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ORIGINAL ARTICLE

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Maturation of dendritic cells from ovarian cancer patients

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Abstract *Purpose*: Dendritic cells (DC) are the most potent antigen-presenting cells of the immune system. We have shown that DC, defined as $LN^{-}DR^{+}$ leukocytes from the ascites of patients with ovarian or peritoneal carcinoma, have the cell surface characteristics of immature cells. Moreover, p70 interleukin-12 has not been detected in the ascites of ovarian cancer patients in vivo. In the current study, we examined the effects of in vitro treatment of DC from peripheral blood and ascites samples of patients with ovarian cancer with either cytokines or proteolytic enzymes (polyenzyme preparation). *Methods*: Mononuclear leukocytes from the ascites of 15 patients or peripheral blood from 9 patients with epithelial ovarian cancer were cultured with tissue culture medium containing either papain, trypsin and chymotrypsin for 5–7 days or recombinant human granulocyte-macrophage colony-stimulating factor, tumor necrosis factor α and interleukin-4 (complete medium) or tissue culture medium alone. *Results*: Phenotypic analysis of DC obtained on days 5–7 of the culture showed higher proportions of $CD83^{+}$, $CD40^{+}$ and $CD80^{+}$ cells when cultured with cytokines or enzymes as compared with DC cultured with medium alone. Stimulation of allogeneic T cells was detected by the mixed leukocyte reaction (MLR) and higher concentrations of IL-12 were detected after growing these cells in the presence of cytokines or enzymes as compared to tissue culture medium alone. *Conclusion*: Our studies demonstrate for the first time that DC obtained from the peritoneal cavity and peripheral blood of ovarian cancer patients after culturing in the presence of a polyenzyme preparation, will undergo maturation. Further studies are warranted to determine whether polyenzyme preparations facilitate DC maturation in vivo.

Keywords Dendritic cells · Ovarian cancer · Polyenzyme preparation

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Introduction

Epithelial ovarian carcinoma (EOC) is the most common cause of death among all gynecologic malignancies in the United States and has a 5-year survival rate of 25% [2]. The addition of paclitaxel to platinum has improved progression-free survival for patients with EOC. Recent developments in bioimmunotherapy approaches, including recombinant cytokines, costimulation, and tumor vaccination, have increased the understanding of such agents in the treatment of ovarian cancer [6].

Dendritic cells (DC) are one of the most important antigen-presenting cells because they have the capability of presenting antigen-derived peptides in the context of the major histocompatibility complex (MHC) and express costimulatory antigens that provide the second signal necessary for T-cell activation [12]. In contrast, tumor cells are not efficient at antigen presentation since they lack functional costimulatory antigens and cell-surface MHC expression is often downregulated.

We have previously identified a mononuclear leukocyte (MNL) population in the blood and peritoneal cavity of ovarian carcinoma patients that have the cell-surface characteristics of immature DC [11]. These cells are $LN^{-}DR^{+}$ leukocytes and have low CD11c expression and lower maturation marker expression in comparison to MNL of healthy donors. The objectives of the

present study were to determine whether a polyenzyme (PE) preparation including chymotrypsin, papain and trypsin, which have previously shown immunostimulatory activity, may also facilitate the maturation of DC obtained from ovarian cancer patients. This PE preparation has previously been shown to alter the expression of cell surface adhesion molecules [9], to stimulate cytotoxic activity of granulocytes [18], and to reduce transforming growth factor beta (TGFβ) production in tumor-associated macrophages from ovarian cancer patients [19]. The PE preparation has been shown in an animal experimental model also to inhibit tumor growth [16]. The oral intake of PE in patients with multiple myeloma is associated with a decreased concentration of soluble tumor necrosis factor alpha (TNFα) receptors [5], a suggestion of prolonged patient survival and has been shown to have an acceptable toxicity profile in phase I studies [13].

In the present study we examined the in vitro effects of treating DC in peripheral blood samples and in ascites obtained from ovarian cancer patients using cytokines or proteolytic enzymes (PE preparation) on leukocyte surface differentiation markers and on DC function (MLR and IL-12 production). Previous studies by Gabrilovich et al. [7] have shown that cytokines can activate tumor-suppressed DC in vitro. This is the first study showing that PE enhances DC1 type activity.

Material and methods

Patients and normal donors

Peritoneal fluids samples from 15 EOC patients (all originally stage III), including peritoneal washings from 4 patients, were obtained during diagnostic or therapeutic paracentesis. Peripheral blood specimens were obtained from 9 of 15 patients and from 3 normal female donors of similar age. Peritoneal washings were obtained during abdominal surgery from EOC patients who did not produce ascites. Washings were obtained immediately upon entry into the abdomen to avoid blood contamination. The mean age of the patients was 55 years. Clinical and pathological characteristics are shown in Table 1. Of the 15 patients, 7 were chemotherapy naive and 8 had been off treatment for at least 3 months at the time of the testing.

Specimen preparation

Fluids obtained from peritoneal ascites or washings were collected in sterile bottles into which heparin (10 U/ml) was added. In addition, peripheral blood was collected into heparinized Vacutainer tubes. Cells were sedimented from the ascites by centrifugation at 800 *g* for 10 min. The cell-free supernatant of ascites was stored at –70°C for further investigation. The cell pellet was resuspended in Hanks' balanced salt solution (Gibco, Grand Island, N.Y.) at a concentration of $1–5\times10^6$ cells/ml. A 20-ml aliquot of the cell suspension was layered onto a 10 ml Ficoll-Hypaque gradient (specific gravity 1.077 g/cm^3) in a 50-ml conical centrifuge tube (Falcon, Becton Dickinson, Franklin Lakes, N.J.) and centrifuged at 650 *g* for 30 min at room temperature to remove contaminating granulocytes and red blood cells. The interface, containing a mixture of MNL and tumor cells, was carefully collected and washed twice with Hanks' balanced salt solution. The total number of cells and percent viability were determined by trypan blue dye exclusion. MNL were then separated from tumor cells by discontinuous density- gradient centrifugation by layering the cells ($1–5\times10^6$ cells/ml in Hanks' balanced salt solution) onto discontinuous gradients consisting of 3 ml of 100% and 3 ml of 75% Ficoll Hypaque (diluted with RPMI medium) and centrifuging at 400 *g* for 20 min at room temperature. MNL were harvested from the 75%/100% interface.

The peripheral blood specimens were diluted with one part Hanks' balanced salt solution, layered over Ficoll Hypaque gradient and centrifuged at 800 *g* for 30 min. After collection, the MNL were washed twice with Hanks' balanced salt solution and resuspended in either phosphate-buffered saline (PBS) (for flow cytometry) or RPMI-1640 medium (for culturing) containing 20 m*M* HEPES, 2 m*M* glutamine and 10% heat-inactivated fetal bovine serum designated as the medium. Cell viability was determined by trypan blue dye exclusion.

Generation of DC

MNL at 1×10^6 cells/ml were plated into six-well flat-bottomed culture plates (Falcon) and incubated in complete medium alone, in medium containing 100 ng/ml of recombinant human granulocyte-

Table 1 Clinical and pathological characteristics of patients (*NS* not staged, *PD* poorly differentiated, *WD* well differentiated)

Patient no.	Age (years)	Pathology	Disease stage	Grade	Previous treatment
1	70	Undifferentiated carcinoma	IIIc	PD	None
2	49	Papillary serous carcinoma	IIIc	PD	Paclitaxel, carboplatin
3	47	Papillary serous carcinoma	IIIc	WD	IFNγ + IL-2, carboplatin, paclitaxel, IL-12
4	62	Papillary serous carcinoma	III	PD	Cisplatin + paclitaxel
5	65	Papillary serous adenocarcinoma	III	PD	None
6	67	Serous carcinoma	III	PD	None
7	59	Metastatic adenocarcinoma	III	PD	None
8	53	Mixed carcinoma	IIIc	PD	Paclitaxel, cisplatin, carboplatin, topotecan, tamoxifen
9	53	Papillary serous adenocarcinoma	III	PD	Carboplatin, gemcitabine, navelbine, VP16, tamoxifen
10	50	Papillary serous adenocarcinoma	III	PD	None
11	22	Mixed germ tumor of the ovary	IIIc	WD	None
12	37	Papillary serous adenocarcinoma	IIIc	PD	Paclitaxel + carboplatin
13	55	Undifferentiated adenocarcinoma	III	PD	Vitamin A, paclitaxel + carboplatin
14	42	Low-grade serous carcinoma	III	WD	None
15	58	Serous carcinoma of the ovary	III	PD	Carboplatin + paclitaxel

macrophage colony-stimulating factor (rhGM-CSF; Genzyme Diagnostics, Cambridge, Mass.), 10 ng/ml TNFα (Genzyme Diagnostics) and 50 ng/ml IL-4 (Genzyme Diagnostics) [3], or in medium containing a mixture (10 μg/ml) of the enzymes papain, trypsin and chymotrypsin (WOBE-MUGOS; Mucos Pharma, Geretsried, Germany) for 5–7 days at 37°C in a humidified atmosphere containing 5% CO_2. The concentration of PE used in our in vitro experiments was within the range of concentrations of PE components that have been detected in the serum of animals and patients who were treated at a dose of six tablets per day (=16 mg/kg) of PE [13]. WOBE-MUGOS was supplied as a sterile endotoxin-free lyophilized preparation containing (per 180 mg) 100 mg papain, 40 mg trypsin and 40 mg chymotrypsin. Sterile reconstitutes of WOBE-MUGOS in Hank's balanced salt solution were used. The cultures were replenished with fresh medium and cytokines or enzymes every 3 days, and the cells were monitored by light microscopy to detect those having morphologic characteristics of DC.

The supernatants of cultures were collected and stored at –70°C for IL-12 determination. After washing, phenotypic and functional mixed leukocyte reaction (MLR) analysis of cells was performed.

Fluorescence-activated cell sorter (FACS) analysis of DC by tricolor flow cytometry

FACS analysis of DC by tricolor flow cytometry was performed as previously described [11, 14]. DC were identified as cells lacking the lineage-associated markers of T cells, B cells, monocytes, granulocytes or NK cells, i.e. they were $CD3^-$, $CD19^-$, $CD20^-$, $CD14^-$, $CD11b^-$, $CD16^-$ and $CD56^-$, and expressing HLA-DR. Briefly, 1×10^6 MNL were incubated for 30 min at 4°C with a cocktail of fluorescein isothiocyanate (FITC)-conjugated lineage-specific monoclonal antibodies (mAbs) reactive against CD3, CD19, CD20, CD14, CD11b (Caltag, Burlingame, Calif.), CD16 (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) and CD56 (Coulter Corporation, Miami, Fl.). These cells were also labeled simultaneously with anti-HLA-DR PerCP (Becton Dickinson Immunocytometry Systems) as well as PE-conjugated mAbs that recognized CD80 (Pharmingen, San Diego, Calif.) or CD83 (Coulter Corporation) or CD40 (Biosource International, Camarillo, Calif.). Cells labeled with PerCP-, PE- and FITC-conjugated isotype control mAbs (Caltag and Becton Dickinson) that were nonreactive to human cells were used as a control. The cells were washed with PBS and fixed with 1% paraformaldehyde. A live gate was set on larger cells which included $LN^-HLA\text{-}DR^+$ cells, and 1000–2000 gated events were routinely collected as previously described [11]. A representative example of the FACS histogram with and without stimulation showing DC (LN^-DR^+) is shown in Fig. 1.

Mixed leukocyte reaction

Stimulator cells (DC-MNL obtained after culture for 5 to 7 days) were washed twice with medium and irradiated at 3000 cGy using a cesium source and then added in a graded concentration (resulting in stimulator-to-responder ratios of 1:1, 1:5, and 1:10), to 1×10^5 allogeneic nylon wool-nonadherent T cells in 96-well round-bottomed plates and incubated for 5 days. Controls included stimulator cells and T cells grown in separate wells. The proliferation of T cells was measured using a nonradioactive cell-proliferation tetrazolium assay (E4Y Biomedica, Vienna, Austria) according to the manufacturer's instructions and as described previously [4]. Briefly, after coincubating the stimulator cells and allogeneic T cells for 5 days, 20 μl of substrate containing tetrazolium salt (E4Y Biomedica) was added to each well and the plates were incubated for another 3 h at 37°C in a humidified atmosphere containing 5% CO_2. The culture plates were then removed from the incubator and

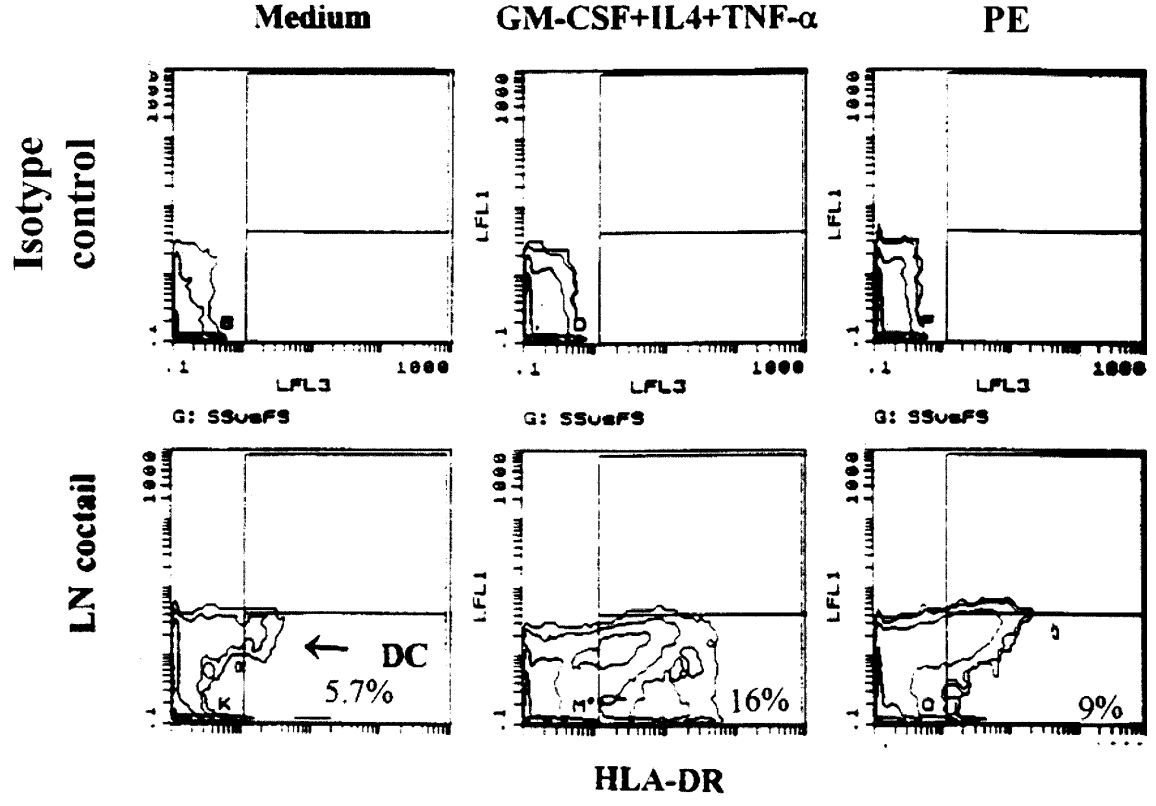

Fig. 1 Representative examples of FACS histograms with and without stimulation showing the percentage of DC (LN^-DR^+) in cultured cells from ascites of an ovarian cancer patient (patient no. 8). MNL obtained from ovarian cancer patients were cultured in vitro for 5–7 days with PE (10 μg/ml), control medium or GM-CSF + TNF-α + IL-4 (100 ng/ml GM-CSF + 10 ng/ml TNF-α + 50 ng/ml IL-4)

the contents gently mixed by tipping the plates at all four sides. The absorbance was then read immediately at wavelengths of both 490 and 650 nm using an automatic microplate reader. Each group was performed in triplicate. The results are expressed as fold proliferation of T cells. In addition, IL-12 production was determined by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Biosource International). This assay detects the p70 heterodimer, p40 monomer and p40 homodimer.

Statistical analysis

The Wilcoxon signed ranks test and Mann-Whitney *U*-test were used for statistical analysis. Results were considered significant when the *P*-value was less than 0.05.

Results

Flow cytometry

Table 2 shows the median and mean (±SEM) proportion of DC (defined as LN^-DR^+ leukocytes) as well as the proportion of DC that expressed CD83, CD80 and CD40 in uncultured ascites obtained from ovarian cancer patients. FACS analysis of these DC in ascites revealed low proportions of DC in peritoneal MNL, and also a low proportion of DC that expressed CD83 and CD80. The proportion of DC that expressed CD40, the receptor for CD40L, was variable, ranging from 0.9% to 56%.

A representative example of the FACS histogram showing DC surface differentiation and costimulatory antigens (above background of isotype control) is shown in Fig. 2.

The median proportion of DC was significantly increased ($P < 0.001$) when the peritoneal MNL were incubated with GM-CSF + TNF-α + IL-4 (11.2 ± 3.2%) or PE (11.0 ± 1.9%) compared with that when MNL were incubated with control medium alone (2.8 ± 0.7%; Fig. 3). The expression of the following cell surface differentiation antigens, CD83, CD80 and CD40, were determined following in vitro culture of the DC containing MNL in rGM-CSF rTNF-α/rIL-4 vs PE vs medium alone, respectively (Fig. 4). Under these culture conditions, respectively, the proportions of $CD83^+$ DC were 19.6 ± 10.4%, 17.8 ± 8.1% and 0.8 ± 0.5%, the proportions of $CD80^+$ DC were 25.5 ± 9.6%, 31.1 ± 10.5% and 6.5 ± 3.0%, and the proportions of $CD40^+$ DC were 48.9 ± 8.4%, 45.0 ± 12.0% and 26.7 ± 8.3%. DC cultures that were grown in GM-CSF/TNF-α/IL-4 or with PE showed significantly higher proportions of cells that expressed CD83, CD80 and CD40 (Fig. 4).

Table 2 Percentage of gated DC (LN^-DR^+) cells that expressed CD83, CD80 and CD40 (*nd* no data)

Patient no.	DC	CD83	CD80	CD40
1	0.2	4.7	nd	nd
2	0.0	0.2	nd	nd
3	0.0	0.0	0.0	0.9
4	1.7	1.6	0.0	nd
5	5.5	0.0	0.0	12.0
6	2.6	1.1	28.0	53.0
7	0.0	nd	nd	56.0
8	3.7	0.0	0.5	2.2
9	6.8	0.0	12.0	52.0
10	1.8	nd	5.6	26.0
11	6.1	nd	4.1	nd
12	2.2	0.0	nd	nd
13	0.0	nd	nd	nd
14	4.1	nd	nd	nd
15	7.4	0.0	7.9	11.7
Median	2.2	0.0	4.1	19.0
Mean	2.8	0.8	6.5	26.7
SE	0.7	0.5	3.0	8.3
n	15	10	9	8

MLR

The MLR of T lymphocytes from healthy donors stimulated with MNL obtained from peripheral blood of an allogeneic healthy donor and from the blood and ascites of ovarian cancer patients is shown in Fig. 5. Cells were used at a stimulator-to-responder ratio of 1:10. The results are expressed as fold proliferation of T lymphocytes. MNL obtained from uncultured blood and ascites of ovarian cancer patients displayed significantly lower stimulatory capacity in MLR compared with those obtained from the peripheral blood of allogeneic healthy donors. Also, MNL from ascites were significantly less stimulatory ($P < 0.05$) than MNL from the blood of ovarian cancer patients. In addition, Fig. 6 shows the proliferative response of allogeneic T lymphocytes obtained from healthy donors to cytokines or PE-treated MNL obtained from blood or ascites of ovarian cancer patients. The results are expressed as fold increase in proliferation after stimulation with treated allogeneic MNL in comparison with control MNL (medium only). MNL from both ascites and blood displayed a significant increase ($P < 0.05$) in stimulatory activity after culturing with GM-CSF + TNF-α + IL-4 (1.3 ± 0.11-fold and 1.79 ± 0.33-fold increase in stimulatory activity in MNL from blood and ascites, respectively). A similar increase in stimulatory activity was observed after culturing MNL with PE (1.88 ± 0.31-fold and 2.02 ± 0.4-fold increase in stimulatory activity in MNL from blood and ascites MNL, respectively; Fig. 6).

IL-12 production

The production of IL-12 (p70 or p40) by peritoneal MNL was low (16 ± 8 pg/ml) following culture in control medium alone but was increased significantly ($P < 0.05$) after culture with GM-CSF + TNF-α + IL-4 (99 ± 60 pg/ml; Fig. 7). Even higher levels of IL-12 were elicited by culture with PE (316 ± 58 pg/ml). The IL-12 concentration in supernatants of MNL obtained from blood was also low (11 ± 6 pg/ml) after culture with medium alone. However, IL-12 production increased

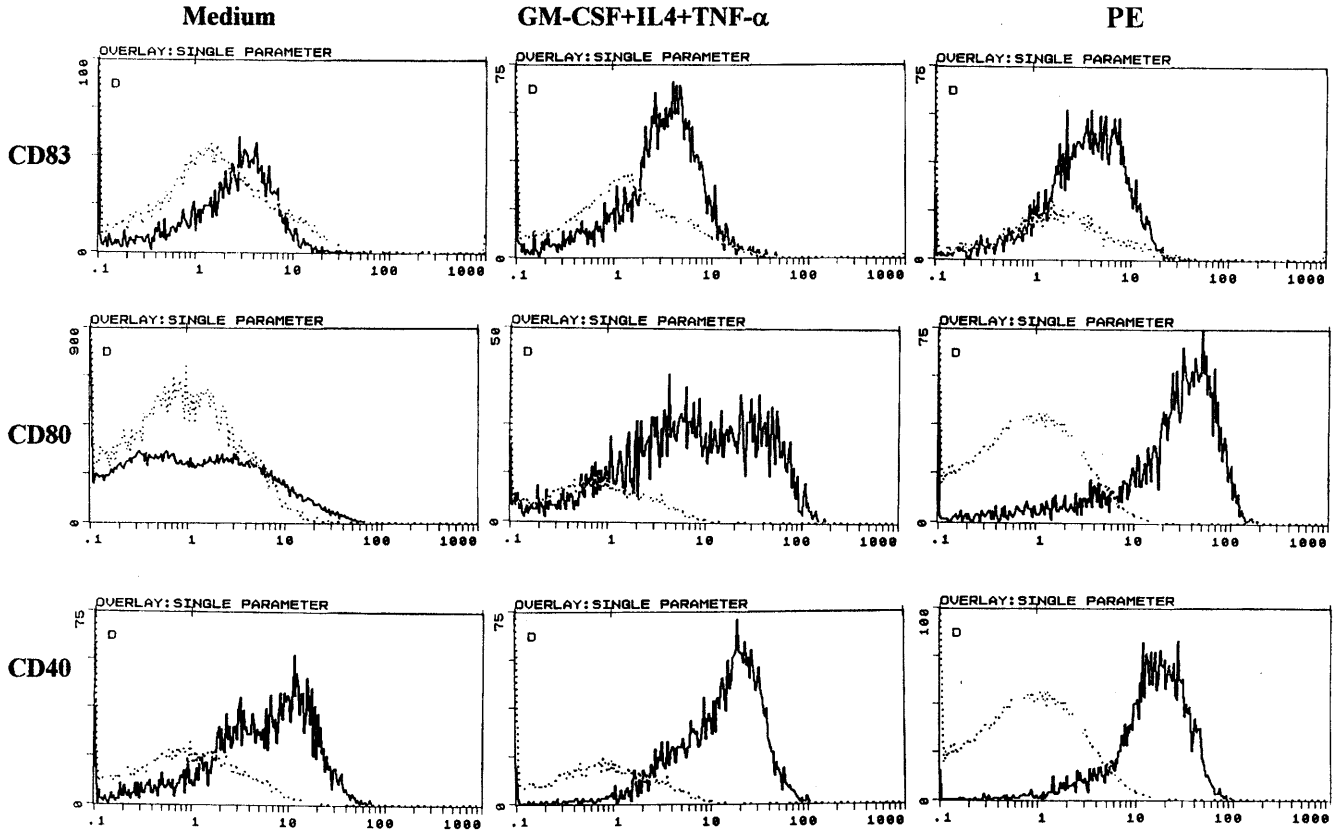

Fig. 2 Representative examples of FACS histograms (patient no. 9) showing the percentage of DC expressing surface-differentiation and costimulatory antigens above the background of the isotype control after culturing with PE, GM-CSF + TNF-α + IL-4, or control medium. The *dotted line* shows the isotype control, the *black line* test sample

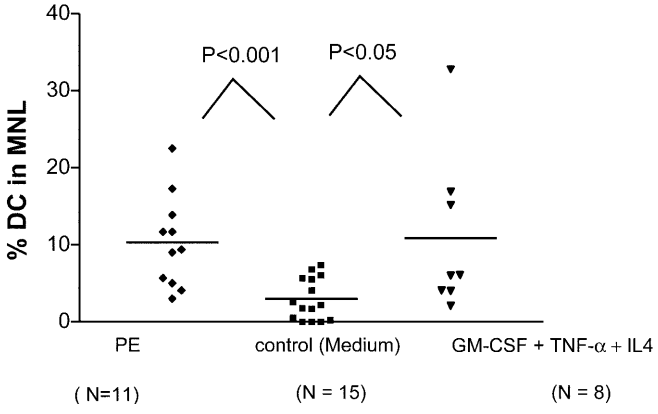

Fig. 3 Median percentage of DC (LN^-DR^+) in peritoneal MNL obtained from ovarian cancer patients following in vitro culture with PE (10 μg/ml), control medium or GM-CSF + TNF-α + IL-4 (100 ng/ml GM-CSF + 10 ng/ml TNF-α + 50 ng/ml IL-4). The *horizontal bars* represent the mean

significantly ($P < 0.05$) after culturing with PE (429 ± 70 pg/ml). GM + TNF-α + IL-4 also stimulated IL-12 production, although the levels were not significantly different from control medium.

Discussion

We showed for the first time that DC obtained from the peritoneal cavity of patients with ovarian cancer can undergo further differentiation following treatment with a PE preparation. Phenotypic analysis of DC showed higher proportions of $CD83^+$, $CD40^+$ and $CD80^+$ cells after culturing with cytokines or a PE preparation as compared with medium alone. Human mature blood DC express CD83, a member of the immunoglobulin superfamily. $CD83^+$ cells appear to be strong stimulators of cells in allogeneic MLR [21].

Functional analysis of $CD83^+$ cells revealed the presence of MHC class II antigen, CD80 antigen (which is a ligand for the CD28/CTLA-4 receptor on lymphocytes), and CD40 (the receptor for CD40L). These molecules have functionally important roles in adaptive immunity and contribute to the specialized costimulatory activity of professional presenting cells. Additionally, CD83 appears to serve as a useful marker for the maturation status of DC [21]. In addition, we showed that culturing DC in the presence of rh-GM-CSF + TNF-α + IL-4, or with a PE preparation led to an increase in the proportion of DC obtained from ascites of ovarian cancer patients, increased production of IL-12, and increased MLR of allogeneic T lymphocytes. The allogeneic lymphocyte proliferation activity

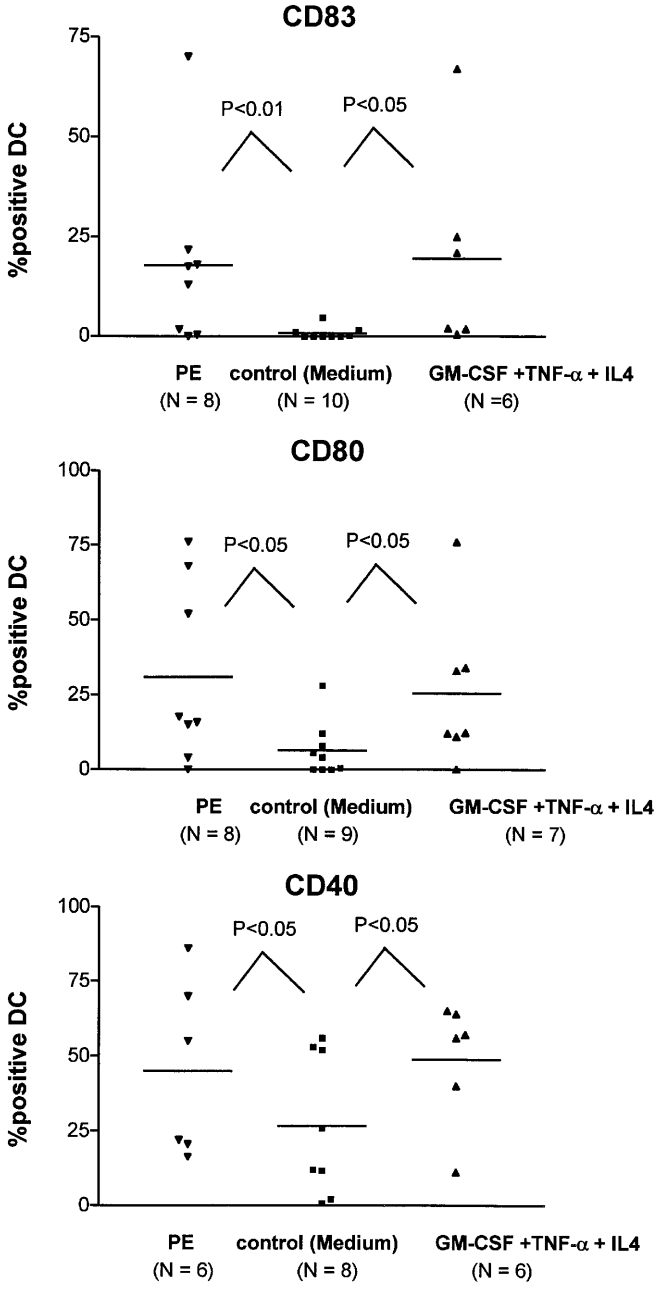

Fig. 4 The percentage of DC ($LN^{-}DR^{+}$) in ascites expressing CD83, CD80 and CD40 after culturing with PE (10 μg/ml), control medium, and GM-CSF + TNF-α + IL-4 (100 ng/ml GM-CSF + 10 ng/ml TNF-α + 50 ng/ml IL-4). The *horizontal bars* represent the mean

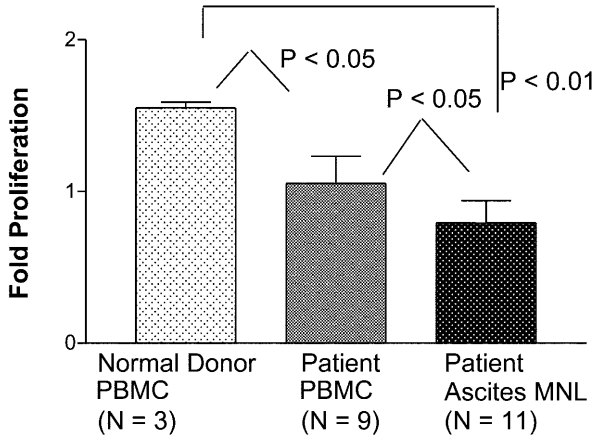

Fig. 5 Proliferative response of T lymphocytes from healthy donors to stimulation by MNL from the peripheral blood of an allogeneic healthy donor or uncultured MNL from the blood and ascites of ovarian cancer patients without stimulation. Cells were used at a stimulator-to-responder ratio of 1:10. The results are expressed as the fold proliferation of the T cells. The columns represent the means ± SEM

of ascites and blood MNL of ovarian carcinoma patients was lower in comparison with that obtained from the peripheral blood of healthy donors. Immunosuppressive factors in the tumor microenvironment may have contributed to this effect, although the specific factors responsible have not been determined.

Certain cytokines, including IL-10 and TGFβ, are known to have an inhibitory effect on the maturation of DC [17, 20]. DC that have undergone differentiation in the presence of IL-10 have an impaired capacity to induce a Th1-type response in vivo [15]. These cytokines have been detected in malignant effusions in ovarian cancer patients [6, 10]. The differentiating effect of PE preparation on DC obtained from ovarian cancer patients may possibly involve a reduction in TGFβ activity because proteolytic enzymes are known to reduce TGFβ by converting the protease inhibitor α2-macroglobulin from the "slow" form of α2-macroglobulin into the "fast" form. In this way, the fast form binds and irreversibly inactivates TGFβ [8]. Additionally, the culturing of tumor- associated macrophages with a PE preparation is associated with reduced TGFβ transcript expression and protein production. TGFβ may also decrease certain functions of DC [1, 20]. It is possible that a decrease in TGFβ expression and production could contribute to maturation of DC after treatment of MNL with enzymes and indirectly stimulate Th1 response. Also incubation of cells with an anti-TGFβ mAb has been shown to result in an increase in the proportions of DC as well as higher proportions of CD83 and CD80 in DC obtained from the peritoneal cavity and blood of ovarian cancer patients (data not shown).

Our studies demonstrate for the first time that DC obtained from the peritoneal cavity and blood of ovarian cancer patients, cultured in the presence of a PE preparation, will undergo differentiation to cells that show high expression of cell-surface differentiation antigens and IL-12 production. Both the cytokine cocktail and PE preparations produced similar results in our experiments in relation to upregulation of cell-surface molecules and MLR. However, the addition of PE resulted in significantly higher concentrations of IL-12 (p70 heterodimer and p40) in vitro. These findings may be important to ongoing multicenter clinical trials with PE in which it is being utilized as an oral agent that has an acceptable level of toxicity. In future studies it will be

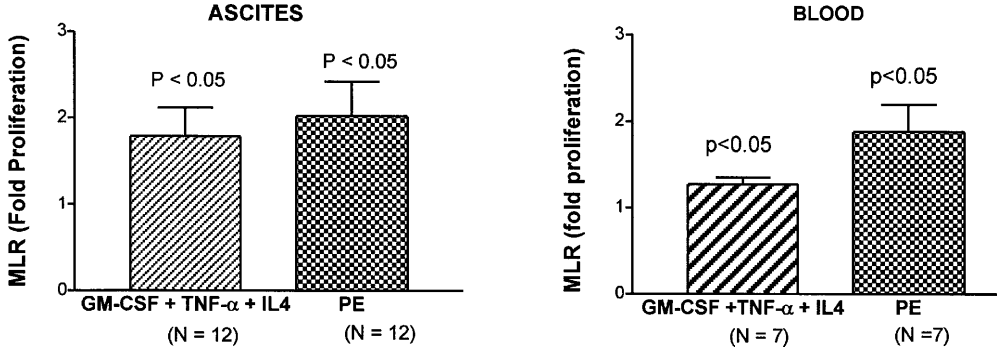

Fig. 6 Proliferative response of T lymphocytes obtained from a healthy donor to MNL from the blood and ascites of ovarian cancer patients after treatment with PE or GM-CSF + TNF-α + IL-4. The results are expressed as the fold increase after stimulation in comparison to controls (cultured with medium only). The columns represent the means ± SEM. The *P*-values indicate the significance of the increases compared with controls cultured with medium only

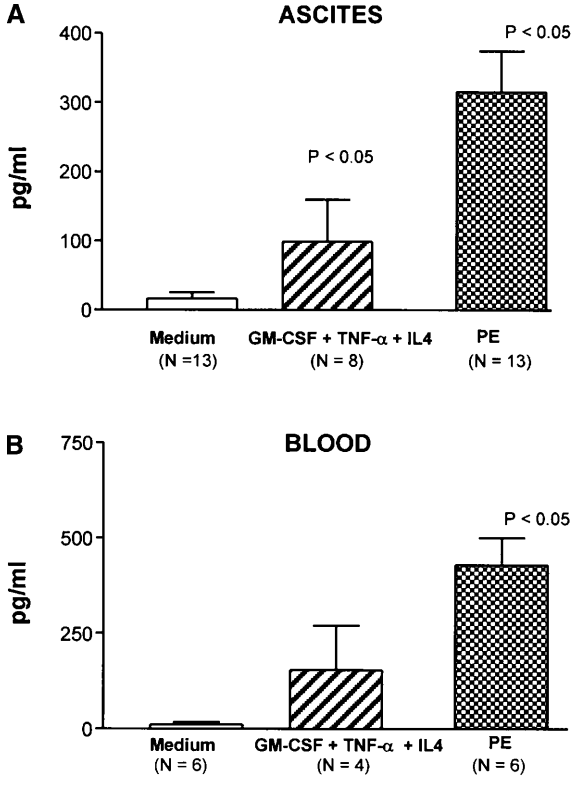

Fig. 7A, B IL-12 production (mean ± SEM) by (**A**) peritoneal MNL or (**B**) blood MNL obtained from ovarian cancer patients cultured with PE, GM-CSF + TNF-α + IL-4, or medium alone. The columns represent the means ± SEM. The *P*-values indicate the significance of the increases compared with controls cultured with medium only

important to determine whether PE facilitates Th1-type responses.

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