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Restoration of tumor specific human leukocyte antigens class I-restricted cytotoxicity by dendritic cell stimulation of tumor infiltrating lymphocytes in patients with advanced ovarian cancer

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Abstract. Santin AD, Bellone S, Palmieri M, Bossini B, Cane' S, Bignotti E, Roman JJ, Cannon MJ, Pecorelli S. Restoration of tumor specific human leukocyte antigens class I-restricted cytotoxicity by dendritic cell stimulation of tumor infiltrating lymphocytes in patients with advanced ovarian cancer. *Int J Gynecol Cancer* 2004;**14:**64–75.

Despite the large number of potentially cytotoxic tumor-infiltrating (TIL) and tumor-associated (TAL) lymphocytes accumulated in the peritoneal cavity ascitic fluid and tumor tissue, advanced ovarian cancer is a progressive disease, suggesting that TIL and TAL populations eventually become functionally suppressed *in vivo*. Dendritic cells (DC) are the most powerful professional antigen presenting cells known in humans and recently, ovarian tumor antigen pulsed DC have been shown to elicit tumor specific human leukocyte antigens (HLA)-class I-restricted cytotoxicity from the peripheral blood of advanced ovarian cancer patients. In this study, we have evaluated the potential of tumor antigen-pulsed fully mature DC stimulation in restoring tumor-specific cytotoxicity in anergic TIL populations from advanced ovarian cancer patients. In addition, we have compared tumor-specific T-cell responses induced by tumor antigen-loaded DC in TIL to those induced in TAL and peripheral blood lymphocytes (PBL). DC stimulation induced powerful cytotoxicity against autologous tumor target cells in TIL-derived CD8+ T-cells from all patients tested, while autologous Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (LCL) were not lysed. Killing of autologous tumor cells was higher by CD8+ T-cells from TIL compared to PBL and TAL ($P < 0.01$) and was more strongly inhibited by anti-HLA class I MAb ($P < 0.05$ compared to PBL and TAL). Phenotypically, all cytotoxic T lymphocyte (CTL) populations were CD3+/CD8+, with variable levels of CD56 expression. Finally, although a marked Type 1 cytokine bias [ie, interferon-gamma/interleukin-4 (IFN-γ^{high}/IL-4^{low})] was observable in all DC-stimulated CD8+ T-cell populations, TIL derived CD8+ T-cells showed a higher percentage of IFN-γ

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positive cells compared to TAL and PBL. Taken together, these data show that tumor lysate-pulsed DC can consistently restore strong CD8+ CTL responses from TIL against autologous ovarian cancer cells. DC-stimulated TIL may represent a superior source of tumor-specific CTL for adoptive T-cell immunotherapy for advanced ovarian cancer.

KEYWORDS: IFN-γ, IL-4, ovarian cancer, tumor infiltrating lymphocytes.

Because ovarian cancer remains localized in the abdominal cavity even in the advanced stages of the disease, it has been suggested that the growth of this malignancy could be related to a local phenomenon of immunosuppression[1,2]. Supporting this hypothesis, local deficiency of several antitumor immune effector mechanisms have been previously reported in the peritoneal cavity of patients with advanced stages of ovarian cancer[1–7]. Although the causes responsible for these impaired immune responses are not yet completely understood, several mechanisms have been suggested to account for tumor-induced subversion of the immune system, including soluble tumor-derived inhibitory factors[8–11], tolerogenic or T-cell-deleting presentation of antigens by ovarian tumor cells[12,13], or activation of inhibitory regulatory elements of the immune system[14].

Dendritic cells (DC) are highly potent antigen presenting cells of bone marrow origin that can stimulate both primary and secondary T- and B-cell responses[15,16]. Recently, the combination of GM-CSF and interleukin-4 (IL-4) has been shown to generate large numbers of myeloid monocyte-derived DC[17–19]. However, these DC are still immature. Although they may effectively capture antigens, immature DC lack full T-cell-stimulatory activity and are sensitive to the immunosuppressive effects of several immunoregulatory cytokines that can be produced by tumors[16,20]. In contrast, when full maturation is induced by appropriate stimuli, such as monocyte-conditioned medium, LPS, or a cocktail of inflammatory cytokines[16,20,21] DC demonstrate a reduced ability to phagocytose antigens, but show a significantly higher production of key cytokines (eg, IL-12), increased resistance to the immunosuppressive effects of IL-10, increased expression of T-cell adhesion and costimulatory molecules, and increased expression of chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T-cells[16,20]. It is thus apparent that the maturation stage of DC is critical for their optimal use in immunization strategies[22–24].

One of the most important components for a successful tumor specific adoptive immunotherapy is the activation and expansion of large amount of tumor specific lymphocytes. In earlier studies, lymphocytes derived from ascitic fluid and infiltrating tumor tissues of ovarian cancer patients have been shown to contain tumor specific cytotoxic T lymphocyte (CTL) precursors that can be stimulated to specifically kill autologous ovarian cancer cells[25–27]. However, in several of these reports, T-cells derived from ascitic fluid tumor-associated lymphocytes (TAL) and tumor tissue (tumor-infiltrating lymphocytes; TIL) have been shown to be defective in several immunologic functions when compared to peripheral blood lymphocytes (PBL) including (a) poor ability to proliferate in presence of mitogens, phorbol esters, or low doses of IL-2, (b) cytotoxic activity against autologous or allogenic tumor cells, (c) effector activity in assays of natural killing, and (d) expression of cytoplasmic CD3-ζ chain, which is essential for T-cell receptor (TcR) signaling[27]. Importantly, however, in some cases, poor immune responses by explanted T lymphocytes could be normalized upon *in vitro* culture with high doses of recombinant IL-2[28], or by T-cell stimulation with anti-CD3 and anti-CD28[29]. It is therefore possible that when properly stimulated by tumor-antigen pulsed mature DC, which are endowed with high expression of human leukocyte antigens (HLA) class I and II antigens and costimulatory molecules[19], TAL or TIL may reverse their anergic state and become a superior source of tumor specific cytotoxic lymphocytes for adoptive T-cell immunotherapy for advanced ovarian cancer.

In this study, we have evaluated and compared the phenotype and cytotoxic activity of tumor lysate-pulsed autologous DC stimulated CD8+ T lymphocytes derived from PBL, TAL, and TIL from advanced ovarian cancer patients. Here we show that tumor antigen-pulsed fully mature DC have the potential to reverse the anergic state of TIL and TAL and that TIL constitute a superior source of tumor specific CD8+ CTL compared to PBL and TAL for potential use in adoptive immunotherapy for ovarian cancer.

Materials and methods

Patients

Three patients who had undergone total abdominal hysterectomy bilateral salpingo oophorectomy and omentectomy for advanced ovarian cancer provided tumor

tissue, ascitic fluid, and peripheral blood lymphocytes. Specimens were obtained at the time of surgery through the Division of Gynecologic Oncology and the Pathology Department at the University of Arkansas for Medical Sciences (UAMS), Little Rock, AR, under approval of the Institutional Review Board. All patients had stage III ovarian cancer with serous papillary histology. Patients 1 was of age 65, while Patients 2 and 3 were 80 and 72 years old, respectively.

Tumor cell lines

Fresh autologous tumor cells were obtained from multiple ovarian tumor biopsies from all patients. Fresh tumor cell lines were maintained in RPMI 1640 (Invitrogen, Grand Island, NY), supplemented with 15% autologous ascitic fluid at 37°C, 5% CO_2. Briefly, single cell suspensions were obtained by processing solid tumor samples under sterile conditions at room temperature. Viable tumor tissue was mechanically minced in RPMI 1640 to portions no larger than 1–3 mm^3 and washed twice with RPMI 1640. The portions of minced tumor were then placed into 250 ml trypsinizing flasks containing 30 ml of enzyme solution [0.14% Collagenase Type I (Sigma, St. Louis, MO) and 0.01% DNAse (Sigma, 2000 KU/mg)] in RPMI 1640, and incubated on a magnetic stirring apparatus overnight at 4 °C. Enzymatically dissociated tumor was then filtered through 150 μm nylon mesh to generate a single cell suspension. The resultant cell suspension was then washed twice in RPMI 1640 plus 10% ascitic fluid. All experiments were performed with fresh or cryopreserved tumor cultures which had at least 90% viability and contained >99% tumor cells.

Collection and isolation of PBL from ovarian cancer patients

Peripheral blood was collected in heparinized tubes at the time of surgery and before any blood transfusion and processed as previously described[30,31], to separate the mononuclear leukocytes from red blood cells by Ficoll-Hypaque density gradient centrifugation. The leukocytes were subsequently harvested, washed twice in phosphate buffered saline (PBS, pH 7.2), and resuspended in RPMI 1640 plus 5% human AB serum (Gemini BioProcust, Calabasas, CA) before being stained with monoclonal antibodies or used in DC-stimulation experiments.

Isolation of TAL

Ascitic fluid specimens (250–900 ml) were obtained at the time of surgery. Thorough hemostasis was performed at the time of abdominal incision to avoid contamination of ascitic fluid lymphocytes with PBL. Briefly, centrifuged cell pellets from ascitic samples were washed twice in RPMI 1640, placed on discontinuous Ficoll-Hypaque (75/100%) density gradients, and centrifuged again to harvest TAL and tumor cells as previously described by Whiteside *et al.*[32]. Enriched TAL preparations were then washed twice in RPMI 1640 plus 10% autologous plasma and either immediately activated with Phorbol 12-myristate 13-acetate (PMA) and ionomycin for intracellular cytokine studies, or incubated overnight at 37°C in tissue culture flasks before collecting the TAL from the non-adherent population.

Isolation of TIL

Single cell suspensions were obtained by processing solid tumor samples as described above. The resultant cell suspension containing tumor cells and TIL was then washed twice in RPMI 1640 plus 10% autologous plasma and placed on discontinuous Ficoll-Hypaque (75/100%) density gradients, and centrifuged again to harvest TIL and tumor cells as described above for TAL[32]. Enriched TIL preparations were then washed twice in RPMI 1640 plus 10% autologous plasma and either immediately activated with PMA and ionomycin for intracellular cytokine studies, or incubated overnight in tissue culture flasks at 37°C before collecting the TIL from the non-adherent population.

DC cultures and generation of ovarian tumor-specific T-cells

The derivation of DC from the patients' peripheral blood mononuclear cells (PBMC), and their subsequent use for generation of ovarian tumor-specific T-cells, was carried out essentially as described[30,31]. Ovarian tumor-specific CD8+ T-cells were derived from all three advanced ovarian cancer patients from lymphocytes obtained from the different anatomical sites. Briefly, DC were generated from plastic-adherent PBMC by culture in AIM-V medium (Invitrogen) plus 800 U/ml GM-CSF (Immunex, Seattle, WA) and 1000 U/ml IL-4 (R&D Systems, Minneapolis, MN). Cultures were fed by half-changes of AIM-V plus GM-CSF and IL-4 every 2 days. Final maturation of monocyte-derived DC was induced by exposure during the last 48 h of culture (ie, day 6–day 8) to TNF-α (1000 U/ml), IL-1β (500 U/ml) (R&D Systems), and PGE2a (0.5 μM/ml) (Sigma). The DC purity (ie, cells strongly expressing HLA-DR+, CD86+, CD83+, CD80+, CD40+, and CD14–) ranged from 62 to 90%

of the total cell population, as previously characterized by our laboratory[19]. After final maturation, DC were harvested for pulsing with ovarian tumor lysate obtained from 5 to 10×10^6 autologous tumor cells cultured in RPMI 1640 plus 15% autologous ascites and lysed by 3–4 freeze cycles (in liquid nitrogen) and thaw cycles (room temperature) as previously described[31]. The cationic lipid DOTAP (Boehringer Mannheim, Indianapolis, IN) was used to deliver the tumor lysate into cells as described[31]. Ovarian tumor-specific CTLs were generated by culturing responder PBMC, TAL and TIL ($10–20 \times 10^6$ cells/well in 6-well culture plates) (Costar, Cambridge, MA) in AIM-V with tumor lysate-pulsed autologous DC (ratios from 20 : 1 to 30 : 1 responder PBMC : DC). The cultures were supplemented with recombinant human IL-2 (10 U/ml; Aldesleukin, Chiron Therapeutics, Emeryville, CA) and restimulated once with tumor antigen-pulsed DC after 10–14 days. At day 21–28, CD8+ T-cells were separated from the bulk cultures by positive selection with CD8-Dynabeads (Dynal Inc., Lake Success, NY) and further expanded in number for 10–14 days using autologous or allogeneic irradiated PBL (5000 cGy) (1×10^6 cells/well) and anti-CD3 monoclonal antibody (MAb) (0.2 μg/ml) in AIM-V plus 5% heat inactivated autologous plasma in 24-well plates (Costar) before being assayed for CTL activity. In some experiments, Dynabead-separated CD8+ T-cells were restimulated once more with autologous lethally irradiated (ie, 10,000 cGy) tumor cells at a 20:1 lymphocyte/tumor ratio or tumor lysate pulsed DC before being tested in cytotoxicity assays or being further expanded with allogeneic irradiated PBL (5000 cGy) (1×10^6 cells/well) and anti-CD3 MAb.

Flow cytometric analysis of surface antigens, intracellular CD3ζ chain, and cytokines by ovarian tumor-specific CTL from PBL, TAL, and TIL

Flow cytometric analysis of superficial antigens and intracellular cytokine expression was conducted essentially as previously described[31,32]. Briefly, flow cytometry for superficial antigen expression was performed using directly conjugated MAbs against the following markers: CD3, pan T-cells; CD4, T helper/inducer; CD8, T cytotoxic/suppressor; CD19, B cells; CD56, NK/K cells; CD25, the IL-2 receptor; anti-HLA-DR; and anti TcR-α/β (all from Becton Dickinson, San Jose, CA). Control isotype-matched FITC- or PE-conjugated MAb were also obtained from Becton Dickinson. For evaluation of the level of CD3ζ chain in CD8+ T-cells, PBL, TAL, and TIL before and after *in vitro* stimulation with ovarian tumor lysate pulsed DC were harvested, stained with anti-CD8-FITC (Becton Dickinson), washed, and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. Cells were then washed and permeabilized by incubation in PBS plus 1% BSA and 0.5% saponin (S-7900, Sigma) for 10 min at room temperature. Experimental and control cells were stained with anti TcRζ-PE (2H2D9-IgG1k), or isotype control (Immunotech, Marseille, France). After staining, cells were washed twice with PBS plus 1% BSA and 0.5% saponin, once with PBS plus 0.5% BSA, and fixed a second time with 2% paraformaldehyde in PBS. All analyses were conducted with a FACScan, utilizing cell Quest software (Becton Dickinson).

For intracellular cytokine staining for interferon-gamma (IFN-γ) and IL-4 expression, CD8+ T-cells obtained from PBL, TAL, and TIL at the beginning of culture were activated by 50 ng/ml PMA and 500 ng/ml ionomycin for 6 h. 10 μg/ml Brefeldin A was added for the final 3 h of incubation. Controls (non-activated cultures) were incubated in the presence of Brefeldin A only. Cells were superficially stained with anti-CD8-FITC or anti-CD8-PE Mab and then washed and permeabilized by incubation in PBS plus 1% BSA and 0.5% saponin for 10 min at room temperature. Activated and control cells were stained with FITC-anti-IFN-γ and/or PE-anti-IL-4 and isotype-matched controls (FITC-anti-Igγ2a and PE-anti-Igγ1) from Becton Dickinson. Purified DC-stimulated CD8+ T-cell populations (ie, day 21–28 of culture) were also analyzed by two color flow cytometry for simultaneous expression of IFN-γ and IL-4 at the single cell level. After staining, cells were washed twice with PBS plus 1% BSA and 0.5% saponin, once with PBS plus 0.5% BSA, and fixed a second time with 2% paraformaldehyde in PBS.

Cytotoxic activity

A 6-h chromium (^{51}Cr) release assay was performed as previously described[30,31] to measure the cytotoxic reactivity of DC-ovarian tumor antigen-stimulated T lymphocytes. The Epstein–Barr virus (EBV)-transformed lymphoblastoid B-cell line (LCL) derived from the same cancer patients that provided the primary tumor cell targets was established by coculture of PBMC with EBV-containing supernatant from the B95.8 cell line in the presence of 1 μg/ml cyclosporin A (Sandoz, Camberley, UK) and was maintained in RPMI 1640 supplemented with 10% human AB serum. To determine the molecular basis of target cell lysis, ^{51}Cr-labeled tumor targets were preincubated with MAbs specific for monomorphic HLA class

I (W6/32, 50 μg/ml) or isotype control Mab. The effector cells and ^{51}Cr-labeled targets were then incubated in a final volume of 200 μl/microwell at 37°C with 6% CO_2.

Statistical analysis

Data were analyzed using Student's *t*-test. All data were expressed as mean percentages of positive cells ± standard deviation (SD). In all tests, the difference was considered significant when *P*-values were <0.05.

Results

Phenotypic analysis of lymphocytes from peripheral blood, ascitic fluids, and tumor tissue

Flow cytometric analysis was used to determine the phenotype of the populations of lymphocytes obtained from PBL, TAL, and TIL before and after the *in vitro* stimulation with autologous ovarian tumor antigen loaded DC. The proportions of CD3+, CD8+, and CD4+ T-cells in PBL, TAL, and TIL from the three ovarian cancer patients evaluated in this study before DC activation are described in Table 1. CD3+ T-cells were the major lymphocyte population in each tissue, with the percentage of CD4+ T-cells greater than the percentage of CD8+ T-cells in PBL and TAL ($P < 0.01$) (Table 1). In contrast, in all three patients CD8+ T-cells and CD4+ T-cells were present in equal numbers in TIL (Table 1). The PBL had the highest CD4+ to CD8+ ratio (mean = 3.1:1), with TAL exhibiting a mean ratio of 2.2:1 and TIL a ratio of 1:1 (Table 1). Starting lymphocyte populations were also different in the percentage of CD56 and CD19 positive lymphocytes, with PBL containing more CD56+ and CD19+ cells compared to TAL and TIL ($P < 0.05$ and $P < 0.05$ for both CD56 and CD19). The expression of CD56 on T lymphocytes was further analyzed by two color immunofluorescence (Table 1). By this technique, the CD8+ T-cells were compared for coexpression of CD56. PBL contained more CD8+/CD56+ T-cells compared to TAL and TIL ($P < 0.05$ and $P < 0.05$ for both TAL and TIL).

After 21–28 days' culture of tumor lysate pulsed DC-stimulated PBL, TAL, and TIL, the striking majority of cells were found to be TcR-αβ+/CD3+ T-cells (90–98%). PBL and TAL contained more CD4+ over CD8+ T-cells (range from 55–88% and 42–65%, for PBL and TAL, respectively). In contrast, in the TIL populations derived from all three patients the percentage of CD8+ T-cells was the predominant over that of CD4+ T-cells (range from 55–78%). Enriched populations of CD8+ T-cells were isolated at this time, and analyzed for expression of CD56 after a further two or more weeks of culture. A variable proportion of CD8/CD56-antigen positive cells was detected in all CD8+ T-cells derived from PBL, TAL, and TIL. However, as representatively shown for Patient 1 (Fig. 1), tumor lysate DC-stimulated TIL consistently expressed a higher percentage of CD8+/CD56+ T-cells compared to PBL ($P < 0.01$) and TAL ($P < 0.05$). Further analysis revealed the populations of CD8+ T-cells to be CD25+, HLA-DR+, and CD16– (data not shown).

Expression of intracellular TcRζ chain in ovarian tumor antigen-stimulated T-cells

To evaluate whether the expression levels of TcRζ chain vary significantly in PBL, TAL, and TIL collected from ovarian tumor patients before and/or after DC-stimulation, we again took advantage of flow cytometry. As shown in Table 2, the mean fluorescence intensity of TcRζ chain expression was significantly higher in CD8+ T-cells derived from PBL compared to those from TAL and TIL ($P < 0.05$) in

Table 1. Subpopulations of mononuclear cells in PBL, TAL, and TIL from advanced ovarian cancer patients

	Patient 1*			Patient 2*			Patient 3*		
Specific MAb	PBL	TAL	TIL	PBL	TAL	TIL	PBL	TAL	TIL
CD3	62	32	51	59	42	49	69	39	44
CD4	46	21	24	46	28	23	50	27	25
CD8	15	10	25	13	12	25	17	12	21
CD19	14	3	2	10	4	3	12	2	2
CD56	11	4	3	12	4	2	9	3	2
CD8/CD56	5	2	3	7	3	2	6	1	2
CD4/CD8 ratio	3.0	2.1	1.0	3.5	2.3	0.9	2.9	2.3	1.2

PBL, peripheral blood lymphocytes; TAL, tumor-associated lymphocytes; TIL, tumor-infiltrating lymphocytes.
Values are percentage of PBL, TAL and TIL positive for a specific marker used in the cell staining.
*Percentage of Phenotype.

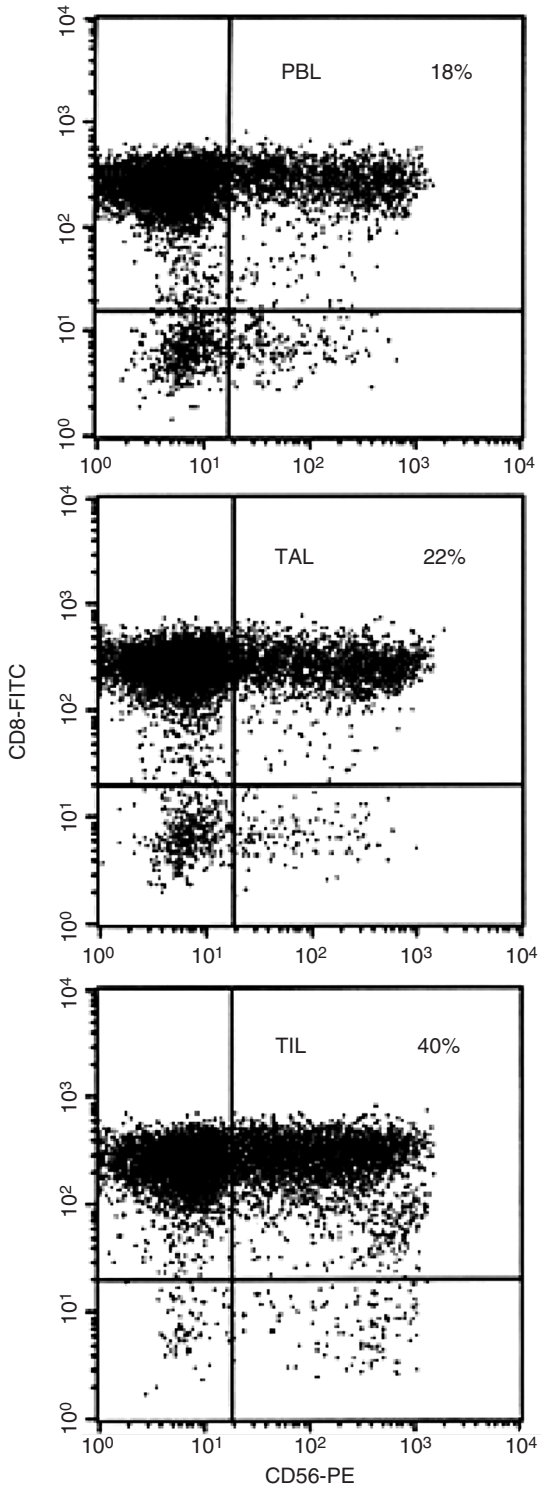

Fig. 1. Two-color flow cytometric analysis of CD56 expression by ovarian tumor-specific CD8+ T-cells in peripheral blood lymphocytes, tumor-associated lymphocytes and tumor-infiltrating lymphocytes from a representative patient after *in vitro* stimulation with tumor antigen-loaded dendritic cells.

all three patients. However, after DC stimulation and expansion, no significant differences were found in

Table 2. Expression of TcRζ chain in CD8+ T-cells derived from PBL, TAL, and TIL from advanced ovarian cancer patients

	Patient 1 %	MFI	Patient 2 %	MFI	Patient 3 %	MFI
PBL	100	30	100	49	100	38
TAL	95	18	99	22	96	26
TIL	97	11	97	12	94	14

MFI, mean fluorescence intensity; PBL, peripheral blood lymphocytes; TAL, tumor-associated lymphocytes; TIL, tumor-infiltrating lymphocytes.
Lymphocyte population at the beginning of culture.

TcRζ chain expression among CD8+ T-cells derived from PBL compared to TAL and TIL (data not shown).

Tumor-specific CD8+ cytotoxic responses in PBL, TAL, and TIL-derived CD8+ T-cells

Cytotoxicity assays on tumor antigen-loaded DC-stimulated CD8+ T-cells were conducted after a minimum of 2 weeks after *in vitro* separation of pure populations of CD8+ T-cells derived from PBL, TAL, and TIL. HLA class I-restricted lysis of autologous tumor cells was detectable in all lymphocyte populations studied (Fig. 2). The results represent the mean of not less than three assays for each patient. Cytotoxic activity of DC-stimulated CD8+ T-cells at an effector:target cell ratio of 20:1 ranged from 12 to 36%, mean 28% (PBL), from 18 to 42%, mean 32% (TAL) and from 30 to 66%, mean 55% (TIL) for Patient 1; from 20 to 38%, mean 25% (PBL), from 18 to 39%, mean 29% (TAL) and from 32 to 56%, mean 48% (TIL) for Patient 2, and from 16 to 32%, mean 23% (PBL), from 15 to 30%, mean 25% (TAL) and from 40 to 71%, mean 60% (TIL) for Patient 3. In all cases low or negligible cytotoxicity was observed against autologous EBV-transformed LCL and against natural killer-sensitive K562 cells (Fig. 2). Blocking studies indicated that in all cases tumor-specific lysis by CD8+ T-cells was inhibited by MAb specific for HLA class I, the range of inhibition being from 15 to 51% (PBL), from 25 to 61% (TAL), and from 35 to 95% (TIL) for the three patients. In contrast with the tumor specific cytotoxicity consistently observed with DC-stimulated CD8+ T-cells from TIL, populations of freshly isolated TIL (and TAL) showed no cytotoxicity against autologous tumor targets.

Intracellular cytokine expression by ovarian tumor antigen-specific T-cells

To determine the patterns of Type 1 and Type 2 cytokine expression in the populations of CD8+ T

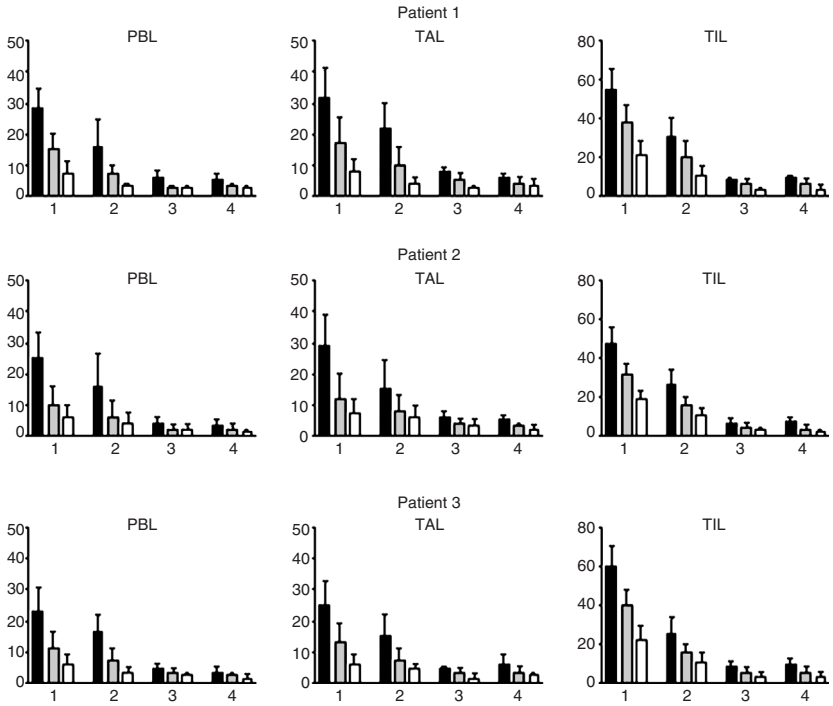

Fig. 2. Tumor specific CD8+ cytotoxic T lymphocytes responses induced in peripheral blood lymphocytes, tumor-associated lymphocytes and tumor-infiltrating lymphocytes by tumor lysate-pulsed dendritic cells in three patients with advanced ovarian cancer, measured in a 6 h ^{51}Cr-release assay. Percentage lysis (±standard deviation) at a 5:1 (empty columns), 10:1 (gray columns) and 20:1 (black columns) effector/target cell ratio is shown. Anti-human leukocyte antigens class I blocking antibody (W6/32) and isotype control Mab (data not shown) were used at 50 μg/ml. Patients 1–3; 1, autologous tumor; 2, autologous tumor + W6/32 anti-class I MAb; 3, lymphoblastoid cell line control; 4, K562. Killing of autologous tumor cells by TAL compared to PBL and TAL ($P < 0.01$). Killing inhibition by anti-HLA class I MAb in TIL compared to PBL and TAL ($P < 0.05$).

lymphocytes collected from PBL, TAL, and TIL, two color flow cytometric analysis of intracellular IFN-γ or IL-4 expression by CD8+ T-cells was performed before and after *in vitro* culture with ovarian tumor antigen-loaded autologous DC. As described in Table 3, at the beginning of culture, significantly more CD8+ T-cells from TIL expressed IFN-γ after stimulation with PMA and ionomycin compared to TAL and PBL-derived CD8+ T-cells ($P < 0.05$). In contrast, IL-4 expression was found at low, but significantly higher levels in PBL ($P < 0.05$) when compared to TAL and TIL (Table 3). When pure populations of CD8+ T-cells were tested after separation and further *in vitro* expansion, significantly higher numbers of CD8+ T-cells from TIL were found to contain intracellular IFN-γ compared to CD8+ T-cells derived from TAL and PBL (Fig. 3). IL-4 was again significantly higher in CD8+ T-cells derived from PBL when compared to the expression of CD8+ T-cells obtained from TAL and TIL (Fig. 3). Similar results were consistently obtained in several repetitive analyses for all patients. Unactivated (ie, resting) CD8+ T-cells failed to stain for IFN-γ or IL-4, and similarly, FITC-anti-IgG2a and PE-anti-IgG1 isotype controls did not stain either activated or unactivated CD8+ T-cells (data not shown).

Discussion

DC are the most potent antigen-presenting cells known in humans and play a crucial role during the priming and reactivation of antigen specific immune responses[16–23]. This unique function as well as the recent standardization of DC culture conditions *in vitro* has provided the opportunity to evaluate their potential for the immunological treatment of cancer

Table 3. Percentage of IFN-γ and IL-4 positive CD8+ T-cells from PBL, TAL, and TIL from advanced ovarian cancer patients

	Patient 1 IFN-γ	IL-4	Patient 2 IFN-γ	IL-4	Patient 3 IFN-γ	IL-4
PBL	12	5	20	4	14	4
TAL	22	2	32	1	25	2
TIL	43	1	49	1	46	1

IFN-IFN-γ interferon-gamma; IL-4, interleukin-4; PBL, peripheral blood lymphocytes; TAL, tumor-associated lymphocytes; TIL, tumor-infiltrating lymphocytes.
Lymphocyte population at the beginning of culture.

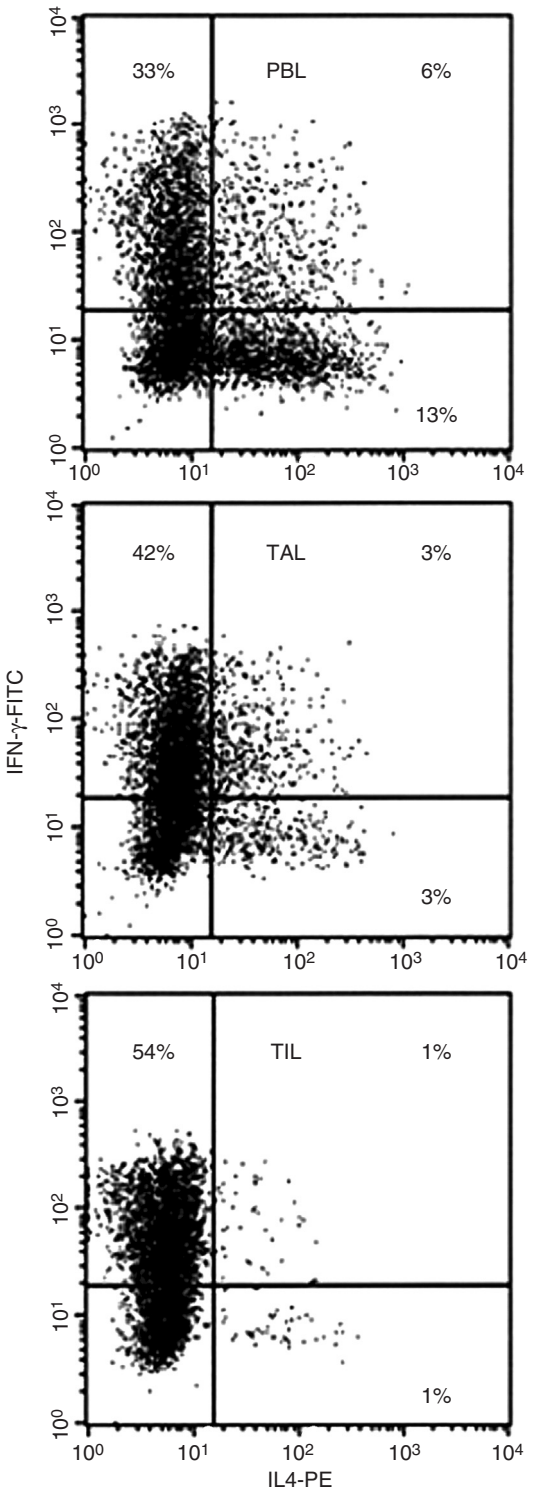

Fig. 3. Representative dot plot analysis of intracellular interferon-γ and IL4 expression by CD8+ T-cells from peripheral blood lymphocytes, tumor-associated lymphocytes and tumor-infiltrating lymphocytes for an individual patient after *in vitro* culture with tumor antigen-loaded dendritic cells. Lymphocytes were activated by PMA and ionomycin as described in the method section. Numbers in the quadrants represent the percentage of CD8+ cytokine positive T lymphocytes.

patients unresponsive to standard treatment modalities. Consistent with this view, several human phase I/II trials have been initiated using tumor antigen pulsed DC, and promising clinical results have been reported, in the absence of significant toxicity[33–38]. However, it is now apparent that the maturation stage of DC is critical for their optimal use in immunization strategies[22–24]. Indeed, *in vivo* clinical studies have shown that monocyte-derived mature DC rapidly generate broad T-cell immunity in healthy subjects vaccinated with less than 3×10^6 antigen-pulsed autologous DC[22,23]. In contrast, the administration of immature DC has been shown to result in inhibition of pre-existing effector T-cell function[24], with recent experiments also providing direct evidence that antigen loaded immature DC may silence T-cells, either by peripheral deletion, or expansion of regulatory T-cells[39].

Patients with advanced ovarian cancer have often been shown to progressively develop impaired immune responses against autologous tumor cells, preceding the development of a more generalized state of immunosuppression[2–6,8–10]. In several studies, diminished T-cell function has been correlated with specific alterations in the T-cell signal transduction pathways, deletion of tumor-specific T lymphocytes by tumor cells, and induction of tolerance against tumor antigens[1–10]. Although the mechanisms responsible for these impaired immune responses are yet not completely understood, it has been recently shown that a population of tolerogenic macrophages from tumor-bearing hosts have the capability to down-regulate cytotoxic T-cell responses against tumor cells as well as to abrogate expression of CD3-ζ chain by contact–dependent interactions[40,41]. Furthermore, a population of immature DC endowed with low or absent expression of CD80 (B7.1), low expression of CD86 (B7.2), and the ability to secrete high levels of immunosuppressive cytokines (ie, IL-10 and TGF-β) have been recently identified in the peritoneal cavity of patients harboring advanced ovarian cancer[14]. It is therefore possible that tumor-associated tolerogenic DC may play a major role in the subversion of the immune system commonly detected in the peritoneal cavity of ovarian cancer patients[14]. Adoptive transfusions of tumor-specific *in vitro* activated T-cells, which avoid the potential problems associated with inducing a CTL response *in vivo*, might therefore be a more effective approach for control of tumor growth in patients harboring advanced stage ovarian cancer.

Although the identity and distribution of antigenic peptides and CTL epitopes presented by freshly

isolated epithelial ovarian tumors are still poorly known, recent studies have unequivocally shown that multiple tumor antigens do exist that can be recognized by CTLs and used as target molecules to induce autologous tumor cell killing *in vitro*[25,26,42]. Consistent with this view, we have recently shown the *in vitro* induction from peripheral blood of tumor specific CD8+ CTL in advanced ovarian cancer patients using unfractionated tumor-derived antigens either as whole tumor cell lysate or as acid-eluted HLA class I-associated peptides isolated from tumor cells[30,31]. Tumor infiltrating lymphocytes represent a population of antigen-experienced CD8+ T-cells which have been previously demostrated to be endowed with 50–100 times stronger antitumor activity compare to *in vitro* activated peripheral blood lymphocytes[43]. TIL could therefore represent a superior source of tumor specific CTL to be used in the adoptive immunotherapy for advanced ovarian cancer. However, the immunocompetence of human TIL and the possibility to consistently restore their tumor specific lytic activity *in vitro* remain an unresolved issue.

In this study, as a basis for the development of more effective immunotherapy protocols for the treatment of this disease, we performed a careful phenotypic and functional analysis of lymphocytes derived from PBL, TAL, and TIL before and after *in vitro* stimulation with tumor antigen-pulsed autologous mature DC.

We found TIL to be a consistently superior source of tumor specific CTL compared to TAL and PBL. Indeed, although not cytotoxic immediately after collection (data not shown), suggesting that a state of anergy exists *in vivo*, induction of higher cytotoxic activity against autologous ovarian tumor cells was consistently noted in TIL populations from all three patients when compared to CD8+ T-cells derived from PBL and TAL. The lack of significant cytotoxicity against HLA-identical autologous LCL confirmed that, although TIL-derived CTLs were highly cytolytic against autologous tumor cells, they failed to kill autologous control target cells expressing different antigens. Furthermore, the fine specificity of the TIL-derived populations of CTL was also confirmed by the significantly higher block in cytotoxic activity detected using anti-HLA class I Mab when compared to TAL or PBL-derived CTL, and by their minor cytotoxic activity against K562 natural killer sensitive target cells.

It is worthy noting that while CD3+ T-cells were the major lymphocyte population detected in each tissue, lymphocytes used at the time of *in vitro* stimulation with tumor antigen-pulsed DC were predominantly CD4+ T-cells in PBL and TAL, but not in TIL (CD4:CD8 ratio: 3.1 versus 2.2 versus 1.0, respectively). After *in vitro* restimulation with antigen loaded fully mature DC, CD4 T-cells remained predominant in PBL and TAL cultures, while in TIL cultures a predominant population of CD8+ T-cells was consistently noted. CD56+/CD16+/CD3– NK cells and CD8+/CD56+ T-cells were predominant in PBL compared to TAL and TIL at the beginning of culture. However, after *in vitro* stimulation and expansion of CD8+ T-cells, TIL contained a significantly higher percentage of CD8+/CD56+ T-cells compared to TAL and PBL. These results were found to correlate with a significantly higher cytotoxic activity in the CD8+ CTL populations derived from TIL compared to those derived from TAL and PBL. Taken together these data support the view that, as previously described by us[30,31] as well as others[44] in CD8+ T-cells derived from peripheral blood, CD56 expression on CD8+ T-cells derived from TIL is an activation antigen associated with higher cytotoxic function, rather than a lineage-specific marker.

In agreement with previous reports[4,7] we found a lower relative expression of TcRζ chain in freshly isolated CD8+ T-cells derived from ovarian TIL and TAL compared to PBL. However, no significant difference in TcRζ chain expression compared to PBL was detected after *in vitro* stimulation of TAL and TIL with fully mature ovarian tumor antigen loaded DC. These data therefore demonstrate that tumor antigen loaded-DC stimulation may provide culture conditions able to induce restoration of TcRζ chain expression in TAL and TIL to the level detected in activated PBL populations.

Type 1 IFN-γ producing T-cells are believed to promote the development of cell-mediated immunity against viral infection as well as the control of tumors[45,46]. Consistent with this view, induction of *in vivo* antitumor immune responses, as well as effective adoptive cellular immunotherapy, have been reported to be more dependent on the induction of a host immune response triggered by Type 1 cytokines (ie, IL-2, IFN-γ, GM-CSF) than the *in vitro* cytotoxic activity of the responder or transferred lymphocytes[47,48]. Recently, a significant dysfunction of Type 1 T-cell responses concomitant with an abnormally elevated production of Type 2 cytokines in several human neoplasias has been reported[49] suggesting that tumor progression may be associated with a preferential and unprotective Type 2 T-cell bias. In our previous work comparing the phenotype of lymphocytes derived from PBL, TAL, and TIL in several advanced ovarian cancer patients[50], IFN-γ

expression was predominant in the CD8+ T-cell populations derived from TIL. Consistent with these data, in all three patients studied in this report, IFN-γ expression was predominant in TIL compared to the PBL and TAL-derived CD8+ T-cell populations. IL-4+ T-cells were detected in low numbers and mainly in PBL. After *in vitro* stimulation with tumor antigen-pulsed DC, a greater proportion of IFN-γ^+ cells was again detected in TIL as compared to TAL and PBL. This pattern of cytokine expression further supports the concept of a specific recruitment and accumulation of a high concentration of antigen-experienced tumor specific CD8+ T lymphocytes in the ovarian tumor tissue[50,51]. Furthermore, these data clearly suggest that the lack of *in vitro* cytotoxic activity commonly detected in fresh TAL and TIL population is not secondary to a Th2 cytokine switch.

In conclusion, we showed that lymphocytes derived from TIL may constitute a superior source of tumor specific HLA class I-restricted CTL compared to TAL and PBL for adoptive immunotherapy for advanced ovarian cancer. Although poor immune responses by explanted T lymphocytes might be expected, it is possible that this *in vivo* defective immunologic response against ovarian tumors may be normalized by *in vitro* T-cell stimulation with professional antigen-presenting cells such as fully mature DC. These results, combined with the fact that the most critical component of successful adoptive immunotherapy of cancer is the identification and isolation of large numbers of lymphocytes with potent and specific antitumor activity, warrant further research efforts for the development of improved protocols to counteract mechanisms leading to impaired TAL and TIL function, and thus exploit these populations for use in adoptive cellular immunotherapy of advanced ovarian cancer.

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References

1 Badger A, Oh S, Moolten F. Differential effects of an immunosuppressive fraction from ascites fluid of patients with ovarian cancer on spontaneous and antibody dependent cytotoxicity. *Cancer Res* 1981;**41:**1133–9.
2 Berek JS, Bast RC, Lichtensteint A *et al.* Lymphocyte cytotoxicity in the peritoneal cavity and blood of patients with ovarian cancer. *Obstet Gynecol* 1984;**64:** 708–14.
3 Heo DS, Whiteside TL, Kanbour A, Herberman RB. Lymphocytes infiltrating human ovarian tumors. I. Role of Leu-19 (NKH1) -positive recombinant IL-2-activated cultures of lymphocytes infiltrating human ovarian tumors. *J Immunol* 1988;**140:**4042–9.
4 Rabinowich H, Suminami Y, Reichert TE *et al.* Expression of cytokine genes or proteins and signaling molecules in lymphocytes associated with human ovarian carcinoma. *Int J Cancer* 1996;**68:**276–84.
5 Merogi AJ, Marrogi AJ, Ramesh R, Robinson WR, Fermin CD, Freeman CM. Tumor–host interaction: analysis of cytokines, growth factors, and tumor infiltrating lymphocytes in ovarian carcinomas. *Hum Pathol* 1997;**28:**321–31.
6 Pisa P, Halapi E, Pisa EK *et al.* Selective expression of interleukin-10, interferon-γ, and granulocyte-macrophage-colony stimulating factor in ovarian cancer biopsies. *Proc Natl Acad Sci USA* 1992;**89:**7708–12.
7 Nakagomi H, Petterson M, Magnusson I *et al.* Decrease expresssion of the signal transducing ζ chains in tumor infiltrating T-cells and NK-cells of patients with colorectal cancer. *Cancer Res* 1993;**53:**5610–2.
8 Hirte H, Clark DA. Generation of lymphokine-activated killer cells in human ovarian carcinoma ascitic fluid: identification of transforming growth factor β as a suppressive factor. *Cancer Immunol Immunother* 1991;**32:**296–302.
9 Gotlieb WH, Abrams JS, Watson JM, Velu TJ, Berek JS, Martinez-Maza O. Presence of IL-10 in the ascites of patients with ovarian and other intra-abdominal cancers. *Cytokine* 1992;**4:**385–90.
10 Granger G, Gatanaga T, Burger R, Grosen E, DiSaia P. TNF LT, IL-1 natural inhibitors (soluble receptors and receptor antagonists) in women with ovarian cancer. In: Sharp F, Mason WP, Blackett T, Berek J, eds. *Ovarian Cancer 3.* London: Chapman & Hall, 1995:115–9.
11 Santin AD, Hermonat PL, Ravaggi A, Cannon MJ, Pecorelli S, Parham GP. Secretion of vascular endothelial growth factor in ovarian cancer. *Eur J Gynecol Oncol* 1999; **3:**177–81.
12 Ioannides CG, Whiteside TL. T cell recognition of human tumors. implications for molecular immunotherapy of cancer. *Clin Immunol Immunopath* 1993;**66:**91–106.
13 Rabinowich H, Torsten RE, Kashii Y, Gastman BR, Bell MC, Whiteside TL. Lymphocytes apoptosis induced by Fas-ligand-expressing ovarian carcinoma cells. Implications for altered expression of T cell receptor in tumor associated lymphocytes. *J Clin Invest* 1998;**101:** 2579–88.
14 Melichar B, Savary C, Kudelka AA *et al.* Lineage negative HLA-DR positive cells with the phenotype of undifferentiated dendritic cells in patients with carcinoma of the abdomen and pelvis. *Clin Cancer Res* 1998;**4:** 799–809.
15 Young JW, Inaba K. DCs as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. *J Exp Med* 1996;**183:**7–11.
16 Schuler G, Steinman RM. Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J Exp Med* 1997;**186:**1183–7.
17 Romani N, Gruner S, Brang D *et al.* Proliferating dendritic cell progenitors in human blood. *J Exp Med* 1994; **180:**83–90.
18 Sallusto F, Lanzavecchia A. Efficient presentation of soluable antigen by cultured human dendritic cells is

maintanined by granulocyte/macrophage colony stimulating factor plus interleukin-4 and down regulated by turner necrosis factor alpha. *J Exp Med* 1994;**17:**1109–16.
19 Santin AD, Hermonat PL, Ravaggi A *et al.* Kinetics of expression of surface antigens during the differentiation of human dendritic cells versus macrophages from monocytes *in vitro*. *Immunobiol* 1999;**200:**187–204.
20 Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;**392:**245–52.
21 Jonuleit H, Kuhn U, Muller G *et al.* Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol* 1997;**27:**3135–42.
22 Dhodapkar MV, Steinman RM, Sapp M *et al.* Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J Clin Invest* 1999;**104:**173–80.
23 Dhodapkar MV, Krasovsky J, Steinman RM, Bhardwaj N. Mature dendritic cells boost functionally superior CD8+ T-cell in humans without foreign helper epitopes. *J Clin Invest* 2000;**105:**R9–R14.
24 Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* 2001;**193:**233–8.
25 Ferrini S, Biassoni R, Moretta A, Bruzzone M, Nicolin A, Moretta L. Clonal analisys of T lymphocytes isolated from ovarian carcinoma ascitic fluid, phenotype and functional characterization of T-cell clones capable of lysing autologous carcinoma cells. *Int J Cancer* 1995; **36:**337–43.
26 Peoples GE, Schoof DD, Ravan-Andrews V, Goedegebuure PS, Eberlein TJ. T-cell recognition of ovarian cancer. *Surgery* 1993;**114:**227–34.
27 Whiteside TL. Tumor infiltrating lymphocytes in human malignancies. Austin, TX: Medical Intelligence Unit, R.G. Landes, 1993.
28 Tartour E, Latour S, Mathiot C *et al.* Variable expression of CD3-zeta chain in tumor-infiltrating lymphocytes (TIL) derived from renal-cell carcinoma: relationship with TIL phenotype and function. *Int J Cancer* 1995;**63:**205–12.
29 Chen YM, Yang WK, Whang-Peng J *et al.* Restoration of the immunocompetence by IL-2 activation and TCR-CD3 engagement of the *in vivo* anergized tumor-specific CTL from lung cancer patients. *J Immunoth* 1997;**20:**354–64.
30 Santin AD, Ravaggi A, Bellone S, Pecorelli S, Cannon MJ, Parham GP. Induction of ovarian tumor-specific CD8+ cytotoxic T lymphocytes by acid-eluted peptide-pulsed autologous dendritic cells. *Obstet Gynecol* 2000;**96:**422–30.
31 Santin AD, Hermonat PL, Ravaggi A *et al.* Induction of tumor-specific HLA class I restricted CD8+ cytotoxic T lymphocytes by ovarian-tumor antigen pulsed autologous dendritic cells in patients with advanced ovarian cancer. *Am J Obstet Gynecol* 2000;**183:**601–9.
32 Whiteside TL, Miescher S, MacDonald HR, Von Fliedner V. Separation of tumor infiltrating lymphocytes from tumor cells in human solid tumors. A comparison between velocity sedimentation and discontinuous density gradient. *J Immunol Meth* 1986;**90:**221–33.
33 Hsu FJ, Benike C, Fagnoni F *et al.* Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nature Med* 1996;**2:**52–8.
34 Nestle FO, Alijagic S, Gilliet M *et al.* Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nature Med* 1998;**4:**328–32.
35 Thurner B, Haendle I, Roder C *et al.* Vaccination with Mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999;**190:**1669–78.
36 Kugler A, Stuhler G, Walden P *et al.* Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. *Nature Med* 2000;**6:**332–6.
37 Geiger JD, Hutchinson RJ, Hohenkirk LF *et al.* Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression. *Cancer Res* 2001;**61:**8513–9.
38 Santin AD, Bellone S, Gokden M, Cannon MJ, Parham GP. HPV-18 E7 pulseddendritic cells in a patient with metastatic cervical cancer. *N Engl J Med* 2002;**346:**1752–3.
39 Steinman RM & Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci USA* 2002;**99:** 351–8.
40 Aoe T, Okamoto Y, Saito T. Activated macrophages induce structural abnormalities of the T cell receptor-CD3 complex. *J Exp Med* 1995;**181:**1881–6.
41 Kono K, Salazar-Onfry F, Petterson M *et al.* Hydrogen peroxide secreted by tumor-derived macrophages down-modulates signal-transducing zeta molecules and inhibits tumor-specific T cell and natural killer cell-mediated cytotoxicity. *Eur J Immunol* 1996;**26:**1308–13.
42 Gong J, Nikrui N, Chen D *et al.* Fusions of human ovarian carcinoma cells with autologous or allogeneic dendritic cells induce antitumor immunity. *J Immunol* 2000;**165:**1705–11.
43 Rosenberg SA, Packard BS, Aebersold PM *et al.* Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma: a preliminary report. *N Engl J Med* 1988;**319:**1676–80.
44 Pittet MJ, Speiser DE, Valmori D, Cerottini J-C, Romero P. Cytolytic effector function in human circulating CD8+ T cells closely correlated with CD56 surface expression. *J Immunol* 2000;**164:**1148–52.
45 Romagnani S. Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. *Int Arch Allergy Immunol* 1992;**98:**279–85.
46 Takashi N, Iwakabe K, Sekimoto M *et al.* Distinct role of antigen-specific T helper Type 1 (Th1) and Th2 cells in tumor eradication *in vivo*. *J Exp Med* 1999;**190:**617–24.
47 Barth RJJ, Mule JJ, Spiess PJ, Rosenberg SA. Interferon gamma and tumor necrosis factor have a role in tumor regression mediated by murine CD8+ tumor infiltrating lymphocytes. *J Exp Med* 1991;**173:**647–54.
48 Goedegebuure PS, Zuber M, Leonard-Vidal DL *et al.* Reactivation of murine tumor-infiltrating lymphocytes with solid phase anti-CD3 antibody: *in vitro* cytokine production is associated with *in vivo* efficacy. *Surg Oncol* 1994;**3:**79–89.
49 Clerici M, Shearer GM, Clerici E. Cytokine disregulation in invasive cervical carcinoma and other human neoplasias: time to consider the TH1/TH2 paradigm. *J Nat Cancer Inst* 1998;**90:**261–2.

50 Santin AD, Ravaggi A, Bellone S *et al.* Phenotypic and functional analysis of tumor infiltrating lymphocytes from ascitic fluid and peripheral blood lymphocytes in patients with advanced ovarian cancer. *Gynecol Obstet Invest* 2001;**51:**254–61.

51 Yamamoto K, Masuko K, Takahashi S *et al.* Accumulation of distinct T cell clonotypes in human solid tumors. *J Immunol* 1995;**154:**1804–9.

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