy requires knowledge of the appropriate tumor antigenic peptides and is restricted to MHC-matched patients.[6–8] Recently, particular attention has been devoted to the small population of Vγ9Vδ2 T cells in the peripheral blood (1–10% of lymphocytes), endowed with powerful antitumor properties. Vγ9Vδ2 T cells detect phosphate molecules [phosphoantigens (PAgs)] overexpressed by tumor cells in an MHC-independent manner. Isopentenyl pyrophosphate (IPP), an intermediate metabolite of the mevalonate pathway,[9–12] has been proposed as an endogenous PAg that triggers activation of the Vγ9Vδ2 TCR. In addition to the TCR, Vγ9Vδ2 T cells express natural killer receptors (NKR), such as NKG2D and CD226 (also called DNAM-1 for the DNAX accessory molecule-1). NKG2D contributes to the cell lysis of target cells bearing the MHC class I-related chain A/B (MICA/B), UL16-binding protein (ULBP), and DNAM-1 binds the nectin-2 (CD112) and necl-5 (CD155) ligands.[10,13] The significant lytic abilities of Vγ9Vδ2 T cells were shown to be mediated primarily by the perforin degranulation pathway.[14] Human cancer cells, including renal, colonic, hepatic, lymphoma and myeloma, are reportedly recognized and lysed by Vγ9Vδ2 T cells *in vitro*.[15–19] However, to our knowledge, no data were available regarding EOC.

Both the synthetic PAg agonists [bromohydrine pyrophosphate (BrHPP); Phosphostim™, Innate-Pharma, Marseille, France] and the aminobisphosphonates (zoledronate; Novartis), which promote intracellular accumulation of IPP by blocking the mevalonate pathway, can be used to activate Vγ9Vδ2 T cells.[20] The results of first human trial aimed at treating lymphoid malignancies by stimulating Vγ9Vδ2 T cells *in vivo* with aminobisphosphonate infusions were recently published, and the results were promising.[18] Alternatively, our group and others have reported that Vγ9Vδ2 T cells from the peripheral blood mononuclear cell (PBMC) population can be amplified *ex vivo* by stimulation with PAg agonists or aminobisphosphonates before their *in vivo* use in adoptive immunotherapy.[15,17,21–24] This strategy is especially attractive for EOC because of the localization of the tumor cells in the peritoneal cavity, which allows treatment by a localized infusion. However, two concerns require further investigation. First, the selection of eligible patients for clinical trials needs to be improved because the *ex vivo* expansion of Vγ9Vδ2 T cells has proven to be unsuccessful in about one-third of patients with cancer.[17,25] Second, the influence of the microenvironment on the ability of Vγ9Vδ2 T cells to lyse target cells must be addressed. Indeed, studies have shown that ovarian ascites contain well-known immunosuppressive factors, including IL-6, IL-10, TGF-β and VEGF.[26,27] Moreover, recently, Fournie *et al.*[28] have demonstrated that PGE2 inhibits the natural Vγ9Vδ2 T-cell cytotoxicity triggered by the NKR and TCR through cAMP-mediated PKA Type I-dependent signaling.

Multiple correspondence analysis (MCA) was used on data from 37 patients with EOC and revealed that the percentage of peripheral blood Vγ9Vδ2 T cells and the patients' age are useful for identifying appropriate patients for inclusion in clinical trials. In addition, our data show the presence of high PGE2 levels in ovarian ascites and highlight PGE2 as a major ascites immunosuppressive factor. Notably, we found that pretreating tumor cells with zoledronate counteracts the immunosuppressive environment of ovarian cancer-associated ascites. Thus, this treatment may be an attractive tool for improving Vγ9Vδ2 T-cell immunotherapy.

Material and Methods

Pathological analysis and surgical procedures

On clinical and radiological suspicion of EOC, patients underwent a laparotomy (Department of Gynecology, Teaching Hospital, Rennes) to remove ovaries. An intraoperative microscopic examination was performed by a pathologist for all of the ovarian specimens to confirm the carcinoma diagnosis. Samples (5–50 ml of peripheral whole blood, ascites, solid tumor and carcinomatosis) were also collected during the surgery. A patient was included in the study when a final pathological diagnosis confirmed ovarian carcinoma. The protocol was approved by the local institutional review board. Written informed consent was obtained from the patients.

Patient specimens

Blood, ascites and tumor samples were collected from 37 patients with ovarian adenocarcinoma. The mean age at the time of surgery was 67 ± 10 years. The EOC staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification system.[29] The majority of the patients had advanced disease (57% had FIGO stage IIIc disease, and 16% were in relapse). Before and after chemotherapy, 73 and 27% of the samples were collected, respectively. Grades I, II and III were reported in 6, 39 and 55% of the cases, respectively. Poorly, moderately and well-differentiated malignancies were noted in 25, 47 and 28% of the cases, respectively. Serous-papillary adenocarcinoma histology was reported in 65%, endometrioid adenocarcinoma in 22%, mucinous adenocarcinoma in 5%, clear cell adenocarcinoma in 5% and serous adenocarcinoma in 3% of the tumors (Table 1). The disease-free survival was the time between the completion of chemotherapy or final surgery and the first recurrence or progression, which is defined as an increase in tumor size or CA-125 values. The duration of overall survival was the time between the completion of chemotherapy or final surgery and death. The observation time was the time between the end of the treatment (adjuvant chemotherapy or final surgery) and the last contact (death or last follow-up). Data were censored at the last follow-up for patients without recurrence, progression or death.

Ex vivo Vγ9Vδ2T lymphocyte culture

PBMCs were isolated by the density separation method (Unisep®; Novamed, Jerusalem, Israel) from donor ($n = 8$;

Table 1. Clinical, pathological and biological characteristics of the ovarian cancer specimen

	Patient code	Age	Adenocarcinoma subtype	Differentiation	Grade	Stage	Carcinomatosis	Ascites at time of surgery	Chemotherapy	CA-125 (ng/ml)	Number of PBMC at D0 ($\times 10^6$)[1]	Number of δ2T cells at D0 ($\times 10^6$)[1]	Number of δ2T cells at D14 BrHPP ($\times 10^6$)[1]	Number of δ2T cells at D14 Zol ($\times 10^6$)[1]
Population 1														
	O388	71	Endometrioid	Well	2	IIb	No	No	No	315	168	5.0	610	383
	O420	70	Serous/papillary	Moderately	3	IIIc	No	No	No	2,300	136	0.4	230	274
	O400	71	Serous/papillary	Well	nd	Relapse	Yes	Yes	Yes	172	120	0.3	70	220
	O413	84	Endometrioid	Poorly	3	Ia	No	No	No	273	74	0.5	47	113
	O385	63	Serous/papillary	nd	nd	Relapse	Yes	Yes	Yes	nd	127	0.7	76	309
	O387	81	Endometrioid	Well	1	Ia	No	No	No	622	137	0.2	26	104
	O419	63	Mucinous	Well	1	Ia	No	No	No	21	96	0.2	29	53
	O402	67	Serous/papillary	nd	3	IIIc	Yes	Yes	No	452	88	0.4	21	22
	O395	70	Serous/papillary	Well	3	IIIc	Yes	Yes	No	2,015	nd	nd	149	120
	O389	83	Endometrioid	Moderately	2	IIIc	Yes	Yes	No	4,741	332	0.3	18	1
	O374	76	Serous	Moderately	2	IIIc	Yes	Yes	Yes	60	240	0.4	10	21
										1,097 ± 1,512	152 ± 79	0.8 ± 1.5	117 ± 177	147 ± 130
Population 2														
	O407	55	Mucinous	Well	nd	IIIc	Yes	Yes	No	235	204	0.6	626	931
	O423	66	Serous/papillary	Well	2	IIIc	Yes	Yes	No	44	164	0.9	295	972
	O399	62	Serous/papillary	Well	I	Ic	No	No	No	21	163	2.4	949	911
	O416	72	Serous/papillary	Moderately	2	IIIc	Yes	Yes	No	277	100	1.6	1,175	636
	O418	73	Serous/papillary	Moderately	nd	IIIc	Yes	Yes	Yes	30	62	0.7	208	235
	O408	73	Serous/papillary	Moderately	3	IV	Yes	Yes	No	5,348	154	1.6	466	396
	O398	54	Clear cell	nd	2	IIIc	Yes	Yes	No	58	57	0.6	353	154
	O421	65	Serous/papillary	Poorly	3	Relapse	No	No	No	60	133	0.9	350	60
	O422	68	Endometrioid	Well	2	Ia	No	No	No	21	189	11.9	1,216	466
										677 ± 1,754	136 ± 53	2.4 ± 3.6	626 ± 390	529 ± 351
Population 3														
	O390	61	Serous/papillary	Moderately	2	Relapse	Yes	Yes	Yes	316	114	1.7	6,296	1,973
	O406	55	Endometrioid	Moderately	3	IB	No	No	No	210	128	3.0	5,715	2,358
	O403	59	Serous/papillary	nd	nd	IV	Yes	Yes	No	450	56	0.3	938	869
	O392	71	Serous/papillary	Poorly	nd	IIIc	No	Yes	No	753	128	1.5	14,309	4,081
	O394	64	Serous/papillary	Moderately	3	IIIc	Yes	Yes	No	536	158	4.6	34,709	55,119

Table 1. Clinical, pathological and biological characteristics of the ovarian cancer specimen (Continued)

	Patient code	Age	Adenocarcinoma subtype	Differentiation	Grade	Stage	Carcinomatosis	Ascites at time of surgery	Chemotherapy	CA-125 (ng/ml)	Number of PBMC at D0 ($\times 10^6$)[1]	Number of δ2T cells at D0 ($\times 10^6$)[1]	Number of δ2T cells at D14 BrHPP ($\times 10^6$)[1]	Number of δ2T cells at D14 Zol ($\times 10^6$)[1]
	O396	54	Serous/papillary	Moderately	2	IIIc	Yes	No	No	697	123	0.6	4,372	3,261
	O401	38	Endometrioid	Moderately	2	Ic	No	No	No	47	212	1.8	4,531	4,421
	O417	53	Serous/papillary	Well	2	IIIc	Yes	Yes	Yes	38	52	0.4	1,108	838
	O414	60	Serous/papillary	nd	3	IV	Yes	No	Yes	12	95	1.2	2,165	1,067
	O411	80	Serous/papillary	Moderately	3	IIIc	Yes	Yes	No	1,704	185	1.0	1,274	1,325
	O397	72	Serous/papillary	Moderately	2	IV	Yes	No	Yes	30	111	0.8	1,280	1,116
	O415	42	Serous/papillary	Poorly	3	IIIc	Yes	No	No	53	496	18.6	2,872	1,356
	O386	70	Serous/papillary	Well	nd	Relapse	Yes	Yes	Yes	1,025	180	0.9	4,042	575
	O404	72	Clear cell	nd	3	Ia	No	No	No	63	70	0.6	542	501
	O391	77	Endometrioid	Poorly	3	IIIc	Yes	Yes	No	3,112	240	4.2	464	1,967
	O393	70	Serous/papillary	Moderately	2	IIIc	Yes	Yes	No	607	88	2.7	2,007	1,459
	O409	72	Serous/papillary	nd	3	Relapse	No	No	Yes	30	nd	nd	1,716	868
										570 ± 798	152 ± 106	2.7 ± 4.4	5,129 ± 8,347	4,891 ± 1,2996

Patients are classified according to the multiple correspondence analysis.
[1]Results for 100 ml of peripheral blood sample.

Etablissement Français du Sang, Rennes, France) and patient with EOC ($n = 37$; Department of Gynecology and Obstetrics, Teaching Hospital, Rennes) blood samples. The PBMCs were resuspended at a concentration of 2×10^6 cells per milliliter in Roswell Park Memorial Institute (RPMI) 1640 (Eurobio, Les Ullis, France) supplemented with 10% fetal calf serum (Gibco Invitrogen Life Technologies, Cergy Pontoise, France), 1% L-glutamine, 50 μg/ml streptomycin and 50 IU/ml penicillin (which will be called the "RPMI complete medium"). The cells were treated once on Day 0 either with 3 μM of BrHPP (a gift from Innate Pharma, Marseille, France) or 1 μM of zoledronate (Zometa®; Novartis, France) and cultured in the RPMI complete medium with 400 IU of IL-2 per milliliter (Proleukin®; Chiron, Suresnes, France) for 2 weeks. Every 3 days, fresh complete medium with 400 IU IL-2 was added, and the cell concentration was adjusted to 0.5×10^6 cells per milliliter. The expansion index (EI) was defined as the number of recovered Vγ9Vδ2 T cells/number of Vγ9Vδ2 T cells seeded at Day 0. Functional activity assays were performed using cell suspensions in which the Vδ2-TCR-positive cells represented greater than 70% of the expanded cells.

EOC primary cell cultures and tumor cell line cultures

The Daudi lymphoid B-cell line and the Raji Burkitt's lymphoma cell line were obtained from the American Type Culture Collection. The EOC cell lines were established in our laboratory from three patients with EOC (O65, O151 and O170). Cells were used within 6 months after resuscitation of frozen aliquots. The EOC primary cell cultures were established from 12 patients (from primary solid tumor, carcinomatosis or ascites). The primary solid tumor or carcinomatosis was cut into small pieces (<1 mm^3) with a scalpel, and the pieces were subjected to enzymatic digestion with 0.23 Wünsch units per milliliter of collagenase (Liberase^{TM} research grade; Roche, Indianapolis, IN). The cultures were performed in the RPMI complete medium.

Cytotoxicity assays and blocking monoclonal antibodies

The expanded Vγ9Vδ2 T cells were tested for cytotoxicity against the EOC cell lines and autologous tumor cells from the primary tumor, carcinomatosis and ascites in a 4-hr standard ^{51}Cr release assay. PBMCs were used as the normal control target. Three thousand target cells labeled with ^{51}Cr sodium chromate (0.2 mCi/10^6 cells; Amersham Saclay, France) were cocultured in complete medium (RPMI) in 96-well U-bottomed plates for 4 hr with Vγ9Vδ2 T cells. The effector to target ratio (E:T) ranged from 1:1 to 60:1. The ^{51}Cr release was assessed in the culture supernatants, using a Top-count gamma counter (Packard Instrument, Gromingen, The Netherlands). The specific lysis (expressed as a percentage) was calculated using the standard formula: [(mean experimental cpm − mean spontaneous cpm)/(mean maximum cpm − mean spontaneous cpm)] × 100. The results are tabulated as the mean of the assays performed in triplicate. In the blocking assays, the effector cells were previously incubated with saturating concentrations of the following specific or isotype control monoclonal antibodies (mAbs) for 60 min: anti-pan-γδ-TCR (IMMU510, 40 μg/ml; Immunotech, Marseille, France), anti-NKG2D (140810, 50 μg/ml; R&D Systems, Lille, France) and anti-DNAM-1 (DX11, 50 μg/ml). In some experiments, the ^{51}Cr release assays were performed in the presence of recombinant human IL-6 (206-IL), IL-10 (1064-IL), VEGF (293-VE) and TGF-β (100-B; all from R&D Systems) and PGE2 (P5640; Sigma) or ascitic supernatants. The ascitic supernatants were obtained by centrifugation (400*g*, 10 min) and were used in equal volumes with the RPMI complete medium. This condition is termed ascites (50%). In some experiments, the tumor cell lines were cultured overnight in the RPMI complete medium and in the presence of zoledronate and were washed extensively before the ^{51}Cr release assay was performed. In PGE2 pathway blocking assays, the Vγ9Vδ2 T cells were incubated with 5 mM of the PKA Type I inhibitor Rp-8Br-cAMP and 50 μM of PGE2 receptor antagonist[30] AH6809 (Cayman Chemical, Ann Arbor, MI) for 1 hr in the RPMI complete medium. The cells were pelleted by gentle centrifugation (110 g for 1 min) before the ^{51}Cr release assay. The lytic units (LUs) were calculated using the formula described by Friberg *et al.*[31] The LUs were used to measure the lytic activity by the potency per lytic batch, where a lytic batch equaled 10^6 effector cells. A regression-line plot of the percentage of lysis/effector cells was used to estimate the number of effector cells required to lyse 20% of the target cells, and this quantity was defined as one lytic unit 20 (LU20). The results were expressed as the number of LU20 for 10^6 effector cells.

Flow cytometry analysis

The cells were incubated for 30 min at 4°C, according to the manufacturer's instructions, with conjugated mAbs against CD3 (UCHT1), CD16 (3G8), CD27 (1A4), CD45RA (alb11), CD45RO (UCHL1), CD112 (R2.477.1), pan-γδ-TCR (IMMU510), Vγ9-TCR (IMMU360) and Vδ2-TCR (IMMU389), all of which were purchased from Immunotech. They were also incubated with mAbs against DNAM-1 (DX11), which was purchased from BD Biosciences (Franklin Lakes, NJ) and with mAbs against CD155 (300907), MICA (159227), MICB (236511), NKG2D (149810), ULBP1 (170818), ULBP2 (165903) and ULBP3 (166510), all of which were purchased from R&D Systems. The isotype-matched murine fluorochrome-conjugated immunoglobulins from the corresponding manufacturer were used as negative controls. The Vγ9Vδ2 T-cell rate was determined within the lymphocyte gate. In the annexin V-affinity assays, the Vγ9Vδ2 T cells were incubated for 4 hr in ascites (50%). The cells were then incubated for 15 min at room temperature with fluorochrome-conjugated annexin-V (BD Biosciences) in the appropriate binding buffer. The immunofluorescence was analyzed on a FACSCalibur cytometer (Becton Dickinson, Mountain View, CA). For the CD107 mobilization assay, 14-day-old expanded Vγ9Vδ2 T cells were stimulated with 3

μM of BrHPP in the RPMI complete medium or in ascites (50%) in the presence of fluorochrome-conjugated anti-CD107 mAbs or the corresponding isotype control and were analyzed by flow cytometry.

Cytokine titration

The quantitative determination of the IL-10 (OptEIA™; Becton-Dickinson), IL-6, VEGF (duoset®; R&D Systems) and PGE2 (R&D Systems) cytokines in the ascitic supernatants was performed using ELISA kits according to the manufacturer's instructions.

Statistical analysis

The MCA[32,33] was performed to identify the eventual linear or nonlinear associations between the expansion of the Vγ9Vδ2 T cells and the clinical and biological characteristics of the patients (Fig. 1). This method establishes an underlying structure to the data, and the ascending hierarchical classification permits the partitioning of the patients into homogeneous groups. To obtain access to the algorithms for performing this analysis, we coded the continuous variables into their quartile values. The clinical criteria depicted in Table 1 were coded as discrete variables. The EI, Vγ9Vδ2 T-cell number and Vγ9Vδ2 T-cell rate at the end of the culture (14 days after the PAg stimulation) were used for partitioning the patients. The associations with the biological and clinical characteristics of the patients before the stimulation were then determined. The variables were compared between the different groups defined in the ascending hierarchical classification using a nonparametric test (Mann-Whitney) for the continuous variables and using the χ^2 test for the categorized variables. The MCA and the ascending hierarchical classification were performed using the Bi ©LOGINSERM 1979/1987 software (Institut National de la Santé et de la Recherche Médicale, Paris, France). The Kaplan-Meier method was used for the survival calculations, with an event being death, regardless of cause, or cancer recurrence. Tick marks are censored data and represent the date of tumor recurrence or the date of last news of patient. The comparison test chosen was the log-rank test with a threshold of $p < 0.05$. The data for Figures 2–5 are expressed as the mean ± standard deviation. The statistical analyses for Figures 2–5 were performed using the Student's *t*-test. A *p*-value of ≤ 0.05 was considered to be statistically significant.

Results

Frequency of Vγ9Vδ2 T cells in the peripheral blood and patients' age are useful criteria for selecting appropriate patients for adoptive immunotherapy

To address the feasibility of *ex vivo* Vγ9Vδ2 T-cell expansion for adoptive immunotherapy in patients with EOC, PBMCs from 37 patients were stimulated with a single BrHPP or zoledronate dose (Table 1). After 14 days of culture, the results showed high heterogeneity in the EI, the final Vγ9Vδ2 T-cell number and the Vγ9Vδ2 T-cell rate. These parameters were used in a MCA aimed at splitting the patients into homogeneous groups. The significant associations with the blood parameters and the clinical characteristics of the patients were then determined. The MCA resulted in classifying the patients into three distinct populations (Fig. 1*a*). Population 1 (pop1) displayed low EI associated with a low Vγ9Vδ2 T-cell count and frequency at the end of the culture. Thus, pop1 was clearly unsuitable for adoptive Vγ9Vδ2 T-cell therapy. Interestingly, the members of pop1 could be distinguished before the PAg stimulation on the basis of the initial Vγ9Vδ2 T-cell rate. Pop2 and pop3 had high Vγ9Vδ2 T-cell counts and frequencies at the end of the culture and both were suitable for adoptive therapy. The Vγ9Vδ2 T-cell count and frequency in pop3 were higher than those of pop2, yet the two populations were not distinguishable before the PAg stimulation by their Vγ9Vδ2 T-cell frequencies at Day 0. No difference was observed between the groups in the number of PBMCs, indicating that the partitioning of patients into the three groups was not related to leukopenia (Table 1). Among the clinical characteristics, advanced patient age was found to be an unfavorable parameter (Table 1). Thus, both the initial Vγ9Vδ2 T-cell rate and the patients' age may be associated to select appropriate patients for Vγ9Vδ2 T-cell adoptive immunotherapy.

The average follow-up duration for the entire population was 7 ± 5 months. The mean overall survival of the entire population was 7 ± 4 months (1–19 months), with a median of 6 months. Disease-free survival is shown in Figure 1*b*. The mean time of recurrence of the entire population was 6 ± 5 months (0–19 months), with a median of 5 months. The mean time of tumor recurrence was longer in pop3 and pop2 than in pop1, suggesting that higher Vγ9Vδ2 T-cell levels are associated with improved outcomes in ovarian cancer. Mean time of tumor recurrence was 8.55 ± 1.15 months for pop3, 6.07 ± 1.14 months for pop2 and only 0.80 ± 0.1 month for pop1, with $p = 0.03$ for pop1 *versus* pop3 and $p = 0.09$ for pop1 *versus* pop2.

Vγ9Vδ2 T cells from the patients with EOC display high cytotoxic activity against autologous ovarian tumor cells

The BrHPP- or zoledronate-expanded Vγ9Vδ2 T cells from the patients with EOC displayed an $CD45RA^{-}CD27^{-}CD45RO^{+}$ effector memory phenotype (Supporting Information Figure). They strongly expressed activating NKR associated with a cytotoxic function, such as NKG2D and CD226 (also called DNAM-1), whereas their CD16 expression was moderate. The cytotoxic assays showed that the Vγ9Vδ2 T cells from the patients with EOC lysed the allogeneic ovarian cell lines as reported with representative data from the O65 cell line (Fig. 2*a*). The ovarian cell lines expressed the CD112 and CD155 DNAM-1 ligands. ULBP2, but not MICA/B or ULBP1/3, was expressed among the NKG2D ligands (Supporting Information Figure and data not shown). The blocking assays performed with anti-pan-γδ-TCR, CD226 or NKG2D mAbs

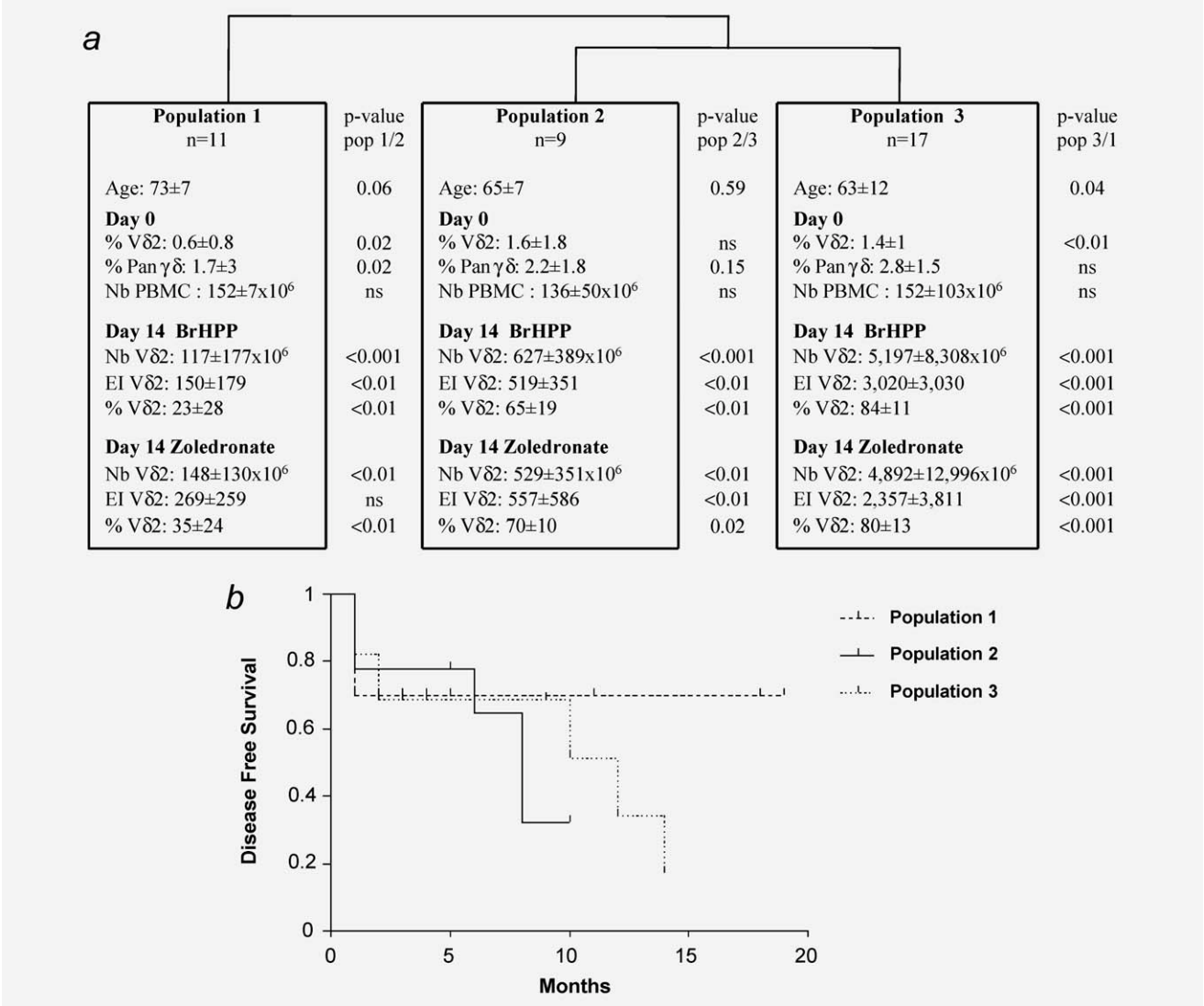
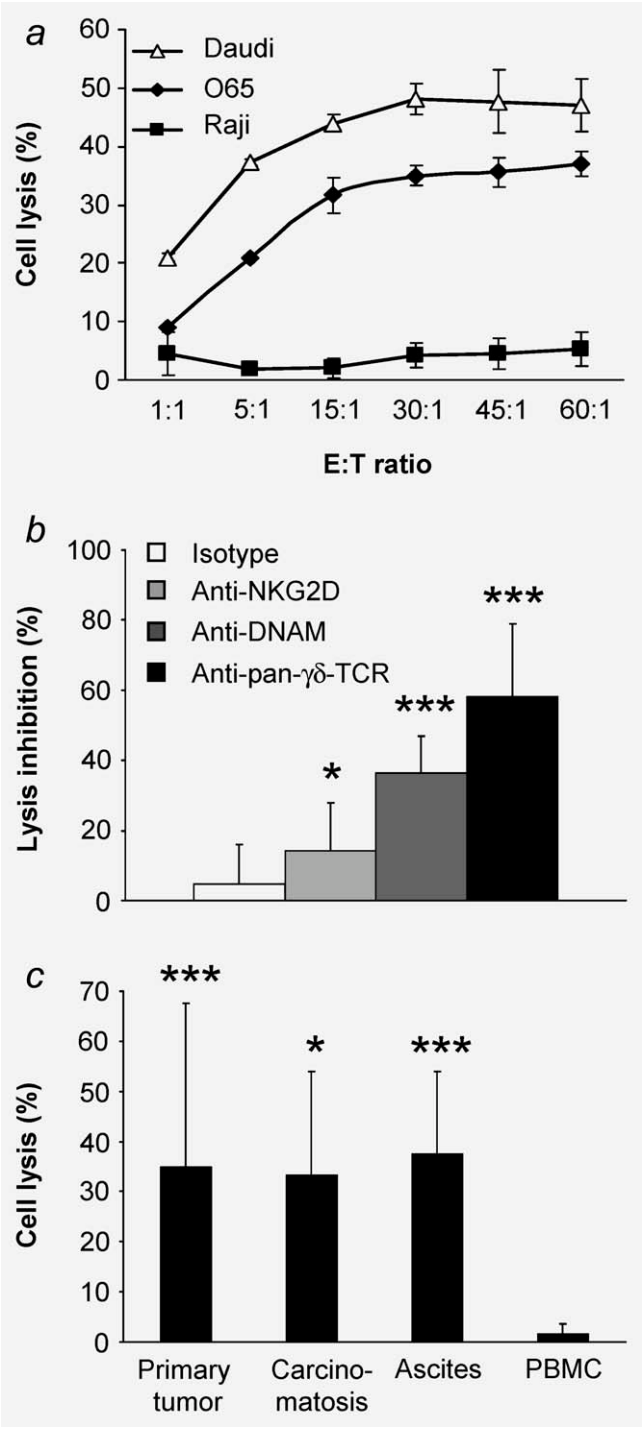


Figure 1. (*a*) Dendrogram of the ascending hierarchical classification of the patients with EOC. PBMCs from patients with EOC ($n = 37$) were stimulated with a single dose of BrHPP or zoledronate. Multiple correspondence analysis (MCA) was performed using the expansion index (EI), the Vγ9Vδ2 T-cell number ($\times 10^6$ and normalized to a 100-ml blood sample) and the Vγ9Vδ2 T-cell frequency at the end of the culture (*i.e.*, 14 days after the PAg or zoledronate stimulation). The MCA classified the patients into three groups (populations 1–3). The populations were characterized by their clinical data (see Table 1), age, pan-γδ-TCR and Vγ9Vδ2 T-cell rates at Day 0 as well as by their EIs and Vγ9Vδ2 T-cell numbers ($\times 10^6$) and rate at the end of the culture with BrHPP (Day-14 BrHPP) and zoledronate (Day-14 zoledronate) stimulation. The data shown are the mean ± SD. Continuous and categorized variables were compared to Mann-Whitney and χ^2 tests, respectively. The *p*-values are indicated on the dendrogram. ns = not significant. (*b*) Disease-free survival of the patients with EOC. Data are the disease-free survival in months after the end of the treatment (chemotherapy or surgery) according to the three populations defined by the multiple correspondence analysis. Tick marks are censored data and represent the date of tumor recurrence or the date of last news of patient. Follow-up was 19 months. Number of recurrence and lost to follow-up are 3 and 7 in pop1 (11 patients), 5 and 4 in pop2 (nine patients) and 8 and 9 in pop3 (17 patients), respectively. Higher rate of lost to follow-up in pop1 is likely to contribute to the lack of progressive decrease of the disease-free survival curve.

revealed that the lysis of the ovarian cell lines was mainly mediated by TCR and DNAM-1 recognition (Fig. 2*b*). Combination of anti-pan-γδ-TCR with anti-DNAM-1 or NKG2D mAbs did not show additive effect (data not shown). Interestingly, the Vγ9Vδ2 T cells from the patients with EOC ($n = 12$) strongly lysed the autologous ovarian cells either from the primary tumors or carcinomatosis or ascites (Fig. 2*c*). The autologous PBMCs used as the normal cell controls were not lysed by the Vγ9Vδ2 T cells. Lysis levels were similar in either autologous or allogeneic context (data not shown).

Ascitic supernatant impairs the cytotoxic activity of Vγ9Vδ2 T cells

To test whether the presence of ascites in the peritoneal cavity negatively influences the lytic properties of the Vγ9Vδ2 T cells, cytotoxic assays were performed in the presence of the ascitic supernatant. Our results showed that the ascitic supernatant dramatically decreased the lysis of the ovarian allogeneic cell lines (Figs. 3*a* and 3*b*). One assay performed in an autologous context using tumor cells, Vγ9Vδ2 T cells and ascites from the same patients led to a similar result (data not shown). A lower percentage of ascitic supernatant (1 or 10%) resulted in only a weak effect on the lysis levels, suggesting a dose dependency (Fig. 3*c*). Similar results were obtained with the Daudi cells, ovarian cell lines (O65 and O170) and EOC primary cell cultures (Fig. 3*e*). The ascitic supernatant did not affect the T-lymphocyte viability. There were 13% ± 8% annexin-V-positive cells after 4 hr-incubation in ascites (50%) *versus* 14% ± 7% after incubation in the RPMI complete medium. Consistent with the impaired cytotoxic activity, the Vγ9Vδ2 T cells displayed reduced CD107 expression in the presence of the ascitic supernatant, indicating a reduced perforin and granzyme degranulation ability (Fig. 3*d*).



PGE2 is a primary contributor to the immunosuppressive effects of ascites in EOC

Ovarian cancer-associated ascites are known to contain immunosuppressive cytokines.[26,27] A quantitative determination in 18 ascitic supernatants showed high levels of VEGF, IL-10 and IL-6 (Fig. 4*a*). The concentrations of VEGF, IL-10 and IL-6 were 1.9 ± 1.2 ng/ml (0.7–4.5 ng/ml), 450 ± 190 pg/ml (210–835 pg/ml) and 8.0 ± 8.4 ng/ml (0.6–28.5 ng/ml), respectively. Moreover, to our knowledge, this is the first report demonstrating the presence in ovarian cancer-associated ascites of PGE2, which has recently been proposed as a strong inhibitor of Vγ9Vδ2 T-cell cytotoxicity, in ascitic supernatant.[28] The concentration of PGE2 ranged from 0.03 to 35 ng/ml, and the mean concentration was 10.2 ± 15.5 ng/ml (Fig. 4*a*).

Figure 2. Vγ9Vδ2 T cells from the patients with EOC efficiently lyse the ovarian tumor cells. The PBMCs were treated at Day 0 with 3 μM of BrHPP and were cultured in a complete medium in the presence of 400 IU of IL-2 per milliliter for 2 weeks. The expanded Vγ9Vδ2 T cells were cocultured for 4 hr with 3×10^3 ovarian tumor cells that had been previously labeled with ^{51}Cr. (*a*) Vγ9Vδ2 T cells from healthy donors were cocultured with the O65 ovarian carcinoma cell line and the Daudi and Raji cell lines at the indicated effector to target (E:T) ratios. The data are the mean ± SD of the cell lysis ($n = 3$). (*b*) Vγ9Vδ2 T cells from healthy donors were incubated with anti-pan-γδ-TCR, NKG2D or DNAM mAbs prior to coculture with the target ovarian cell lines (O65, O151 and O170). The effector to target ratio (E:T) was 30:1. The data are the mean ± SD of cell lysis inhibition compared to the lysis in the absence of mAbs ($n = 8$). Statistically significant compared to the isotype control mAbs at $^*p < 0.05$ and $^{***}p < 0.001$. (*c*) The effector cells were the Vγ9Vδ2 T cells from the 12 patients with EOC. The target cells were autologous ovarian tumor cells from primary tumors ($n = 6$), carcinomatosis ($n = 3$) or ascites ($n = 9$). The effector to target ratio (E:T) was 30:1. Autologous PBMCs were used as normal control cells ($n = 7$). Statistically significant compared to the PBMCs at $^*p < 0.05$ and $^{***}p < 0.001$.

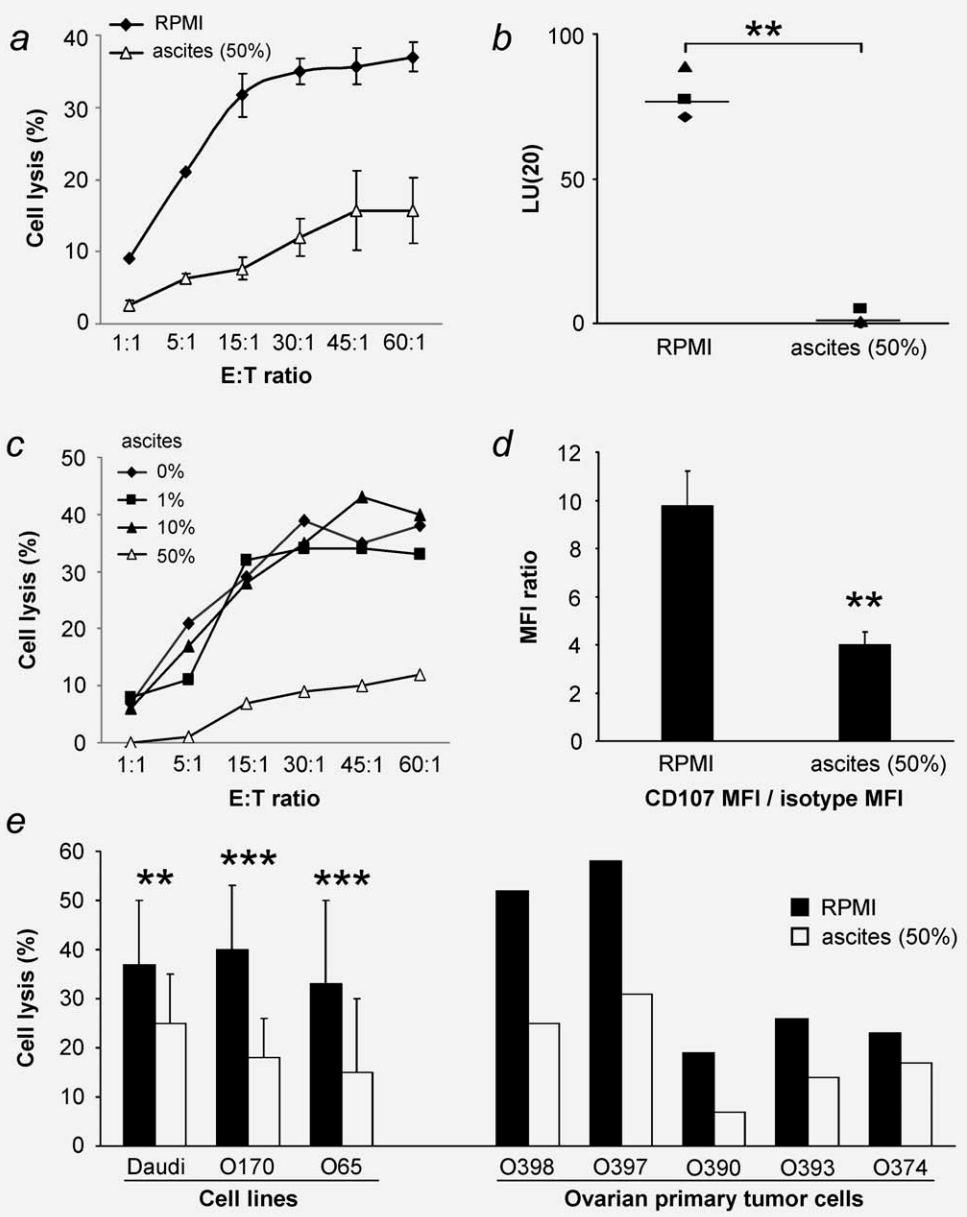


Figure 3. Ascitic supernatant impairs the cytotoxic activity of the Vγ9Vδ2 T cells. The PBMCs were treated on Day 0 with 3 μM of BrHPP and were cultured in a complete medium in the presence of 400 IU of IL-2 per milliliter for 2 weeks. The expanded Vγ9Vδ2 T cells were cocultured for 4 hr with 3×10^3 ovarian tumor cells previously labeled with ^{51}Cr. Cytotoxic assays were performed in the complete medium (RPMI) or in the complete medium and ascitic supernatant in an equal volume ratio (ascites 50%). (*a* and *b*) Vγ9Vδ2 T cells from healthy donors were cocultured with the O65 ovarian cell line in the presence of ascitic supernatants. The data are the mean ± SD of the cell lysis at the indicated effector to target ratio (E:T; $n = 3$; *a*). The data are expressed as lytic units 20 (LU20)/10^6 effector cells ($n = 3$). The median is depicted on the histogram. Statistically significant at $^{**}p < 0.01$ (*b*). (*c*) Vγ9Vδ2 T cells from one healthy donor were cocultured with the O65 ovarian cell line in the complete medium and the ascitic supernatant at different E:T ratios. (*d*) After 14 days of culture, the expanded Vγ9Vδ2 T cells were stimulated by 3 μM of BrHPP in the complete medium or in the ascites (50%) and stained with anti-CD107 for 4 hr. The data are the ratio between the mean fluorescence intensity (MFI) of the CD107 and isotype control mAb ($n = 4$). Statistically significant compared to the control RPMI complete medium alone at $^{**}p < 0.01$. (*e*) The target cells were Daudi cells ($n = 19$), ovarian cell lines ($n = 30$ for O65; $n = 17$ for O170) and five EOC primary cell cultures ($n = 1$). The effector to target ratio (E:T) was 30:1. Statistically significant compared to the corresponding RPMI control condition at $^{**}p < 0.01$ and $^{***}p < 0.001$.

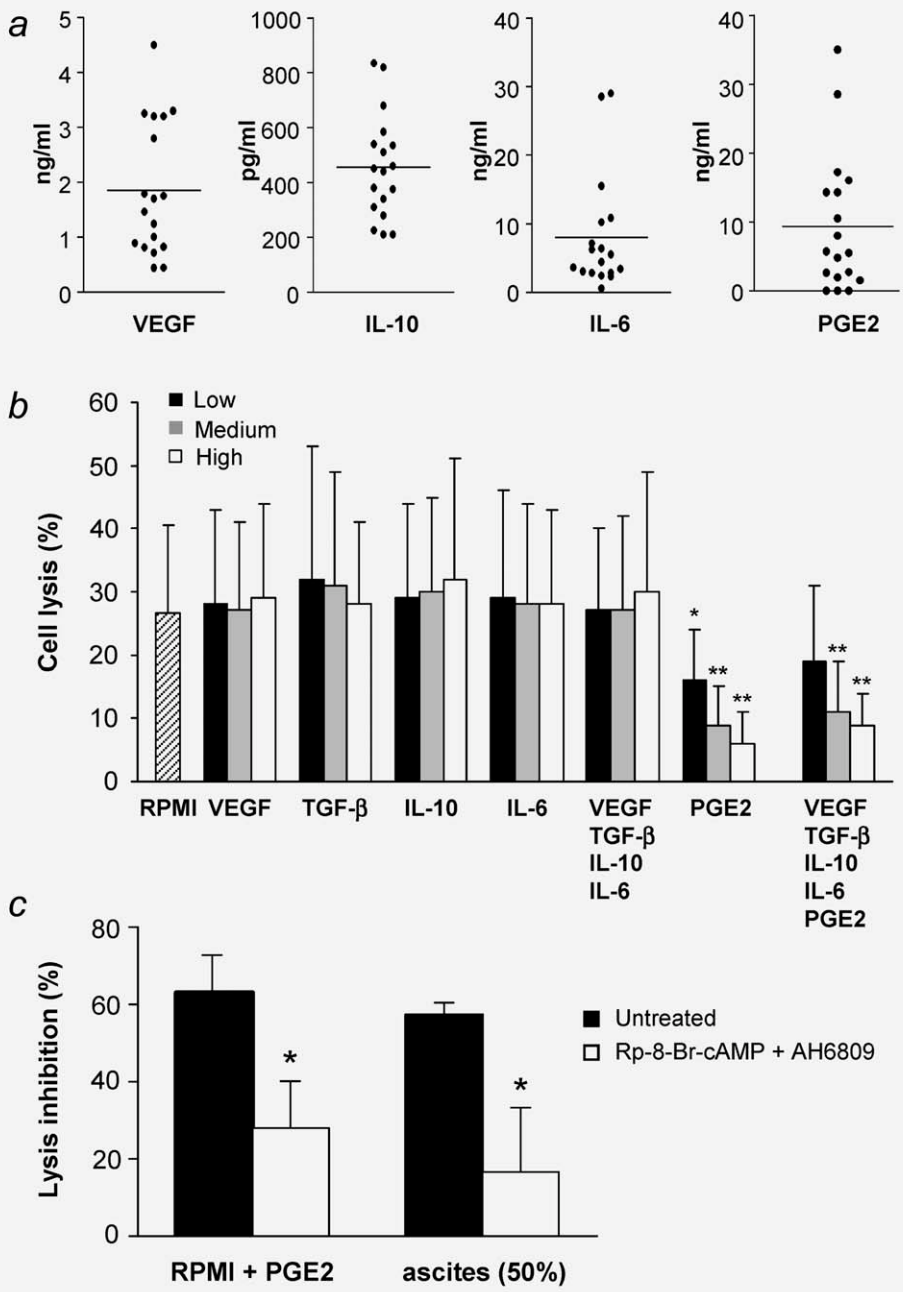


Figure 4. PGE2 in ovarian cancer-associated ascites is a strong inhibitor of the Vγ9Vδ2 T-cell lytic properties. (*a*) Scatter plot of the distribution of cytokine concentration in the ascitic supernatants. VEGF, IL-10, IL-6 and PGE2 were assessed in the ascitic supernatants of 18 patients by ELISA. Data are expressed in nanograms per milliliter for VEGF, IL-6, PGE2 and in pictograms per milliliter for IL-10. Bars represent the mean. (*b* and *c*) Influence of immunosuppressive cytokines and PGE2 on Vγ9Vδ2 T-cell cytotoxicity. The PBMCs were treated on Day 0 with 3 μM of BrHPP and were cultured in the complete medium in the presence of 400 IU of IL-2 per milliliter for 2 weeks. The expanded Vγ9Vδ2 T cells were cocultured for 4 hr with 3×10^3 ovarian tumor cells previously labeled with ^{51}Cr. (*b*) Cytotoxic assays were performed in the presence of IL-6, IL-10, VEGF, TGF-β and/or PGE2 (alone or in combination). Each molecule was used at three different concentrations according to the ascites cytokine levels reported in the literature. Dose-response curves (with low, medium and high concentrations) were calculated with 10, 100 and 1000 ng/ml for PGE2; 1, 10 and 100 ng/ml for IL-6 and VEGF; 0.1, 1 and 10 ng/ml for IL-10 and 0.01, 0.1 and 1 ng/ml for TGF-β. Effector cells were Vγ9Vδ2 T cells from one donor, and the target cells were three ovarian cell lines (O65, O151 and O170; $n = 3$). The effector to target ratio (E:T) was 30:1. Statistically significant compared to the RPMI control condition at $^*p < 0.05$ and $^{**}p < 0.01$. (*c*) Vγ9Vδ2 T cells were pretreated with Rp-8-Br-cAMP (5 mM) and AH6809 (50 μM) for 1 hr in complete RPMI medium. Cells were washed before the ^{51}Cr release assay was performed in the complete medium (RPMI) or in the complete medium and ascitic supernatant ($n = 3$) in an equal volume ratio (ascites 50%). The target cells were the O65 ovarian cell line. The effector to target ratio (E:T) was 30:1. Statistically significant at $^*p < 0.05$.

Interestingly, PGE2 alone, but not VEGF, TGF-β, IL-10 or IL-6, reduced the lytic properties of the Vγ9Vδ2 T cells against ovarian cell lines in a dose-dependent manner (Fig. 4*b*). Furthermore, the inhibition of the cell lysis in the presence of ascitic supernatants was reduced by adding PGE2 pathway inhibitors (Fig. 4*c*).

Zoledronate counteracts the immunosuppressive effects of the ascitic supernatant

Zoledronate is known to sensitize tumor cells to Vγ9Vδ2 T cell cytotoxicity by increasing intracellular IPP level, resulting in direct stimulation of Vγ9Vδ2 T cells through the TCR.[12,34] Accordingly, pretreatment of the tumor cells by low-dose zoledronate enhanced the lysis of the ovarian cell lines by the Vγ9Vδ2 T cells (Figs. 5*a* and 5*b*). This result was not correlated with an increase in the expression of NKG2D ligands (MICA/B and ULBP1/2/3) or DNAM-1 ligands (CD112 and CD155; data not shown). The immunosuppressive effect of PGE2 was reversed by pretreating the tumor cells by zoledronate (Figs. 5*a* and 5*b*). Regardless of the zoledronate concentration, the lysis levels are not significantly different in the presence or absence of PGE2. Interestingly, the zoledronate pretreatment also partially counteracted the influence of the ascites. Zoledronate restored the baseline cytotoxic level to that obtained under control conditions without ascites. However, the increase in the dose of zoledronate did not increase the percentage of lysis beyond the control levels (Fig. 5*c*).

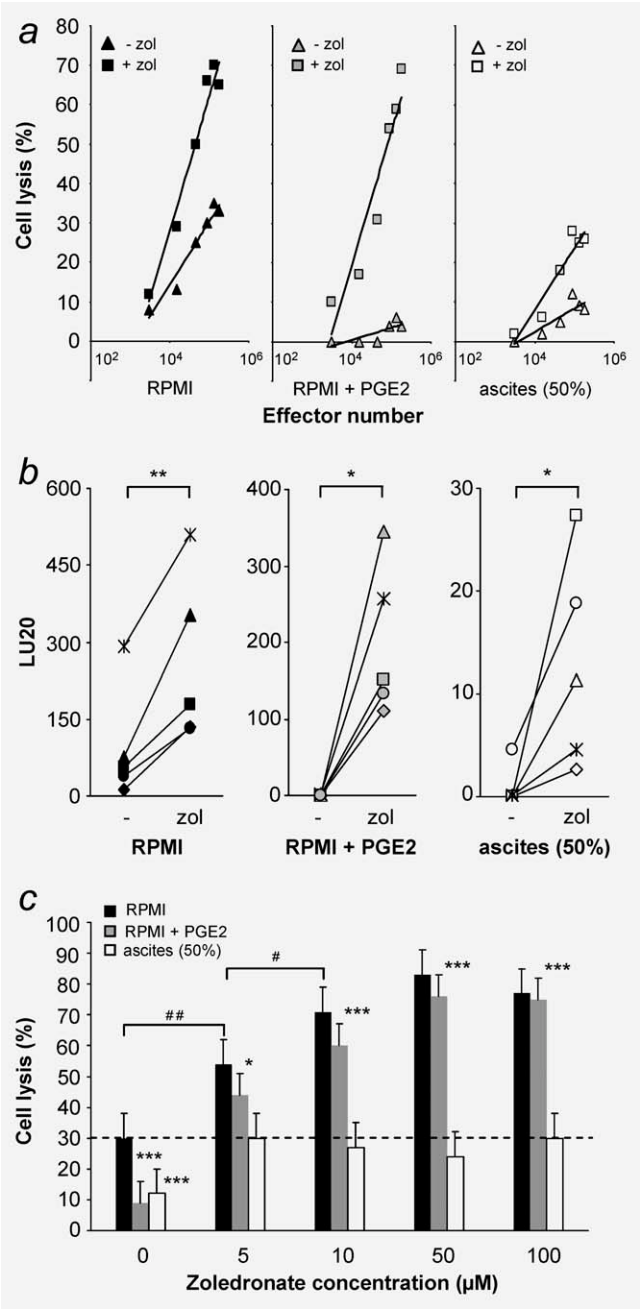


Figure 5. Zoledronate counteracts the immunosuppressive effects of PGE2 and the ascitic supernatant. The PBMCs were treated on Day 0 with 3 μM of BrHPP and were cultured in the complete medium in the presence of 400 IU of IL-2 per milliliter for 2 weeks. The expanded Vγ9Vδ2 T cells were cocultured for 4 hr with 3×10^3 ovarian tumor cells that had been previously labeled with ^{51}Cr. (*a* and *b*) Cytotoxic assays were performed with the Vγ9Vδ2 T cells from healthy donors in the presence of the ascitic supernatant or 1 μg/ml of PGE2. The target cells (O65 ovarian cell line) were pretreated or not overnight with 10 μM zoledronate. The data are reported as the cell lysis (%; $n = 1$; *a*) or as lytic units 20 (LU20)/10^6 effector cells ($n = 5$; *b*). (*c*) Cytotoxic assays were performed with the Vγ9Vδ2 T cells in the presence of the ascitic supernatant or 1 μg/ml of PGE2. The target cells (O65 ovarian cell line) were pretreated or not overnight with zoledronate at the indicated concentration ($n = 3$). The effector to target ratio (E:T) was 30:1. Statistically significant compared to the level of cell lysis under the RPMI control condition in the absence of zoledronate pretreatment at $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Statistically significant at $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

Discussion

Previous studies have highlighted the strong cytotoxic properties of Vγ9Vδ2 T cells against various tumor cells *in vitro*.[15–19] Thus, adoptive Vγ9Vδ2 T-cell therapy has become a promising adjuvant cancer treatment to chemotherapy and surgery. Here, for the first time, we show that *ex vivo* Vγ9Vδ2 T-cell expansion can be accomplished for the majority of patients with EOC and reach suitable cell numbers for adoptive therapy ($\geq 10^9$ cells) from a 100-ml peripheral blood sample.[8,15,24,35] However, as previously observed for other cancers,[17,25] the *ex vivo* expansion protocol using BrHPP or zoledronate stimulation was unsuccessful for one-third of the patients with EOC. This failure calls into question the future use of the protocol in clinical trials. Thus, identifying the predictive factors for failing the Vγ9Vδ2 T-cell expansion is needed. MCA performed with data from 37 patients revealed three distinct patient populations.

Interestingly, the patients in pop1 (11/37), which was characterized by poor Vγ9Vδ2 T-cell expansion, could be identified by their peripheral blood Vγ9Vδ2 T-cell percentage and age. The statistical analysis did not reveal significant differences between the three groups in the number of PBMCs, which indicated that the low frequency of Vγ9Vδ2 T cells in pop1 was not related to leukopenia. Thus, this study suggests that taking into consideration the frequency of Vγ9Vδ2 T cells in the peripheral blood along with the patients' age may be useful for enrolling appropriate patients in clinical trials. It could be an interesting alternative strategy to the prescreening test by small-scale *ex vivo* expansion of Vγ9Vδ2 T cells prior to initiating therapy.

The Vγ9Vδ2 T cells from the patients with EOC displayed high cytotoxic activity *in vitro* against the EOC autologous primary culture and the EOC cell lines. The TCR was of primary importance for the killing of ovarian tumor cells, and the activating NKR DNAM-1 also contributed to the Vγ9Vδ2 T-cell-mediated cytotoxicity. In contrast to the results of previous studies, the contribution of the NKG2D receptor to EOC cell lysis was low.[16,36] Of note, this result is consistent with the weak expression of the NKG2D ligand (MICA/B and ULBP1/2/3) in ovarian cancer cells.

In the setting of a therapeutic intraperitoneal infusion, the cytotoxic activity of the Vγ9Vδ2 T cells may be impaired in an ovarian cancer-associated ascitic environment. Previous studies have revealed the presence of increased concentrations (compared to their serum levels) of inhibitory cytokines, such as IL-6, IL-10, TGF-β and VEGF, in ovarian cancer-associated ascites.[27] Moreover, we found that PGE2 was present in the ascites of our cohort of patients. The high levels of this well-known immunosuppressive factor might result from the overexpression of the COX-1 cyclooxygenase pathway.[37–40] Accordingly, the cytotoxic assays performed with the ascitic supernatant (medium/ascitic supernatant, 50%) showed a dramatic decrease in the Vγ9Vδ2 T-cell cytotoxicity against the ovarian tumor cells. This decrease in the lytic properties was noted for all of the investigated ascites ($n = 13$) and was associated with a reduced capacity for perforin/granzyme degranulation, which is the main mechanism of cell lysis by Vγ9Vδ2 T cells. Toxicity of the ascitic supernatant against the Vγ9Vδ2 T cells was ruled out because the annexin-V assays attested to the equivalent T-cell viabilities. Interestingly, among the immunosuppressive factors evaluated in the cytotoxic assays, PGE2, but not IL-6, IL-10, VEGF or TGF-β, led to decreased levels of tumor cells lysis. Although other immunosuppressive factors may play a role, this result clearly suggests that PGE2 is a primary contributor to the immunosuppressive effect of ovarian cancer-associated ascites. The PGE2 pathway blocking experiments confirmed the important role of PGE2 in this inhibition. Our results question the additive strategies to counteract the immunosuppressive effects of ascites and to restore satisfactory lysis levels in the ovarian cancer cells. We demonstrated that pretreatment of the ovarian tumor cells with zoledronate reverses the immunosuppressive effects of PGE2. The lytic levels were similar in the presence or absence of PGE2 for all of the zoledronate concentrations studied in the dose-curve assay. Interestingly, the zoledronate pretreatment also partially counteracted the immunosuppressive effect of the ascitic supernatant. In contrast to PGE2, dose escalation with zoledronate (5–100 μM) was not associated with an increase in the lytic level, and the maximal effect of zoledronate was obtained between 5 and 10 μM. This result suggests that the ascitic supernatant contains additional inhibitory factors that are not counteracted by the pretreatment with zoledronate. Zoledronate has recently been suggested to possess direct antitumor activity[41,42] and the ability to trigger apoptosis by inhibiting the prenylation of the small G proteins involved in cell signaling or through an apoptosis-intrinsic pathway via activation by AppI, an AMP-conjugated form of IPP.[43–45] In our experiments, 12 hr of zoledronate pretreatment did not induce the direct killing of the tumor cells. This result was supported by the annexin-V assays, which indicated a mechanism other than zoledronate-induced apoptosis. The cell lysis sensitization that results from inhibiting farnesyl pyrophosphate synthase in the mevalonate pathway and the subsequent IPP accumulation in the tumor cells is a more likely mechanism for zoledronate's action.[10,12,25] However, further investigation is needed to unravel the effects of zoledronate on the various ascites components. In addition, recent publications lend support to using *in vivo* zoledronate infusion to decrease the immunosuppressive influence of the cancer microenvironment. Indeed, zoledronate has been shown to reduce ascites formation in a mouse xenograft model of human ovarian carcinoma.[46] Moreover, zoledronate displays antiangiogenic activity and can decrease the secretion of immunosuppressive (TGF-β, FGF-β and metalloproteinases) and proinflammatory (IL-1β, IL-6 and IL-8) factors.[42,47–50]

In a previous clinical trial designed to evaluate phosphoantigen administration in B-cell malignancies, a correlation was observed between the *in vivo* expansion of Vγ9Vδ2 T cells and the objective antitumor response.[18] An adoptive transfer immunotherapy assay has also been conducted in metastatic renal cell carcinoma by Kobayashi *et al.*[21] and in lung carcinoma by Nakajima *et al.*[51] These studies found that autologous Vγ9Vδ2 T cells are well tolerated and induce an antitumor effect. Our current results highlight the notion that the frequency of Vγ9Vδ2 T cells in the peripheral blood and the age of the patients may be useful predictive markers with which to select patients for inclusion in future clinical trials. They also support the presensitization of tumor cell targets by zoledronate *in vivo* to enhance the Vγ9Vδ2 T-cell lytic activity. An intraperitoneal approach should have an important advantage in adoptive immunotherapy because it provides the opportunity to deliver a high concentration of zoledronate *in situ* during the presensitization phase, which can be followed by a Vγ9Vδ2 T lymphocyte infusion in close proximity to the tumor cells. Taken together, our results provide a rationale for clinically evaluating intraperitoneal injections of Vγ9Vδ2 T lymphocytes in ovarian carcinoma cases.

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