Eur J Med Res (2006) 11: 7-12 © I. Holzapfel Publishers 2006

Deficiences in Phenotype Expression and Function of Dentritic Cells from Patients with Early Breast Cancer

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Abstract
Purpose: Monocytes derived from patients with early breast cancer (EBC) have shown functional deficiencies. These functional deficiencies are characterized by changes in phenotype and morphology. We have expanded these investigations to dendritic cells generated from monocytes from patients with early breast cancer.
Patients and Methods: Peripheral blood from 36 patients with EBC and from 26 healthy age-matched women was drawn and prepared for *ex vivo* generation of dendritic cells (DC) by incubation with granulocyte/macrophage-colony stimulating factor (GM-CSF) and interleukin 4 (IL4). The phenotype of DC was examined by flow-cytometry. T cell - proliferation was induced with tetanus toxoid pulsed autologous dendritic cell.
Results: Dendritic cells generated from monocytes from EBC-patients showed a significantly lower expression of the phenotype-associated antigens CD1a, CD83, CD80, CD86 and CD54 than the dendritic cells from healthy controls. T cell - proliferation in response to TT-pulsed autologous dendritic cells was significantly decreased when induced with dendritic cells from patients with early breast cancer, when compared to healthy controls. Morphologically, only dendritic cells from healthy women possessed prominent dendrites indicating maturity.
Conclusions: These findings indicate that dendritic cells generated from monocytes from patients with early breast cancer express an immature phenotype, exhibit immature morphology and show functional deficits when compared to the cells derived from healthy age-matched controls. Whether these findings offer a potential target for therapeutic interventions remains to be elucidated.

Key words: dendritic cells, breast cancer, phenotype, costimulatory molecules, antigen presentation.

Abbreviations: DCs: dendritic cells; EBC: early breast cancer; MHC: major histocompatibility complex; TT: tetanus toxoid; PBMC: peripheral blood mononuclear cells; ICAM-1: intercellular adhesion molecule-1 (CD54); CD80: B7-1; CD86: B7-2; APC: antigen presenting cell; FCS: fetal calf serum; IL-: interleukin; TNF: tumour necrosis factor; GM-CSF: granulocyte/macrophage – colony stimulating factor; LPS: lipopolysaccharide; BSA: bovine serum albumin; FITC: fluorescein isothiocyanate; HBSS: Hanks balanced salt solution; MFI: mean channel fluorescence intensity.

Introduction

Dendritic cells (DC) are of crucial importance for the initiation of primary immune responses by their high potency for antigen presentation. DC undergo a maturation process characterized by changes in phenotype and morphology. Phenotypic changes include an increase in the expression of the specific surface antigen CD83 [1]. In addition, co-stimulatory antigens, namely CD40, CD80, CD86, CD18/CD11a (LFA-1) and CD58 (LFA-3) [2], antigens associated with the major histocompatibility complex (MHC) including CD1a, which is important for antigen capture and processing [3], and intercellular adhesion molecules CD54 (ICAM-1), CD102 (ICAM-2) and CD50 (ICAM-3) are expressed. Morphological changes of DC are indicated by the presence or absence of prominent dendrites. Their appearance depends on the degree of cell maturation, because immature DC do not possess dendrites.

Similar to other antigen-presenting cells (APCs), DC interact with T cells by presentation of processed antigen via the T-cell receptor in the presence of CD3 and MHC-associated antigens, but also by DC-associated co-stimulatory molecules, namely CD80 and CD86, which deliver a secondary signal to specific T-cell-associated ligands CD28 and CD152 (CTLA-4). The intact interaction between co-stimulatory molecules and their ligands is mandatory for an appropriate immune response, whereas the absence of co-stimulatory signals results in anergy or induction of apoptosis [4]. Among other entities, cell death was suggested to be operational in patients with early breast cancer due

to the absence of co-stimulatory molecules CD80, CD86 and CD54 on monocytes. Therefore, this absence of co-stimulatory molecules might contribute to the development of tolerance towards malignant cell clones [5]. Furthermore, the absence of these co-stimulatory molecules has been shown to result in defective autologous T cell proliferation following exposure to tetanus toxoid (TT)-pulsed monocytes derived from patients with early breast cancer [5].

The present investigation expands these investigations to DC due to their crucial role for antigen presentation. It is hypothesized that their potential to overcome the mentioned monocyte defect might result in a therapeutic modality for the manipulation of the immune system in patients with early breast cancer. There is evidence that a maturation defect of DC might exist in patients with very limited malignancies including melanoma [6] and hepato-cellular carcinoma [7]. Conversely, DC derived from patients with renal carcinoma were found to express high concentrations of the co-stimulatory molecule CD86 [8]. In early breast cancer, recent investigations have vaguely proposed a maturation defect of DC suggested by their decreased ability to produce IL12, their increased ability to induce expression of IL10 and their decreased allo-stimulatory activity as well as rapid apoptosis [9]. This is consistent with recent reports that patients with early breast cancer had significantly higher levels of the immuno-supressive cytokine IL-10 and reduced levels of IL-12, respectively [10, 11]. These findings corroborated previous data on the low expression of CD1a by DC derived from patients with breast cancer [12] and the presence of immature DC within malignant breast tissue [13]. A published phase I clinical trial based upon 10 patients with breast cancer with MUC-1-pulsed DC resulted in the induction of a partial response in one and in a stabilization of the disease in another patient [14]. Future research results will provide the final answer whether this is just anectodal coincidence or part of a larger consistent picture.

Materials and Methods

Patients and Controls

A total of 36 females with stage I or II early breast cancer with a median age of 59 years (range: 25 to 90) were studied. Out of these 36 patients, 17 patients had not received any adjuvant treatment, 8 patients had received adjuvant tamoxifen (20mg/day) and 7 were studied on day 1 of the second cycle of adjuvant chemotherapy with cyclophosphamide, methotrexate and 5-fluorouracil, whereas 4 received other adjuvant chemotherapy. From the 17 patients without adjuvant treatment blood was drawn before surgery to exclude any immunosuppressive effects induced by the intervention itself.

Twenty six healthy age-matched women with a median age of 53 (range: 22-87) years without an individual or family history of breast cancer and without clinical, radiological and serologic evidence of the presence of breast cancer served as parallel controls.

Isolation of Monocytes

Monocytes (peripheral blood mononuclear cells, PBMC) were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) from freshly drawn heparinized peripheral venous blood. The cells were washed three times with 0.9% saline, resuspended and adjusted to 1 x 10^6 cells/mL in RPMI 1640 supplemented with 10% human AB-Serum (all from Gibco, Paisley, UK), 50 IU/mL penicillin and 50 μg/mL streptomycin (HyClone, Cramlington, UK) and plated in Petri dishes. Non-adherent cells were then removed by repeated washing. Purity of cell separation was tested by non-specific esterase staining and purity results indicate a 90-95% pure monocyte cell population, finally.

Generation of Dendritic Cells from Monocytes by Cytokines and Phenotype Analysis

DC were generated from monocytes using a standard protocol [1]. GM-CSF (1000U/mL, Novartis Basel) and IL-4 (1000U/mL, Pharmingen, San Diego, CA) were added on days 2, 4 and 6. The cells were harvested on day 7 of culture.

Analysis of Dendritic Cell Phenotype by FACS

The phenotype of DC was analyzed by flow cytofluorometry. Cell monolayers were briefly trypsinized, vigorously vortexed and washed in phenol red-free Hanks balanced salt solution (HBSS; Gibco, UK) containing 0.3% bovine serum albumin (Sigma, St. Louis, MO) and 0.1% NaN3. Cells were incubated with 50μL 20% human AB-Serum (BIOTEST AG, Dreieich, Germany) in HBSS at room temperature for 30 minutes. Then the cells were washed twice with HBSS and incubated with the respective antibody (see below) at 4 °C for 30 minutes. After 2 more washing cycles, the cells were resuspended in 300μL of HBSS and analyzed immediately. Indirect immunofluorescence and flow cytofluorometry were performed using a FACScan (Becton-Dickinson, Comp. Mountain View, CA) cytofluorometer. According to the manufacturer's instructions, results were calculated using the LYSYS II software. Background fluorescence was assessed using murine IgG1 (20μL per sample, see above). The percentages of positive cells and the fluorescence histograms were recorded for each sample.

Antibodies for FACS Analysis

Fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mouse IgG1) directed against CD54 (R&D System, Minneapolis, MN), CD1a, CD14, CD80, CD83 (B7-1) (all from PharMingen, San Diego, CA) and a PE-labeled monoclonal antibody (mouse IgG1) directed against CD86 (B7-2) and CD 152 (CTLA4; B7-2) (PharMingen) were used. FITC and PE-labeled mouse IgG1 antibodies (PharMingen) were used in control experiments.

Exclusion of Contamination of DC Preparations by T Cells or by Monocytes

The presence of T cells or monocytes in the preparations of DC was controlled by the assessment of expression of antigens CD3, CD4, CD8 and CD14.

Preparation of T Cell-enriched Lymphocytes

Monocytes were separated as described above; T cells were separated after 2 hours. Non-adherent cells were removed by repeated washing, and CD4+ T cells were isolated by magnetic immunoselection. Cells were washed twice, mixed with sheep anti-mouse IgG-coated beads (Dynabeads M450, Hamburg, Germany) at a final concentration of 1 x 10^7 beads/mL with 10^7 cells and incubated for 60 minutes at 4 °C under continuous rotation. CD4+ T cells bound to magnetic beads and were removed with a permanent magnet (HPC-1, Dynal A.S., Oslo, Norway). The remaining cells were collected, washed twice, and analyzed by flow-cytometry. Cell purities abound 90% - 95% CD4+ cells were considered sufficient.

Antigen Presentation and Assessment of CD4+ T Cell Proliferation

Triplicate cultures containing adherence-purified DC (1 x 10^4/well) and CD4+ T cells (1 x 10^5/well) were set up in flat-bottomed microtiter plates (Falcon, Lincoln Park, New Jersey), and 25ng of Tetanus Toxoid (TT)/mL (ID Biomedical Corpration, Vancouver, Canada) were added. Cells were kept suspended in RPMI 1640 medium supplemened with 10% (vol/vol) heat inactivated human AB serum, 100 IU/mL penicillin and 100µg /mL streptomycin for 5 days at 37 °C in a humidified atmosphere containing 5% CO_2. (^{3}H)Thymidine (Amersham International Life Science, Buckinghamshire, UK) at a concentration of 0.5 µCi/well for the final 16 hours. Then the cells were harvested with the Harvester Micromate 196 onto Glass Fiber Filters and the incorporation of (^{3}H) thymidine into DNA was measured by a Direct Beta Counter-Matrix 96 (all purchased from Packard, Groningen, Netherlands).

Microscopic Evaluation

DC derived from patients and controls were examined by light microscopy (Olympus CK2, Tokyo, Japan) for length, location and form of dendrites.

Statistics

Student's t-test was used for statistical analysis. A two-sided p-value of <0.05 was considered to indicate statistically significant differences. Age data was displayed as median and minimum and maximum. All other data are expressed as mean ± standard deviation (SD).

Results

Examination of Purity of DC Preparations

All preparations of DC were checked for contamination by T cells or monocytes by expression of CD3, CD4, CD8 and CD14 antigens. Only CD3- CD4- CD8- and CD14 negative cell preparations were used for further experimental evaluations. Purity of DC was 90-95% by definition of CD4-CD8- antigen expression. As described below, the presence of monocytes was monitored in all experiments by CD14 expression. Light microscopy of DC generated from monocytes from healthy controls showed the expected long and spiny processes (>10µm) with large and prominent dendrites (Fig.1A), whereas these cells presented with shortened and badly developed dendrites when derived from patients with early breast cancer (Fig.1B).

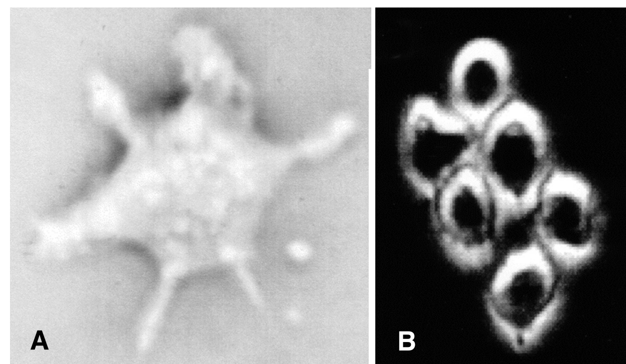

Fig. 1. Morphology of dendritic cells derived from a healthy female (A) and from a patient with early breast cancer (B).

Expression of CD1a and CD83 and of Co-stimulatory molecules CD80, CD86 and CD54 (ICAM-1) on Dendritic Cells Derived from Patients with EBC and Healthy Age-matched Controls

Figure 2 shows FACS histograms of DC from one patient with early breast cancer and from one healthy control. The expression of CD1a and CD83 was significantly lower on DC derived from patients with early breast cancer, than on DC derived from healthy controls (Table 1; $p < 0.0001$ and $p = 0.0001$, respectively). In addition the expression of the co-stimulatory molecules CD80, CD86 and of intercellular adhesion molecule -1 (ICAM-1, CD54) also was significantly lower on DC derived from patients with early breast cancer, than on those derived from healthy controls (Table 1; $p = 0.001$, $p<0.0001$ and $p<0.0001$, respectively). Adjuvant treatment (tamoxifen, chemotherapy) did not influence these results (data not shown). Generation of DC from monocytes with 2- and 5-fold concentrations of cytokines and prolonged incubation times varying between 24 and 72 hours yielded identical results (Table 1) thus ruling out kinetic effects on maturation. Two different methods for the generation of DC from monocytes [1, 15] were used and proved equal (data not shown).

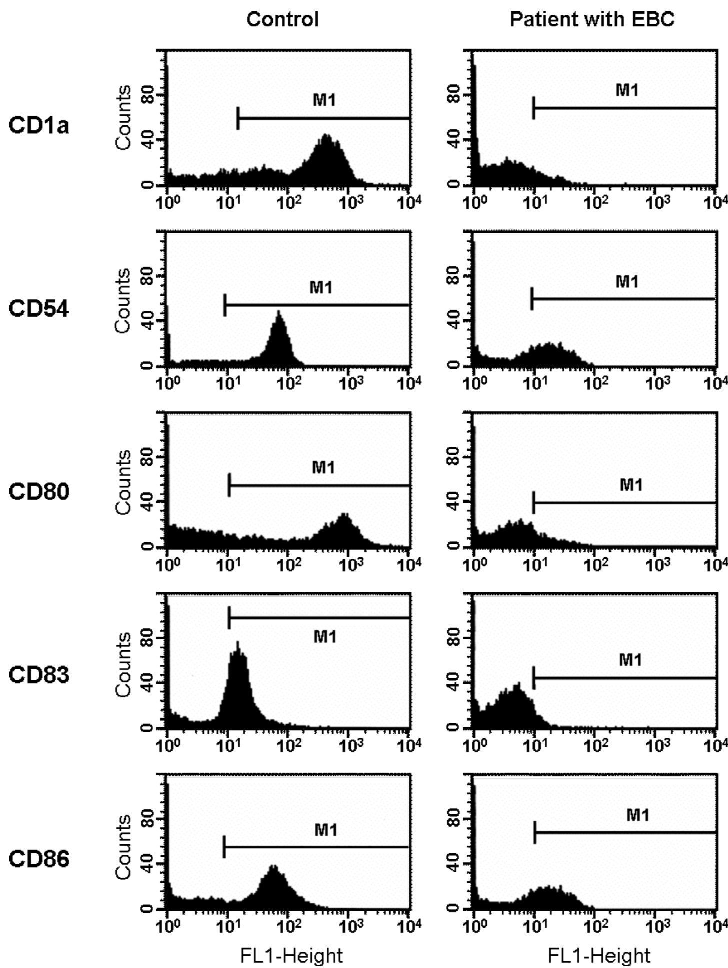

Fig. 2. FACS histograms of dendritic cells (DC) stained with typical markers of DC maturity of a patient with EBC in comparison to DCs from a healthy control female. M1 indicates number of positive cells with a cut-off of 5% for separation against unspecific background counts.

Table 1. Phenotype of dendritic cells generated from monocytes from patients with early breast cancer (EBC) and from healthy age-matched control females.

	CD1a	CD54	CD80	CD83	CD86
Healthy controls, n = 26	59.1 ± 34.9#	58.5 ± 23.3	36.9 ± 19.2	53.5 ± 27.2	50.2 ± 30.3
Patients with EBC, n = 36	7.2 ± 18.2	10.8 ± 21.2	5.1 ± 10.3	2.3 ± 3.6	9.8 ± 18.0
Comparison p-value	<0.0001	<0.0001	<0.001	<0.0001	<0.0001

mean percentage of positive cells ± SD

Tetanus Toxoid Induced CD4+ T Cell Proliferation

CD4+ T cell proliferation was measured after exposure to TT-pulsed DC generated from monocytes from patients with early breast cancer. Patients with EBC had a significantly lower T cell proliferation than healthy controls in an identical experimental setting (Fig. 3). Adjuvant treatment (tamoxifen or chemotherapy) did not influence T-cell proliferation in these experiments (data not shown).

Dicussion

We found that DC generated from monocytes from patients with early breast cancer showed a significantly lower expression of CD1a, CD83, CD80, CD86 and CD54 than those of healthy age-matched controls. Be-

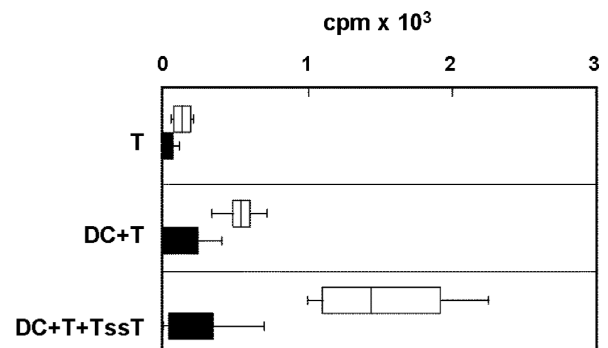

Fig. 3. Significantly decreased T cell proliferation by autologous dendritic cells derived from patients with EBC, as compared to healthy age-matched control females. DT derived from patients showed significantly decreased presentation of the used antigen Tetanus Toxoid (Tsst) to T cells (T): Tsst+DC+T.

cause these antigens characterize mature DC these findings indicate phenotypic immaturity. This indication of immaturity of DC derived from patients with EBC was strengthened by morphological immaturity indicated by a defect in dendrite formation demonstrated by light microscopy. Additionally, TT-pulsed DC generated from monocytes from patients with early breast cancer had reduced ability to induce appropriate proliferation in T- cells.

In previous experiments monocytes derived from patients with early breast cancer presented with an expression defect of co-stimulatory molecules CD80, CD86 and CD54, which might contribute to the development of tolerance towards malignant cell clones [5]. The absence of these co-stimulatory molecules has been shown to result in defective autologous T cell proliferation following exposure to tetanus toxoid pulsed monocytes derived from patients with EBC [5].

The described characteristics of DC found in addition to previously described defects in co stimulatory antigen expression and antigen presentation by monocytes derived from patients with early breast cancer may explain the reduced ability of patients with early breast cancer to launch a primary immune response [16, 17] together with a potentially defective induction of cytotoxic T cell function directed against tumor cells [18]. The latter is physiologically mediated by the interaction of the DC-associated antigen CD40 with its corresponding ligand CD40L expressed on activated T lymphocytes in the presence of a co-stimulatory signal [19-21]. The presently described defect in the expression of co-stimulatory molecules on DC could not be abolished by either changes in kinetics or concentrations of cytokines used for the generation of DC. Moreover, our data are consistent with observations by others that breast cancer tissue either contains low numbers or is completely void of DC simultaneously with a decreased expression of antigens CD83 and CD1a [15].

Maturation defects of DC have been described in patients with melanoma [6] and hepato-cellular carcinoma [7] In contrast, DC from patients with renal carcinoma were found to highly express the co-stimulatory molecule CD86 [8]. In early breast cancer, recent investigations [22, 23] have also suggested a maturation defect of DC based on their decreased ability to produce IL12, an increased ability to induce expression of the immunosuppressive cytokine IL10 and their decreased allo-stimulatory activity as well as rapid apoptosis [9]. Furthermore, it has been reported recently that patients with early breast cancer had significantly enhanced levels of IL-10 as well as reduced levels of IL-12 [10, 11]. Other corroborating studies have described low expression of CD1a by DC derived from patients with breast cancer [12] and the presence of immature DCs within malignant breast tissue [13]. The physiological consequences of these observations and their relevance for anti-tumor immunity are relatively unclear. Clinically, the application of MUC-1-pulsed DC in a phase I trial has resulted in the induction of one partial response and one disease stabilization in 10 patients with breast cancer [14].

Our results are consistent with a picture of significant immune dysfunction in early breast cancer. We conclude that DC generated from monocytes derived from patients with early breast cancer had an immature phenotype which resulted in a functional deficiency in antigen-induced T cell proliferation. These data add further evidence to the concept of breast cancer representing a systemic disorder which includes a functional impairment of monocytes, T-cells [5], cytotoxic effector cells [18] and – as demonstrated in this study - also DC. Whether these results may have consequences for therapeutic approaches to induce specific anti-tumour immunity in early breast cancer needs further investigations.

Acknowledgement: The authors want to thank Dr. David Parkinson for fruitful discussions and for reviewing the manuscript. Ms. Waclawa Kalinowski delivered expert technical assistance and Dr. Michael Gnant provided the infrastructure for the experimental setup.

References

1. Romani N, Reider D, Heuer M, Ebner S, Kampgen E, Eibl B, et al. (1996). Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. J Immunol Methods 196: 137-151.
2. Banchereau J, Steinman RM (1998). Dendritic cells and the control of immunity. Nature 392: 245-252.
3. Coventry BJ (1999). CD1a positive putative tumour infiltrating dendritic cells in human breast cancer. Anticancer Res 19: 3183-3187.
4. Slavik JM, Hutchcroft JE, Bierer BE (1999). CD28/CTLA-4 and CD80/CD86 families: signaling and function. Immunol Res 19: 1-24.
5. Wolfram RM, Budinsky AC, Brodowicz T, Kubista M, Kostler WJ, Kichler-Lakomy C, et al. (2000). Defective antigen presentation resulting from impaired expression of costimulatory molecules in breast cancer. Int J Cancer 88: 239-244.
6. Enk AH, Jonuleit H, Saloga J, Knop J (1997). Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. Int J Cancer 73: 309-316.
7. Ninomiya T, Akbar SM, Masumoto T, Horiike N, Onji, M (1999). Dendritic cells with immature phenotype and defective function in the peripheral blood from patients with hepatocellular carcinoma. J Hepatol 31: 323-331.
8. Thurnher M, Radmayr C, Ramoner R, Ebner S, Bock G, Klocker HR et al. (1996). Human renal-cell carcinoma tissue contains dendritic cells. Int J Cancer 68: 1-7.
9. Kiertscher SM, Luo J, Dubinett SM, Roth MD (2000). Tumors promote altered maturation and early apoptosis of monocyte- derived dendritic cells. J Immunol 164: 1269-1276.
10. Satthaporn S, Eremin O (2001). Dendritic cells (II): Role and therapeutic implications in cancer. J R Coll Surg Edinb 46: 159-167.
11. Satthaporn S, Eremin O (2001). Dendritic cells (I): Biological functions. J R Coll Surg Edinb 46: 9-19.
12. Coventry BJ, Austyn JM, Chryssidis S, Hankins D, Harris A (1997). Identification and isolation of CD1a positive putative tumour infiltrating dendritic cells in human breast cancer. Adv Exp Med Biol 417: 571-577.
13. Bell D, Chomarat P, Broyles D, Netto G, Harb GM, Lebecque S, et al. (1999). In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. J Exp Med 190: 1417-1426.

14. Brossart P, Heinrich KS, Stuhler G, Behnke L, Reichardt VL, Stevanovic S, et al. (1999). Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. Blood 93: 4309-4317.
15. Coventry BJ, Lee PL, Gibbs D, Hart DN (2002). Dendritic cell density and activation status in human breast cancer -- CD1a, CMRF-44, CMRF-56 and CD-83 expression. Br J Cancer 86: 546-551.
16. Rosen HR, Stierer M, Wolf HM, Eibl MM (1992). Impaired primary antibody responses after vaccination against hepatitis B in patients with breast cancer. Breast Cancer Res Treat 23: 233-240.
17. Zielinski CC, Stuller I, Dorner F, Potzi P, Muller C, Eibl MM (1986). Impaired primary, but not secondary, immune response in breast cancer patients under adjuvant chemotherapy. Cancer 58: 1648-1652.
18. Budinsky AC, Brodowicz T, Wiltschke C, Czerwenka K, Michl I, Krainer M, Zielinski CC (1997). Decreased expression of ICAM-1 and its induction by tumor necrosis factor on breast-cancer cells in vitro. Int J Cancer 71: 1086-1090.
19. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature 393: 478-480.
20. Ridge JP, Di Rosa F, Matzinger P (1998). A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. Nature 393: 474-478.
21. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 393: 480-483.
22. Brown JA, Dorfman DM, Ma FR, Sullivan EL, Munoz O, Wood CR, et al. (2003). Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. J Immunol 170: 1257-1266.
23. Della Bella S, Gennaro M, Vaccari M, Ferraris C, Nicola S, Riva A, et al. (2003). Altered maturation of peripheral blood dendritic cells in patients with breast cancer. Br J Cancer 89: 1463-1472.

Received: September 14, 2005 / Accepted: November 30, 2005

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