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TEXAS MEDICAL CENTER NASA/JOHNSON SPACE CENTER  
COOPERATIVE AGREEMENT PROGRAM NCC 9-36, ROUND I

COVER SHEET FOR FINAL REPORT

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Name of Subcontractor: Cherylyn A. Savary, Ph.D.  
Title: Instructor in Surgical Oncology  
Institution: University of Texas M.D. Anderson Cancer Center  
Name of Project: Role of Dendritic Cells in Immune Dysfunction  
Amount of Grant:   
Date Project Was Completed: June 30, 1997

Grants Officer: Donna S. Gilberg, CPA  
Title: Manager, Sponsored Programs  
Phone: (713)794-1825  
Fax: (713)796-0381

THE UNIVERSITY OF TEXAS  
MD ANDERSON  
CANCER CENTER

*Cherylyn A. Savary, Ph.D.*  
*Instructor in Surgical Oncology*  
*Department of Surgical Oncology, Box 18*  
*Ph. (713) 792-3134*  
*Fax (713) 794-4830*

June 30, 1997

Mary Schiflett, Program Administrator  
Texas Medical Center/NASA  
Cooperative Research Program  
Texas Medical Center  
406 Jesse H. Jones Library Building  
Houston, Texas 77030-2894

Dear Ms. Schiflett:

Please find enclosed the final summary report for Round 1 of our TMC/NASA Cooperative Agreement project entitled "Role of Dendritic Cells in Immune Dysfunction". We believe that we made several new and important observations in the investigations funded by this program - some of our results have been submitted in 2 manuscripts (attached as Appendix 1 and 2).

I am also pleased and excited to announce that some of the observations made in this study led to a new avenue of research which will be funded by a 4-year grant from NRA-96-OLMSA-03. This would not have been accomplished without the funding from the TMC/NASA Cooperative Agreement, and without the support and encouragement shown by you, Dr. Wainerdi and Dr. Pool.

I hope that the format and content of this summary is acceptable, but I will be ready to make any revisions that may be required or suggested.

Thank you.

Sincerely,



Cherylyn A. Savary, Ph.D.

**TEXAS MEDICAL CENTER NASA/JOHNSON SPACE CENTER  
COOPERATIVE AGREEMENT NCC 9-36  
Applications Subcontract Program, Round 1  
Final Report**

Name of Institution: University of Texas M.D. Anderson Cancer Center  
Name of Subcontractor: University of Texas M.D. Anderson Cancer Center  
Title of Project: Role of Dendritic Cells in Immune Dysfunction (Savary)

**Specific Aims:**

1. Application of the bioreactor to enhance cytokine-regulated proliferation and maturation of dendritic cells (DC).
2. Based on clues from spaceflight: compare the frequency and function of DC in normal donors and immunocompromised cancer patients.
3. Initiate studies on the efficiency of cytokine therapy and DC-assisted immunotherapy (using bioreactor-expanded DC) in animal models of experimental fungal infections.

**Studies and Results:**

Several novel and important observations were made during Round 1 which provide new insight into DC immunobiology, DC dysfunction in cancer patients, and characteristics of DC growth in the 3-dimensional culture conditions of the NASA bioreactor. These studies are summarized below. Additional details are provided in the attached manuscripts (Appendices 1 and 2).

*Generation of DC from CD34<sup>+</sup> progenitor cells in NASA bioreactors.* We and others have shown that human DC can be generated from early hematopoietic progenitors (i.e. CD34<sup>+</sup> cells isolated from bone marrow or from G-CSF mobilized peripheral blood) cultured for 1-3 weeks with cytokines in standard 2-dimensional tissue culture conditions. We now report that DC generation and maturation can also be achieved within the 3D environment of the NASA bioreactors (including the HARV, STLV and RCCS-D vessels). These DC were identified primarily by their characteristic morphology (numerous elongated cytoplasmic processes; Fig. 1), but also by their lack of lineage (lin)-associated surface markers, high expression of HLA-DR Class II molecules, and high allostimulatory activity in a mixed lymphocyte reaction - the latter function is considered a hallmark of DC (Fig. 2).

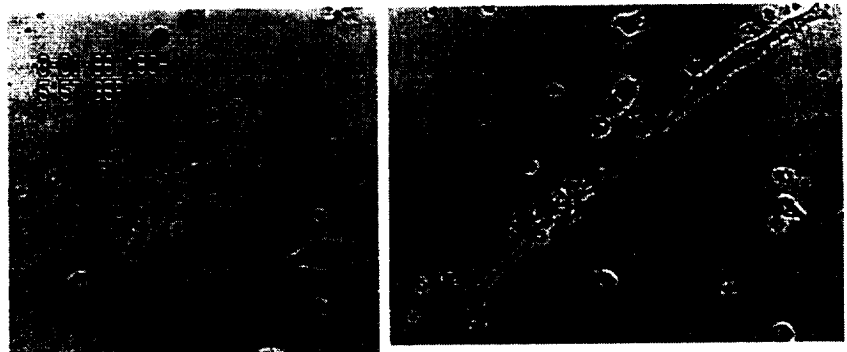
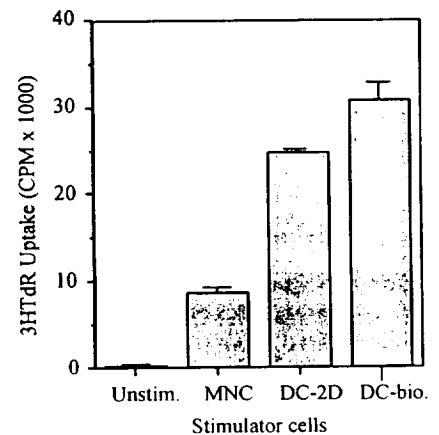
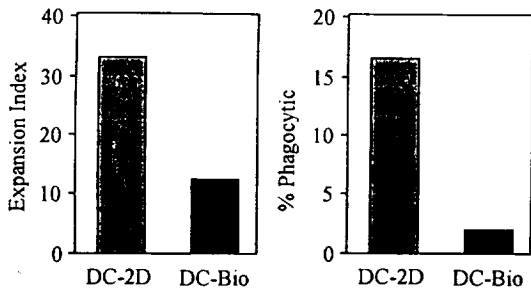


Fig 1. Morphology of DC generated from CD34 progenitors in the NASA bioreactor. Note the thinly veiled cell with long, branched processes in the center of the left panel and the elongated cell spanning the right panel.

However, several intriguing differences were observed when the DC generated in the bioreactors were compared to those generated

**Fig. 2** Superior allostimulatory activity of DC generated in the NASA bioreactor. DC generated in standard 2D and 3D bioreactor (bio) cultures were tested for the ability to stimulate an allogeneic mixed lymphocyte reaction (1:40 DC:lymphocyte ratio). Both DC populations were substantially more efficient stimulators compared to unseparated mononuclear cells (MNC), even when the latter were tested at a ratio as high as 1:1. Unstim= responder cells cultured without stimulators.

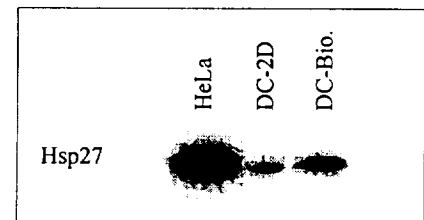




**Fig. 3** Proliferative and phagocytic activity of DC generated in 2D cultures and 3D bioreactors (Bio). Left panel: expansion index = no. cells after 10 days of culture ÷ no. cells at start of culture. Right panel: percentage of DC ingesting *Aspergillus* conidia. Representative experiments are shown.

in 2D: **first**, while cells proliferated in both conditions, the overall cell expansion in the 3D bioreactors within a 10-21 day period was only 25-50% of that achieved in 2D (Fig. 3, left panel); **second**, DC generated in the bioreactor displayed a reduced ability to phagocytose *Aspergillus* conidia (Fig. 3, right panel); **third**, despite these deficiencies in antigen uptake and proliferation, DC generated in 3D bioreactors often displayed higher allostimulatory activity (Fig. 2); **fourth**, cells from 3D cultures displayed higher levels of heat shock proteins (Hsp) (Fig. 4). While Hsp are upregulated by various stressors, as molecular chaperones they are involved in many processes in unstressed cells, including proliferation, antigen presentation, etc. Upregulation of Hsp in DC may reflect alterations in cellular functions.

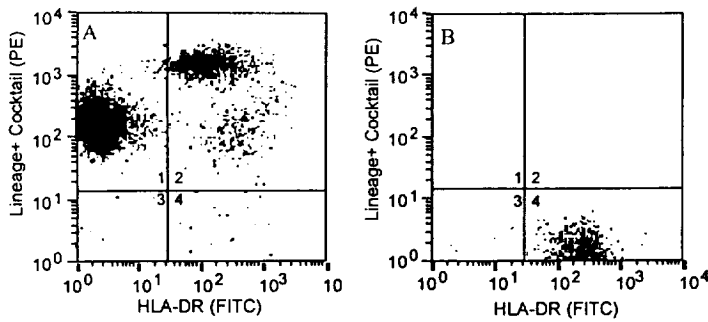
According to current models, DC maturation progresses from a less mature stage characterized by high antigen uptake/processing capabilities and low antigen-presenting function, to a more mature stage in which antigen uptake/processing capabilities are lost as antigen-presenting functions are enhanced. If this model is correct, our data would suggest that the DC generated in the bioreactors may be more mature than those from 2D cultures. As discussed below, this observation is important in light of the general opinion that terminally differentiated DC will be more suitable for therapeutic purposes.



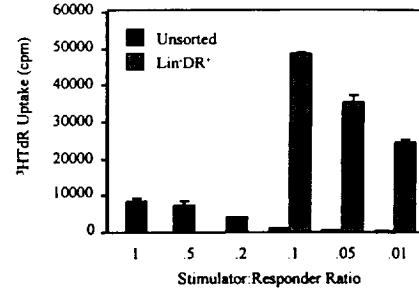
**Fig. 4** Upregulation of heat shock proteins in DC generated in the NASA bioreactor (Bio). Hsp27 was detected in lysates of DC and HeLa cells (positive control) using standard Western blotting techniques.

*Comparison of DC of normal donors and immunocompromised cancer patients* (Appendices 1 and 2). Patients recovering from stem cell transplantation are immunocompromised and thus at high risk of infection. We hypothesized that a deficiency in DC might be an underlying cause of the lymphopenia experienced by these patients, since antigen presenting cells are required for the optimum activation and proliferation of T lymphocytes. Because DC are present in only trace amounts in blood, it is not possible to purify these cells from severely lymphopenic individuals. Therefore, we devised a method to detect DC directly within peripheral blood mononuclear cell (PBMC) samples - a technique that required only small samples of blood and minimal manipulation (i.e. Ficoll-hypaque separation).

First, we used multidimensional flow cytometry to show that magnetically sorted DC isolated from blood of normal donors displayed a morphology, size profile, surface phenotype and functional properties of typical DC described previously by other investigators, i.e. these DC displayed high forward light scatter (Appendix 1); expressed CD4, HLA-DR (MHC-Class II), CD86 (costimulatory molecules), and CD54 (adhesion/costimulatory molecules) surface structures, but lacked the lin markers of T cells, NK cells, B cells, macrophages or granulocytes (Fig. 5 and Table 1). These lin<sup>-</sup>DR<sup>+</sup> cells also displayed high allostimulatory activity (Fig. 6), and showed the typical morphology of DC after culture with GM-CSF and TNF $\alpha$  (Fig. 7). Next, we used a cocktail of antibodies directed against the lin antigens (first color) and anti-HLA-DR (second color), to establish that lin<sup>-</sup>DR<sup>+</sup> DC-like cells could be detected directly in unseparated PBMC samples (Fig. 8). When analyzed by 3-color flow cytometry, these lin<sup>-</sup>DR<sup>+</sup> DC, like the sorted DC, coexpressed CD4, CD86 and CD54 surface antigens (Table 1). Using this technique, we then analyzed PBMC from breast cancer patients (all undergoing some form of chemotherapy) and patients who were 2-6 months post stem cell transplantation. Fig. 9 indicates that the number of circulating DC detected in both groups of patients was significantly lower than that of normal donors.



**Fig. 5** Enrichment of lineage negative HLA-DR<sup>+</sup> (lin<sup>-</sup>DR<sup>+</sup>) leukocytes by immunomagnetic sorting. Unsorted PBMC (Panels A) and cells sorted using a DC isolation kit (Panels B) were analyzed by flow cytometry. DC-like cells were characterized as the lin<sup>-</sup>DR<sup>+</sup> events in quadrant 4. The lineage cocktail consisted of PE-conjugated mAbs recognizing TCRαβ, TCRγδ, CD19, CD56 and CD14 antigens.



**Fig. 6** Allostimulatory activity of immunomagnetically sorted lin<sup>-</sup>DR<sup>+</sup> cells.

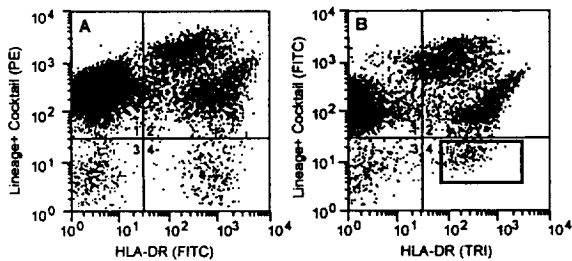


**Fig. 7** Morphology of immunomagnetically sorted lin<sup>-</sup>DR<sup>+</sup> leukocytes.

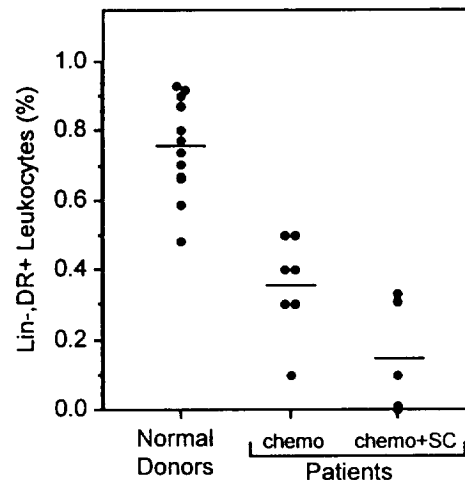
**Table 1.** 3-Color flow cytometric analysis of lin<sup>-</sup>DR<sup>+</sup> leukocytes in unsorted and immunomagnetically sorted peripheral blood specimens.

Expt. No.	Percent of lin-DR+ cells coexpressing the particular antigen				
	CD4	CD54	CD86	CD80	CD1a
PBMC-1	94.6	98.1	93.2	5.3	6.0
2	87.2	93.6	86.5	0.6	0.3
3	92.2	97.4	99.3	1.6	1.3
Sorted 1	-	92.2	99.3	1.6	2.0
2	-	99.3	87.5	4.5	-
3	-	98.8	86.5	3.2	6.7

Lin<sup>-</sup>DR<sup>+</sup> cells in unsorted PBMC or immunomagnetically sorted populations were analyzed for coexpression of several other surface markers using 3-color flow cytometric analysis. Sorted cells were positively selected for CD4 expression and not reanalyzed for this antigen..



**Fig 8** Flow cytometric analysis of lin<sup>-</sup>DR<sup>+</sup> leukocytes within PBMC preparations. **Panel A:** PBMC were labeled with a cocktail of PE-conjugated mAbs recognizing CD3, CD19, CD20, CD16, CD56, CD11b and CD14, and FITC-conjugated anti-HLA-DR mAbs. **Panel B:** The same population was labeled with a cocktail of FITC-conjugated lineage mAbs and TRI-anti-HLA-DR. The boxed lin<sup>-</sup>DR<sup>+</sup> events in quadrant 4 were further analyzed for coexpression of a third marker (results shown in Table 1).



**Fig. 9** Frequency of lin<sup>-</sup>DR<sup>+</sup> cells in PBMC of normal donors and cancer patients. Patients included 7 breast cancer patients who received prior chemotherapy (chemo) and 5 patients who had received stem cell transplants (chemo+SC) 2-6 months prior to testing. Horizontal lines represent the mean.

A similar analysis was performed on peripheral blood and ascites fluid samples of ovarian cancer patients (see manuscript, Appendix 2). In these patients also, the frequency of circulating DC-like lin<sup>-</sup>DR<sup>+</sup> cells in blood was reduced compared to normal donors ( $0.4 \pm 0.3\%$  versus  $0.9 \pm 0.3\%$ , respectively;  $p < 0.02$ ) Interestingly though, a higher proportion of lin<sup>-</sup>DR<sup>+</sup> cells was detected within the ascites fluid compared to blood samples from the same patients ( $4.5 \pm 5.7\%$  versus  $0.5 \pm 0.4\%$ , respectively;  $p < 0.05$ ). However, the ascites-derived DC expressed a the lower density of HLA-DR molecules compared to DC in peripheral blood, suggesting that they might have been negatively affected by factors associated with the tumor environment. All of these studies point to the potential usefulness of multidimensional flow cytometry for evaluation of DC in various tissue compartments.

*Analysis of murine DC generated in the NASA bioreactor.* Our studies of murine DC are still underway, but indicate that these cells can also be cultivated from unseparated bone marrow cell suspensions in the RCCS-D. In fact, a higher proportion of NLDC-145<sup>+</sup> cells (a surface marker expressed by murine DC) has been detected in 3D bioreactor cultures compared to standard 2D conditions (Table 2). Somewhat in contrast to the human studies, we have observed a higher proliferation of murine cells within the bioreactor compared to standard 2D cultures. Future studies will compare the ability of 2D and bioreactor-generated DC to protect mice from infection with *Aspergillus* or *Candida*.

**Table 2.** Generation of murine DC in the NASA bioreactor

Expt. No.	% NLDC-145 <sup>+</sup> cells	
	2D Cultures	3D Bioreactor
1	3	18
2	9	24

**Significance.** DC are considered to be the most potent of antigen presenting cells. Only recently though, has it been possible to expand this rare leukocyte subset *in vitro* in cytokine-supplemented cultures. To our knowledge, our studies are the first of their kind to generate and cultivate DC in a 3D environment. One of our long-term goals is to generate sufficient numbers of fully differentiated DC for *in vivo* supplementation/restoration of patients who we now show have low numbers of circulating DC. These DC could be pulsed *in vitro* with antigen and then infused to stimulate naive or resting T lymphocytes (DC-assisted vaccination). Alternatively, T cells could be collected, exposed to antigen-pulsed DC *in vitro*, and then reinfused.

Despite several advances in the field of DC and the intense interest that has been focused on cultivating these cells, there are several issues that still must be addressed before DC gain widespread clinical application. One particular obstacle has been the inability to define the conditions/stimuli that promote the terminal, irreversible, differentiation of DC. Unless this stage of maturation is achieved, DC might lose their function or revert to a less differentiated state after *in vivo* instillation. This could not only diminish the effectiveness of treatment, but could also induce T cell anergy if critical costimulatory molecules are downregulated. Our observations, that DC generated in the bioreactor may be more differentiated than those obtained in standard 2D cultures is therefore important, because if stable differentiation can be achieved, this may represent a novel approach for generating DC for therapeutic purposes.

While early emphasis was placed on defining conditions for generating massive numbers of DC, it is becoming increasingly evident that it is the quality rather than the quantity of DC which will ultimately determine their therapeutic effectiveness. Therefore, the decreased cell expansion observed in bioreactor-grown DC is not likely to be problematic. Importantly, this system will provide us with the opportunity to analyze in depth the cellular and molecular events associated with DC maturation from its earliest progenitor to terminal differentiation, as this entire process appears to be accomplished in the bioreactor. This information will be important to understanding the mechanism of DC dysregulation in cancer patients, and may be applicable also to understanding changes in DC that might contribute to the blunting of immunity associated with spaceflight.

Our findings that circulating DC are significantly reduced in patients with gynecologic cancers, and in individuals recovering from stem cell transplantation are novel. Future studies are planned to determine whether this effect is related to the disease-state or to the chemotherapy. The functional capabilities of the small numbers of DC-like cells detected in these patients remains to be determined, but the lower levels of DC might contribute to the inability of patients to mount an effective anticancer response and could explain the increased susceptibility of these patients to opportunistic infections. As discussed above, bioreactor-grown DC might eventually be used to correct these deficiencies. Furthermore, our observations suggest that the multidimensional flow cytometric technique described here could be used to monitor DC recovery after transplantation and to evaluate the effect of various treatment regimens or environmental conditions on DC frequency or maturation. Such a tool might also aid in identifying therapies that spare or boost immunity. In fact, DC testing might become routine in any comprehensive immunological evaluation of patients and space travelers.

Our finding of increased Hsp in cells cultured in the bioreactor is intriguing. Upregulation of these stress proteins might reflect a protective response of DC against as yet undefined stressors, some of which might resemble those experienced in microgravity. Since Hsp have been shown to be involved in antigen presentation, changes in Hsp expression could also cause the altered function of DC cultured in the bioreactor. Understanding the role of Hsp in DC maturation might provide new insight into the molecular pathways that may be disturbed in disease or in space. We have received funding to explore this new avenue of research, that represents a spin-off of the present studies, through NRA-96-OLMSA-03.

**Future Studies:** Many of the above described studies are continuing in Round 2. We are presently evaluating DC in patients undergoing cytokine therapy to determine if DC number and function are altered, and how this might relate to the clinical response. We are also conducting an in depth analysis of the maturational status of DC grown in the bioreactor as reflected by changes in particular cell surface markers known to be differentially expressed by less mature *versus* more mature DC, and by functional tests. We will also study the *in vivo* effectiveness of DC generated in the bioreactor to protect against fungal infections in a murine model.

Manuscripts submitted:

Savary CA, Grazziutti ML, Melichar B, Przepiorka D, Freedman RS, Cowart RE, Cohen DM, Anaissie EJ, Woodside DG, McIntyre BW, Pierson DL, Pellis NR, Rex JH. Multidimensional flow cytometric analysis of dendritic cells in peripheral blood of normal donors and cancer patients. *Cancer Immunol. Immunother.* (submitted).

Melichar B, Savary CA, Kudelka AP, Verschraegen C, Kavanagh JJ, Edwards CL, Platsoucas CD, Freedman RS. Lineage negative HLA-DR+ cells with the phenotype of undifferentiated dendritic cells in patients with carcinoma of the abdomen and pelvis. *Cellular Immunol.* (submitted).

Funding resulting from these studies:

NRA-96-OLMSA-03 "Use of NASA Bioreactors in a Novel Scheme for Immunization Against Cancer"

PI: C.A. Savary, Ph.D.; FY 1997 to FY 2000; \$465,000

Other activities/accomplishments during Round 1:

Feb. 1997 - presented results of the present studies at the annual meeting of the Biotechnology Cell Science Program Investigators Working Group.

Student involvement in these studies -

Monica Grazziutti, M.D., Clinical Fellow

Luis Tome, M.D., Clinical Fellow

Alysson Ford, summer student, King Foundation Summer Research Program

Lee Aleksich, summer student, NASA Sharp Plus/Qem Program

Manu Goyal, summer student; NASA Sharp Plus/Qem Program

abstract of project accepted for presentation at the AJAS/AAAS conference (Appendix 3)

**Multidimensional flow cytometric analysis of dendritic cells in  
peripheral blood of normal donors and cancer patients**

**Cherylyn A. Savary, Monica L. Grazziutti, Bohuslav Melichar, Donna Przepiorka,  
Ralph S. Freedman, Richard E. Cowart, David M. Cohen, Elias J. Anaissie,  
Darren G. Woodside, Bradley W. McIntyre, Duane L. Pierson, Neal R. Pellis, John H. Rex**

C. A. Savary (✉)

Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center,  
1515 Holcombe Blvd.- Box 018, Houston, TX, 77030, USA Fax: (713) 794 4830

M. L. Grazziutti • <sup>1</sup>D. M. Cohen • J. R. Rex

Division of Infectious Diseases, Department of Internal Medicine, Center for the Study of  
Emerging and Reemerging Pathogens, The University of Texas Medical School, Houston, TX,  
77030, USA

<sup>2</sup>E. J. Anaissie • R. E. Cowart

Department of Medical Specialties, The University of Texas M. D. Anderson Cancer Center,  
Houston, TX, 77030, USA

B. Melichar • R. S. Freedman

Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center,  
Houston, TX, 77030, USA

D. Przepiorka

Department of Hematology, The University of Texas M. D. Anderson Cancer Center, Houston,  
TX, 77030, USA



D. G. Woodside • B. W. McIntyre

Department of Immunology, The University of Texas M. D. Anderson Cancer Center, Houston,  
TX, 77030, USA

D. L. Pierson • N. R. Pellis

National Aeronautics and Space Administration, Johnson Space Center, Houston, TX, 77058-  
3696, USA

<sup>1</sup>Present address: J32 Omega Dr., Newark, DW, 19713-2076, USA

<sup>2</sup>Present address: Division of Hematology/Oncology, Section of Oncologic Emergencies,  
University of Arkansas for Medical Sciences, Little Rock, AR, 72205, USA

Please send proofs to:

Cherylyn A. Savary, Ph.D.  
Dept. of Surgical Oncology  
The University of Texas M.D. Anderson Cancer Center  
1515 Holcombe Blvd. - Box 018  
Houston, TX 77030  
Ph: (713) 792-3134  
Fax: (713) 794-4830

**Abstract** We studied the potential of multidimensional flow cytometry in evaluation of the frequency and maturation/activation status of dendritic cells (DC) in minimally manipulated peripheral blood mononuclear cell preparations (i.e., Ficoll-Hypaque separated only) of normal donors and cancer patients. A rare subset of HLA-DR<sup>+</sup> leukocytes (<1% of mononuclear cells) was detected in blood of normal donors that displayed all the features of DC: these cells had high forward light scatter characteristics and coexpressed CD4, CD86 and CD54 surface antigens, but lacked the lineage-associated surface markers of T cells, B cells, monocytes, granulocytes or NK cells (i.e. they were CD3<sup>-</sup>, CD19<sup>-</sup>, CD20<sup>-</sup>, CD14<sup>-</sup>, CD11b<sup>-</sup>, CD16<sup>-</sup>, CD56<sup>-</sup>). These physical and phenotypic properties were virtually identical to those of immunomagnetically sorted leukocytes characterized as DC on the basis of morphology, phenotype and high stimulatory activity in allogeneic mixed lymphocyte cultures. Using this flow cytometric approach we observed that the frequency of DC-like cells in peripheral blood mononuclear cell specimens of cancer patients receiving chemotherapy alone or those recovering from stem cell transplantation was significantly lower than that of normal individuals (mean  $\pm$  SE: 0.36  $\pm$  0.05%, 0.14  $\pm$  0.06%, and 0.75  $\pm$  0.04%, respectively). Multidimensional flow cytometric analysis of DC might represent an important new tool for assessing immunocompetency, and for monitoring the effects of therapeutic regimens on the immune system.

**Key words** Dendritic cells • Flow cytometry • Stem cell transplantation • Breast cancer

## Introduction

Dendritic cells (DCs) are a subset of bone marrow-derived leukocytes present in trace amounts in virtually all tissues with the exception perhaps of brain [7]. Because they express MHC Class I and Class II molecules and the costimulatory structures required for optimal activation of naive and memory T lymphocytes, DCs have been considered to be the most efficient of antigen-presenting cells (APC) [7]. The study of DCs has been difficult, though, because of their very low numbers in peripheral blood and other tissues. Another difficulty has been the failure thus far to identify any unique surface marker that could be used to directly enumerate, isolate, and analyze human DCs. Furthermore, evidence suggests that the phenotype of DCs may vary depending on the stage of maturation or activation, and tissue source. As a result, DCs are usually identified (a) *morphologically*, by their numerous cytoplasmic processes [7], (b) *phenotypically*, by their expression of HLA-DR and B7 costimulatory molecules, and lack of surface markers associated with mature NK cells, B cells, T cells, macrophages/monocytes, or granulocytes [2, 6], and (c) *functionally*, by their potent T cell-stimulatory activity [2, 7].

Much of our information on human peripheral blood DCs has come from analysis of DCs that are highly-enriched through rigorous and multiple fractionation procedures [2, 7, 11]. Since DC are estimated to represent <1% of mononuclear cells, such studies require a large amount of peripheral blood. Thus investigations using enriched DC are impractical for many studies involving patients, particularly those with leukopenia. Furthermore, the cell loss that occurs from the multiple manipulations involved in DC enrichment may decrease the accuracy of the results. Finally, DC isolation is too complex and time-consuming for routine analysis of patient samples. Therefore, we investigated the feasibility of using multidimensional flow cytometry to analyze DC directly in peripheral blood mononuclear cell (PBMC) samples obtained with only minimal manipulation, i.e. Ficoll-Hypaque gradient separation. We show that the phenotype and light-scatter properties of these cells are indistinguishable from DC isolated by immunomagnetic sorting.

Finally, we show the usefulness of this technique in evaluating the DC-like subset in peripheral blood specimens from cancer patients.

## **Materials and methods**

### **Preparation of mononuclear cells**

Peripheral blood from normal donors and cancer patients was obtained by venipuncture and collected into heparinized vacutainer tubes. Patients included 7 with breast cancer who were undergoing standard chemotherapy treatments and 6 individuals recovering from autologous or allogeneic stem cell transplantation (1 with acute myelogenous leukemia, 1 with lymphoma, and 4 with breast cancer). In some studies buffy coat samples from normal donors were purchased from a local blood bank. Blood specimens were diluted with 2 parts  $\text{Ca}^{++}/\text{Mg}^{++}$ -free phosphate buffered saline (PBS), layered onto Accu-Prep gradient solution (density = 1.077 g/ml; Accurate Chemical and Scientific Corp., Westbury, NY), and centrifuged at 750 x g for 20 min. The PBMC were collected from the interface and washed 3 times in PBS.

### **Flow cytometry**

Cell surface phenotype was examined by 2- and 3-color immunofluorescence using monoclonal antibodies (mAbs) that were directly conjugated to fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), or Tri-color<sup>®</sup> (TRI). The specificity and source of these mAbs are listed in Table 1. For 2-color flow cytometric detection of DC in PBMC preparations,  $10^6$  cells were incubated for 20 min at 4°C with FITC-conjugated anti-HLA-DR, together with a cocktail of PE-conjugated mAbs reactive against CD3, CD11b, CD14, CD16, CD19, CD20, and CD56. The latter lineage (lin) cocktail was titrated to label simultaneously all NK cells, T cells, B cells,

monocyte/macrophages, and granulocytes. Cells labeled with PE- and FITC- conjugated isotype control mAbs that were nonreactive to human cells were used as a control. The labeled cells were washed, fixed with 1% paraformaldehyde, and analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a single 488 nm argon laser and 3 fluorescence detectors with filter settings for FITC (530 nm), PE (585 nm) and TRI/PerCP (>650 nm). A total of 30,000 to 50,000 events were collected in list mode and analyzed using FACScan Research Software (Becton Dickinson). FSC and SSC gates were set to exclude erythrocytes and debris, and markers set to exclude background fluorescence as established using appropriate isotype controls. In these experiments DC were identified as lin<sup>-</sup>DR<sup>+</sup> leukocytes with high forward light scatter (FCS) properties.

For 3 color flow cytometric analysis of DC, 10<sup>6</sup> cells were labeled simultaneously with a FITC-lin cocktail, TRI-conjugated anti-HLA-DR, and a PE-conjugated mAb recognizing one of the following cell surface determinants: CD1a, CD4, CD54, CD80, or CD86. The cells were washed and fixed with 1% paraformaldehyde. For flow cytometric analysis, a live gate was set on the lin<sup>-</sup>DR<sup>+</sup> DC population (i.e. FITC<sup>-</sup>, TRI<sup>+</sup>), and 1000-2000 gated events routinely collected. This population was then analyzed for co-expression of a third marker identified as positive events (above background fluorescence) within the PE-channel.

#### Immunomagnetic sorting of DCs

Highly-enriched preparations of DCs were obtained by immunomagnetic separation using DC isolation kits obtained from Miltenyi Biotec, Inc., Auburn CA [4]. The isolation procedure included a negative selection step to remove NK cells, T cells, and monocytes, followed by positive selection of CD4<sup>+</sup> DCs. Briefly, PBMC were suspended in cold PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO) at a concentration of 3 x 10<sup>8</sup> cells/ml. The cells were incubated for 10 min at 4°C with an FcR blocking reagent and a cocktail of mAbs recognizing CD3, CD11b and CD16 antigens. The

suspension was then washed, and incubated for an additional 15 min at 4°C with paramagnetic microbeads recognizing the mAb cocktail. This suspension was passed through a type CS iron fiber depletion column placed within a strong magnetic field (SuperMACS, Miltenyi Biotec). Effluent cells that were depleted of macrophages, granulocytes, NK cells and T cells were collected, washed, incubated for 30 min at 4°C with anti-CD4 microbeads, and passed through a type MS iron bead separation column placed within the magnetic field. After extensive washing the column was removed from the magnet and the CD4<sup>+</sup> adherent cells flushed from the column with cold buffer. These cells were then passed through a second positive selection column to further purify the DCs. The purity of the sorted population was analyzed by labeling the recovered cells with FITC-anti-HLA-DR, and a cocktail of PE-conjugated mAbs directed against CD19, TCR $\alpha\beta$ , TCR $\gamma\delta$ , CD56, and CD14 (to detect any residual NK cells, T cells, B cells, or monocytes).

#### Culture of DC

In some experiments, immunomagnetically sorted DCs were suspended in RPMI 1640 medium ( $10^5$  cells/ml) supplemented with 10% heat-inactivated fetal bovine serum (GibcoBRL, Grand Island, NY), 10 mM HEPES buffer, 100  $\mu$ g/ml streptomycin, 100 U/ml penicillin, 50  $\mu$ g/ml gentamicin, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate,  $2 \times 10^{-5}$  M 2-mercaptoethanol (2ME), 50 ng/ml human recombinant GM-CSF (R&D Systems, Minneapolis, MN), and 10 ng/ml highly purified human recombinant TNF $\alpha$  from *E. coli* (Cetus Corp., Emeryville, CA) [10]. The cells were cultured for 5 days at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

### Allogeneic mixed lymphocyte response (MLR)

The MLR was performed as described previously [13]. DC stimulator cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum (Pel Freez, Brown Deer, WI), HEPES buffer, antibiotics, nonessential amino acids, sodium pyruvate, glutamine and 2ME as described above, and irradiated with 3000 cGy delivered from a cesium source. Serial dilutions of the DC were prepared in triplicate in 96 well round-bottomed plates (0.1 ml/well) and an equal volume of medium containing  $1.5 \times 10^5$  allogeneic nylon wool nonadherent T lymphocytes was added to each well (stimulator:responder ratios ranged from 0.01:1 to 1:1). Controls included DC and T cells incubated alone. The plates were cultured for a total of 5 days, with 1  $\mu$ Ci of  $^3\text{H}$ -thymidine ( $^3\text{HTdR}$ ; NEN-DuPont, Boston, MA) added during the final 16 hr of culture. Cells were harvested onto glass fiber filters, and the radioactivity measured by  $\beta$ -scintillation counting. The results are expressed as counts per minute (cpm).

### Digital imaging

Images of cells in cultures were captured as described previously [8] using a VI-470 CCD video camera system (Optronics Engineering, Goleta, CA) attached to a Nikon DIAPHOT-TMD inverted microscope (Nikon Inc., Melville, NY) that was equipped with 10x or 20x objectives. The images were digitized using a QuickCapture frame grabber board (Data Translation Inc., Marlboro, MA).

### Data analysis

Data were analyzed using a Students t-test analysis. Results were considered significant when  $p < 0.05$ .

## Results

### Phenotype of DCs isolated from peripheral blood of normal donors

Freshly isolated peripheral blood DCs are known to be of low buoyant density (<1.077 g/ml) and lack surface markers expressed by T cells, B cells, NK cells, monocytes/macrophages, or granulocytes, i.e. they are CD3<sup>-</sup>CD11b<sup>-</sup>CD14<sup>-</sup>CD19<sup>-</sup>CD16<sup>-</sup>CD20<sup>-</sup>CD56<sup>-</sup> [6, 7, 11]. However, DCs express MHC Class II [7]. Another distinguishing characteristic of freshly isolated peripheral blood DCs is the expression of CD4 surface molecules [1, 6]. A DC isolation kit purchased from Miltenyi Biotec Inc. (Sunnyvale, CA) was used for immunomagnetic sorting of DC from peripheral blood specimens based on the above mentioned characteristics. This isolation was performed in 2 steps according to the manufacturer's recommendations: PBMC were first depleted of T cells, NK cells, and monocytes using a cocktail of mAbs recognizing CD3, CD16, and CD11b, respectively (negative selection step), and then CD4<sup>+</sup> leukocytes were obtained by positive selection [1, 2, 5]. Using this technique, we obtained highly-enriched populations of cells (up to 95% purity) displaying a phenotype consistent with DC as analyzed by flow cytometry, i.e., TCR $\alpha\beta$ <sup>-</sup>TCR $\gamma\delta$ <sup>-</sup>CD19<sup>-</sup>CD14<sup>-</sup>CD56<sup>-</sup>, but HLA-DR<sup>+</sup> (Figs. 1A and 1B). When the light-scatter property of these cells was examined, it was found that their size (as estimated by FSC) was intermediate to that of lymphocytes and monocytes (Figs. 1C and 1D).

### Morphology and function of sorted DCs

Immunomagnetically sorted lin<sup>-</sup>DR<sup>+</sup> cells were placed into culture for 5 days with GM-CSF and TNF $\alpha$ , and then analyzed by light microscopy. As seen in Fig. 2, these cultures contained cells with elongated processes typical of more mature DC. Furthermore, these cells were potent stimulators of a primary allogeneic MLR, as evidenced by their ability to stimulate



significant proliferation of allogeneic T lymphocytes at stimulator-to-responder ratios as low as 0.01:1 (Fig. 3).

#### Detection of DC-like cells among peripheral blood mononuclear cells

Based on the information obtained from the sorting experiments, we next determined if multidimensional flow cytometry could be used to accurately detect  $\text{lin}^-\text{DR}^+$  cells directly in minimally manipulated PBMC preparations (i.e. cells obtained from blood specimens subjected to Ficoll-Hypaque gradient separation only). PBMC from normal donors were labeled simultaneously with a PE-cocktail of mAb recognizing the lin-associated markers CD3, CD11b, CD14, CD16, CD19, CD20, and CD56 (first color), and FITC-conjugated anti-HLA-DR (second color). Cells labeled with isotype control antibodies were included to determine background fluorescence. Because of the low frequency of  $\text{lin}^-\text{DR}^+$  cells, we routinely collected 30,000 to 50,000 events to more easily visualize and gate on this population.

As seen in the representative experiment shown in Fig. 4A, a very small subset of  $\text{lin}^-\text{DR}^+$  leukocytes could be detected using this technique. This population represented  $0.75 \pm 0.04\%$  (mean  $\pm$  SE; n=12) of PBMC from normal individuals. Similar levels of  $\text{lin}^-\text{DR}^+$  cells were observed using instead a cocktail of FITC-conjugated lineage-associated mAb and TRI-anti-HLA-DR (Fig. 4B). By gating on the  $\text{lin}^-\text{DR}^+$  events in quadrant 4 of the bivariate plot and viewing the FSC profile, we observed that this subset was intermediate in size to lymphocytes and monocytes, a characteristic that was similar to the immunomagnetically sorted DC (compare Figs 1D and 4C). Although these cells were rare, this detection technique was highly reproducible as seen by the consistency of  $\text{lin}^-\text{DR}^+$  cells detected in the same sample using the different mAb combinations, as well by the relatively low variability among replicate samples from five other donors tested (Table 2).

To further characterize this  $\text{lin}^-\text{DR}^+$  subset and to compare it more closely with immunomagnetically sorted DC, we used 3-color flow cytometry to analyze the co-expression of

surface molecules that have been associated with DC maturation/activation [6]. PBMC were labeled simultaneously with a cocktail of FITC lin-associated mAbs, TRI-conjugated anti-HLA-DR, and PE-conjugated mAb recognizing CD1a, CD54, CD80, or CD86 surface antigens. A live gate was set on the lin<sup>+</sup>DR<sup>+</sup> cells as shown in Fig. 4B, and these cells analyzed for coexpression of a third surface marker detected by a PE-conjugated mAb. An example of CD86 coexpression by lin<sup>+</sup>DR<sup>+</sup> cells in a PBMC specimen is shown in Fig. 4D. Using this approach it was observed that the phenotype of the majority of lin<sup>+</sup>DR<sup>+</sup> cells was consistent with that described recently for DCs freshly obtained from peripheral blood using other isolation techniques, i.e. CD4<sup>+</sup>CD54<sup>+</sup>CD86<sup>+</sup>CD80<sup>-</sup>CD1a<sup>-</sup> (Table 3) [6]. Furthermore, this phenotype was similar to that of the highly-enriched DC obtained by immunomagnetic cell sorting (Table 3).

As further support that the lin<sup>+</sup>DR<sup>+</sup> cells detected in PBMC were comparable to sorted DC, we spiked a sample of PBMC labeled with a PE-lin cocktail alone with purified FITC-DR<sup>+</sup> DC obtained from the same individual. The sorted DC displayed the same fluorescence and size characteristics as the DC-like cells gated within the whole PBMC population (data not shown).

#### Analysis of DC in PBMC of cancer patients

Having determined that the above described 2-color flow cytometric technique could be used to confidently detect DC-like cells in peripheral blood of normal donors, we applied the same approach to determine the levels of DC in cancer patients. As seen in Fig. 5, the proportion of lin<sup>+</sup>DR<sup>+</sup> leukocytes in PBMC specimens of breast cancer patients who had undergone prior chemotherapy was only  $0.36 \pm 0.05\%$  (mean  $\pm$  SE) which was significantly less than that of normal individuals ( $p < 0.0005$ ). With the exception of patient #3, the majority of lin<sup>+</sup>DR<sup>+</sup> cells detected in these patients, like those of normal donors, coexpressed CD4 and CD54 (Table 4). Also with one exception (patient #2) few or none of the lin<sup>+</sup>DR<sup>+</sup> cells detected in PBMC of these patients coexpressed CD1a or CD80.

Even lower levels of lin<sup>-</sup>DR<sup>+</sup> leukocytes were detected in PBMC samples of cancer patients recovering from stem cell transplantation (Fig. 5). The frequency of DC-like cells in these patients who were 1-5 months post stem cell transplantation was only  $0.14 \pm 0.06\%$  (mean  $\pm$  SE), and was significantly reduced compared to both normal donors and non-transplanted cancer patients ( $p < 0.0005$  and  $< 0.025$ , respectively). In fact, lin<sup>-</sup>DR<sup>+</sup> cells were undetectable in one transplanted patient.

## Discussion

We have evaluated the feasibility of using a simple 2-color flow cytometric technique to detect DC-like cells in mononuclear cell preparations of peripheral blood of normal donors and cancer patients. These cells represented approximately 1% or less of the PBMC fraction, a frequency that is in agreement to that estimated for DC analyzed in a variety of other types of assay conditions [6, 7, 11]. Furthermore, they expressed a phenotype and size that was virtually identical to that of immunomagnetically sorted DCs, as well as to freshly isolated peripheral blood DC described in previous reports [2]. However, because our approach allows for detection of DCs directly in blood samples that have received only minimal manipulation (Ficoll-Hypaque), it allows for a more rapid, and perhaps more accurate assessment of this important APC population compared to other DC enrichment procedures. While other investigators have shown that DC-like cells can be detected in PBMC populations [2], our study is the first to apply multidimensional flow cytometry to begin to analyze in more depth the frequency and maturation/activation status of these cells in blood specimens of cancer patients, including those who are judged as immunodeficient by other criteria (e.g. low numbers of circulating CD4<sup>+</sup> T lymphocytes). Our data suggest that DC testing could become a part of the routine clinical evaluation of immune competency.

We have shown for the first time that while  $\text{lin}^-\text{DR}^+$  leukocytes in PBMC specimens of breast cancer patients who have received prior chemotherapy phenotypically resemble those detected in normal PBMC (i.e.  $\text{CD4}^+\text{CD86}^+\text{CD54}^+\text{CD80}^-\text{CD1a}^-$ ), this population is reduced by approximately 50% in the patients. Studies are planned to determine whether this effect is related to the disease-state or to the chemotherapy. The functional capabilities of the small numbers of DC-like cells detected in these patients remains to be determined, but it was recently reported that DC antigen presentation is compromised in cancer patients [3]. In any case, the lower levels of APCs might contribute to the inability of patients to mount an effective anticancer response. Similarly, the low levels of  $\text{lin}^-\text{DR}^+$  DC-like cells in stem cell transplanted patients could contribute to the increased susceptibility of these patients to opportunistic infections. Our observations suggest that the multidimensional flow cytometric technique described here could be used to monitor DC recovery after transplantation and to evaluate the effect of various treatment regimens or environmental conditions on DC frequency or maturation. Such a tool might also aid in identifying therapies that spare or boost immunity.

We found that the 2-color flow analysis of DC could be performed with only  $2 \times 10^6$  PBMC, a number of cells that was easily obtained from normal donors, but more importantly, also from immunocompromised patients who often have low leukocyte counts. Additional cells were required for the more extensive 3-color analyses. However, as the technology for multicolor analysis is improving and expanding, it should be possible to simultaneously analyze DC within a single sample for co-expression of multiple surface and cytoplasmic markers.

Analysis of human DC will also be significantly facilitated if mAb specific for these populations can be identified. Recently it was shown that some subsets of human peripheral blood DC express CD83 [11-13]. This surface antigen is not specific for DC, and may not detect less mature DC subsets [13]. However, the availability of antibodies to CD83 has been instrumental in analyzing cytokine- and chemokine-gene expression in peripheral blood DC, and discrimination of DC subsets that are susceptible to HIV infection [11-13]. Studies to analyze CD83 expression by the DC detected within PBMC populations are currently in progress.

Until more direct analysis of DC is possible, the exact composition of the lin<sup>-</sup>DR<sup>+</sup> population, and the proportion of cells within this subset that are truly DC-committed cannot be precisely determined. It is possible, for example, that this population may contain DR<sup>+</sup> progenitor cells that are not yet committed to a specific lineage [9]. Whether such cells could be driven towards the DC lineage, however, is an intriguing question with relevance perhaps for the treatment of immunodeficient patients. Nevertheless, the co-expression of MHC Class II and costimulatory molecules by the lin<sup>-</sup>DR<sup>+</sup> cells detected using the multidimensional flow cytometric technique described here indicate that they might possess potent antigen-presenting activity.

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Table 1. Monoclonal antibodies used for cell surface analysis of human dendritic cells

Surface Antigen	Distribution	Source <sup>a</sup>
CD1a	DC; cortical thymocytes	Caltag
CD3	T cells	Caltag
CD4	DC; T-helper/inducer cells; monocytes/macrophages	Caltag
CD11b	Granulocytes; monocytes/macrophages; NK cells	Caltag
CD14	Monocytes/macrophages; some granulocytes	Caltag
CD16	NK cells; granulocytes; activated monocytes/macrophages	B-D, Caltag
CD19	B cells	Caltag
CD20	B cells (except pre-B and plasma cells)	Caltag
CD54 (ICAM-1)	DC; endothelial cells; many activated cells	Caltag
CD56	NK cells; T cell subset	B-D; Coulter
HLA-DR	DC; some B cells, T cells and monocytes	Caltag/B-D
CD80 (B7.1)	DC; activated B cells, T cells and monocytes	Calbiochem
CD86 (B7.2)	DC; activated B cells and monocytes	Calbiochem

<sup>a</sup>Caltag, Burlingame, CA; Becton-Dickinson (B-D), Immunocytometry Systems, San Jose, CA; Coulter Corp. Hialeah, FL; Calbiochem, San Diego, CA.



**Table 2** Detection of linDR<sup>+</sup> leukocytes in PBMC of normal donors

Donor No.	Percent LinDR <sup>+</sup> Cells		
	Test 1	Test 2	Test 3
1	1.0	1.1	1.0
2	0.9	0.9	0.9
3	0.7	0.7	-
4	1.1	0.9	-
5	0.5	0.5	-

LinDR<sup>+</sup> cells in PBMC were detected by 2-color flow cytometry.

**Table 3** 3-Color flow cytometric analysis of lin<sup>+</sup>DR<sup>+</sup> leukocytes in unsorted and immunomagnetically sorted peripheral blood specimens.

Expt. No.	Percent of lin <sup>+</sup> DR <sup>+</sup> cells coexpressing the particular antigen				
	CD4	CD54	CD86	CD80	CD1a
PBMC-1	94.6	98.1	93.2	5.3	6.0
2	87.2	93.6	88.4	0.6	0.3
3	88.2	93.8	93.1	6.0	-
4	92.2	97.4	99.3	1.6	1.3
Sorted 1	-	98.9	79.4	1.4	-
2	-	97.6	91.4	2.2	4.9
3	-	98.8	86.5	3.2	6.7

Lin<sup>+</sup>DR<sup>+</sup> cells in unsorted PBMC or immunomagnetically sorted populations were analyzed for coexpression of several other surface markers using 3-color flow cytometric analysis. The sorted cells had been positively selected using an anti-CD4 mAb and were not reanalyzed for expression of this antigen.

**Table 4** Phenotype of lin<sup>-</sup>DR<sup>+</sup> leukocytes of breast cancer patients

Patient No.	% of Lin <sup>-</sup> DR <sup>+</sup> cells coexpressing:				
	CD4	CD54	CD86	CD80	CD1a
1	83	92	4	1	3
2	87	99	91	30	n.t.
3	43	7	55	0	0
4	68	51	22	0	0
5	65	55	11	5	0
6	85	97	98	0	n.t.
7	54	67	77	0	0

PBMC were analyzed by 3-color flow cytometry as described in the legend of Fig. 4.

**Fig. 1** Enrichment of lin<sup>-</sup>DR<sup>+</sup> leukocytes by immunomagnetic cell sorting. Unsorted PBMC (Panels A and C) from a normal donor and cells sorted using a DC isolation kit as described in the Materials and Methods section (Panels B and D) were analyzed by flow cytometry. Markers were set using PE- and FITC-conjugated isotype control antibodies to exclude >98% of background events. DC-like cells were characterized as the lin<sup>-</sup>DR<sup>+</sup> events in quadrant 4 of the FITC vs PE dot plots in Panels A and B. The lineage cocktail consisted of PE-conjugated mAbs recognizing TCR $\alpha\beta$ , TCR $\gamma\delta$ , CD19,CD56 and CD14 antigens. The light-scatter characteristics of the leukocyte populations are shown in panels C and D. The two major leukocyte populations, i.e. lymphocytes (L) and monocyte/macrophages (M) are labeled in Panel C. The experiment shown is a representative experiment from 3 sorts of PBMC from normal donors.

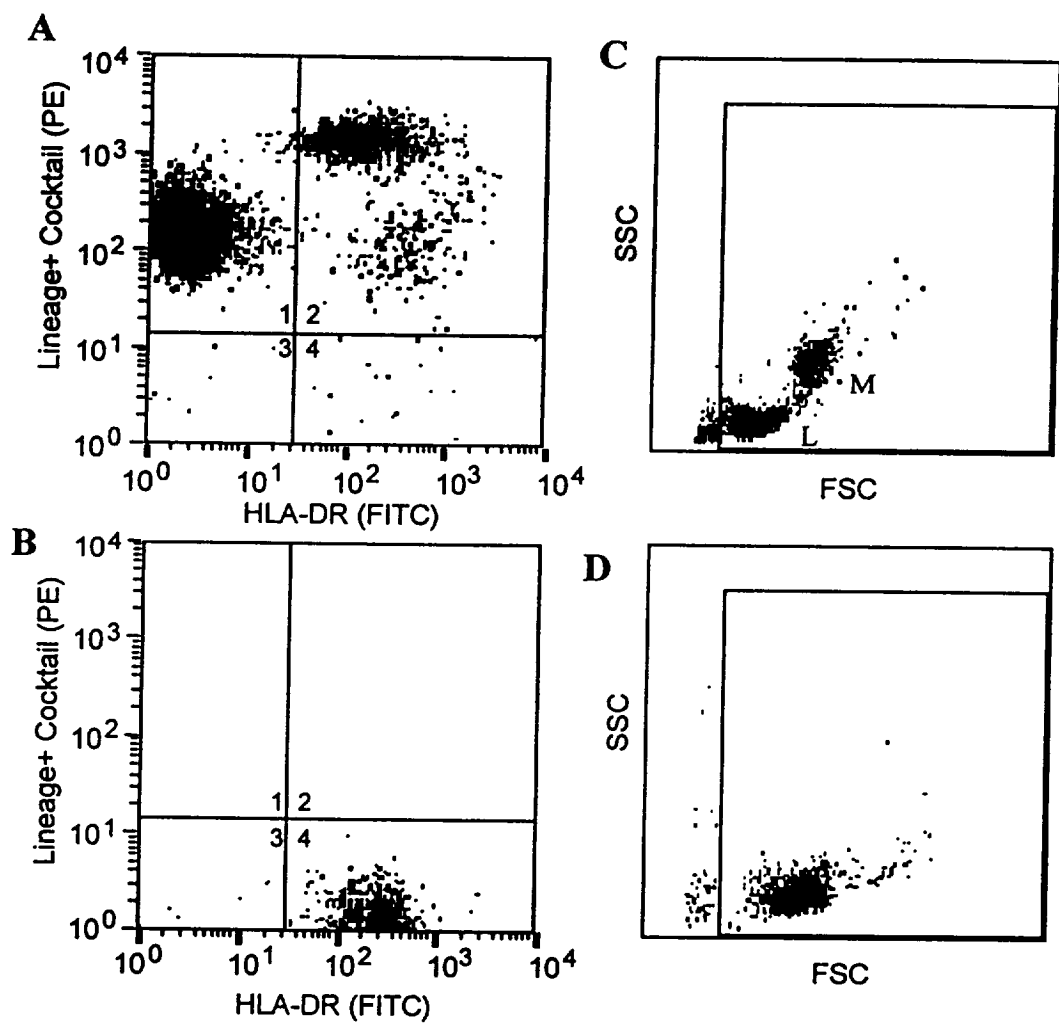
**Fig. 2.** Morphology of immunomagnetically sorted  $\text{lin}^-\text{DR}^+$  leukocytes.  $\text{Lin}^-\text{DR}^+$  leukocytes from PBMC of a normal donor were sorted as described in Fig. 1 and cultured for 5 days with GM-CSF and  $\text{TNF}\alpha$ . Note cells with elongated cytoplasmic processes typical of DC (arrow).

**Fig. 3** Allostimulatory activity of immunomagnetically sorted lin<sup>+</sup>DR<sup>+</sup> cells. Unsorted PBMC and immunomagnetically sorted lin<sup>+</sup>DR<sup>+</sup> leukocytes were tested for the ability to stimulate allogeneic lymphocytes in a standard mixed leukocyte reaction. Bars represent mean  $\pm$ SE of replicate samples. Proliferation of responder cells was evaluated by <sup>3</sup>HTdR uptake measured after 5 days of culture.

**Fig 4** Flow cytometric analysis of  $\text{lin}^-\text{DR}^+$  leukocytes within PBMC preparations. *Panel A:* PBMC of normal donors were labeled with a cocktail of PE-conjugated mAbs recognizing CD3, CD19, CD20, CD16, CD56, CD11b and CD14, and FITC-conjugated anti-HLA-DR mAbs. *Panel B:* The same PBMC population was labeled with a cocktail of FITC-conjugated mAbs recognizing the  $\text{lin}$ -associated antigens listed above and TRI-anti-HLA-DR mAbs. *Panel C:* Forward light-scatter (size) characteristics of the unseparated PBMC (••••) and  $\text{lin}^-\text{DR}^+$  cells contained within the rectangular gate shown in quadrant 4 of the TRI versus FITC dot plot in Panel B (—). *Panel D:* PBMC were labeled simultaneously with FITC- $\text{lin}$  cocktail, TRI-anti-HLA-DR and PE-anti-CD86, or with the same FITC and TRI labeled antibodies and a PE-conjugated isotype control antibody. The histograms represent the proportion of  $\text{lin}^-\text{DR}^+$  cells in the live gate of FITC<sup>-</sup>, TRI<sup>+</sup> events that react with the isotype control (••••) or anti-CD86 (—) mAbs.

**Fig. 5** Frequency of lin<sup>-</sup>DR<sup>+</sup> cells in PBMC of normal donors and cancer patients. The percentage of lin<sup>-</sup>DR<sup>+</sup> leukocytes was determined by 2-color flow cytometry as described in the legend of Fig. 4. Patients included 7 breast cancer patients who received prior chemotherapy (chemo) and 6 patients who had received stem cell transplants (chemo+SC) 1-5 months prior to testing. Horizontal lines represent the mean.





**Figure 1**

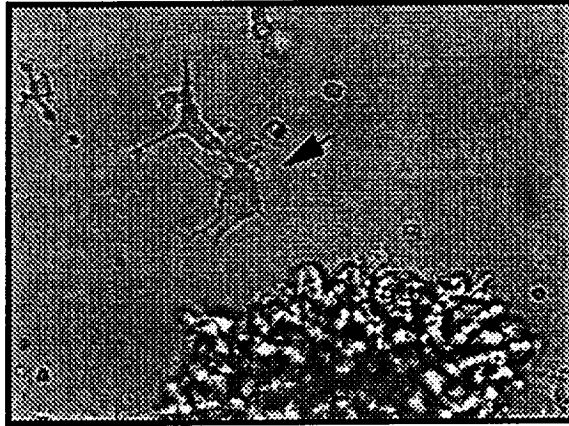
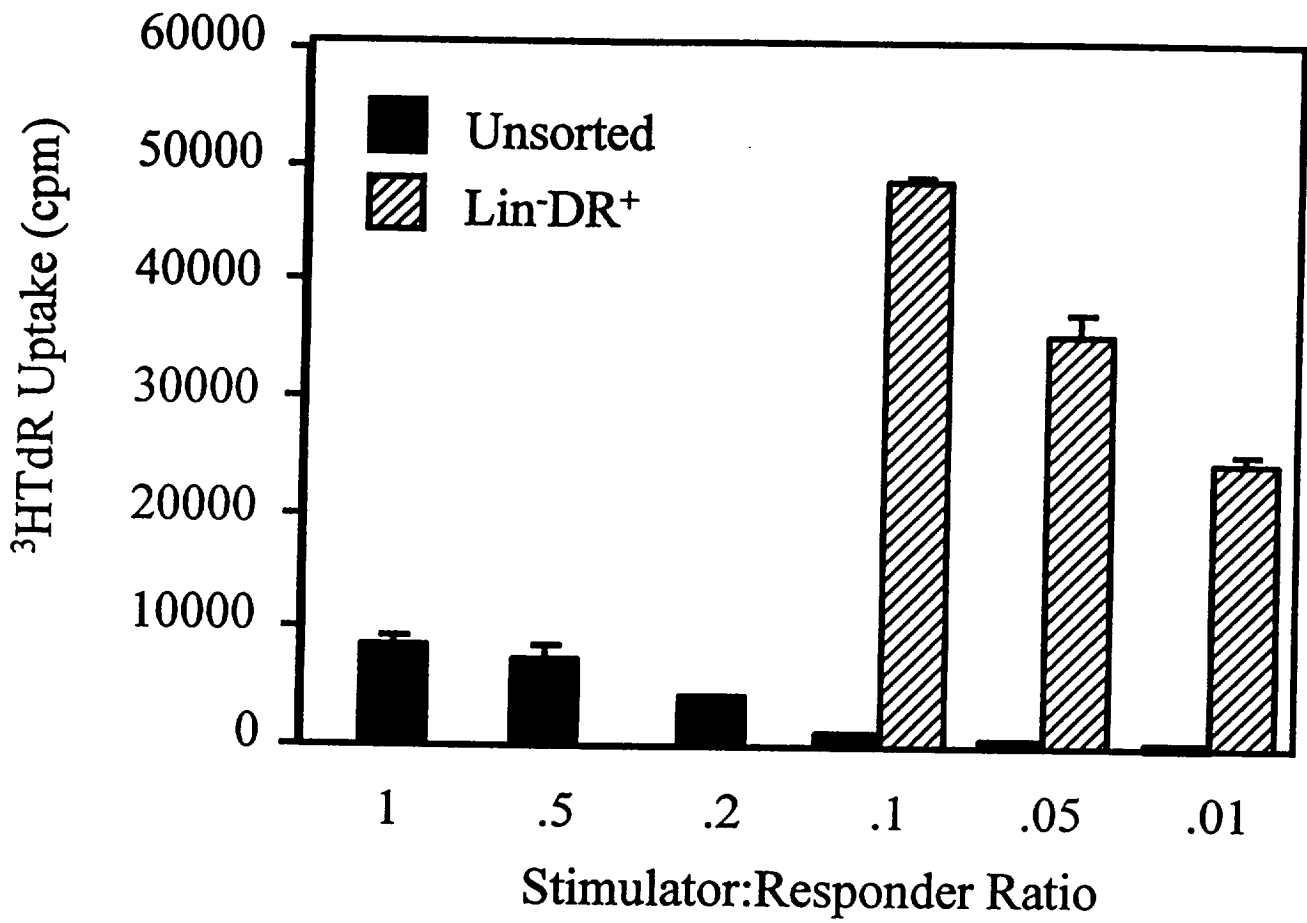
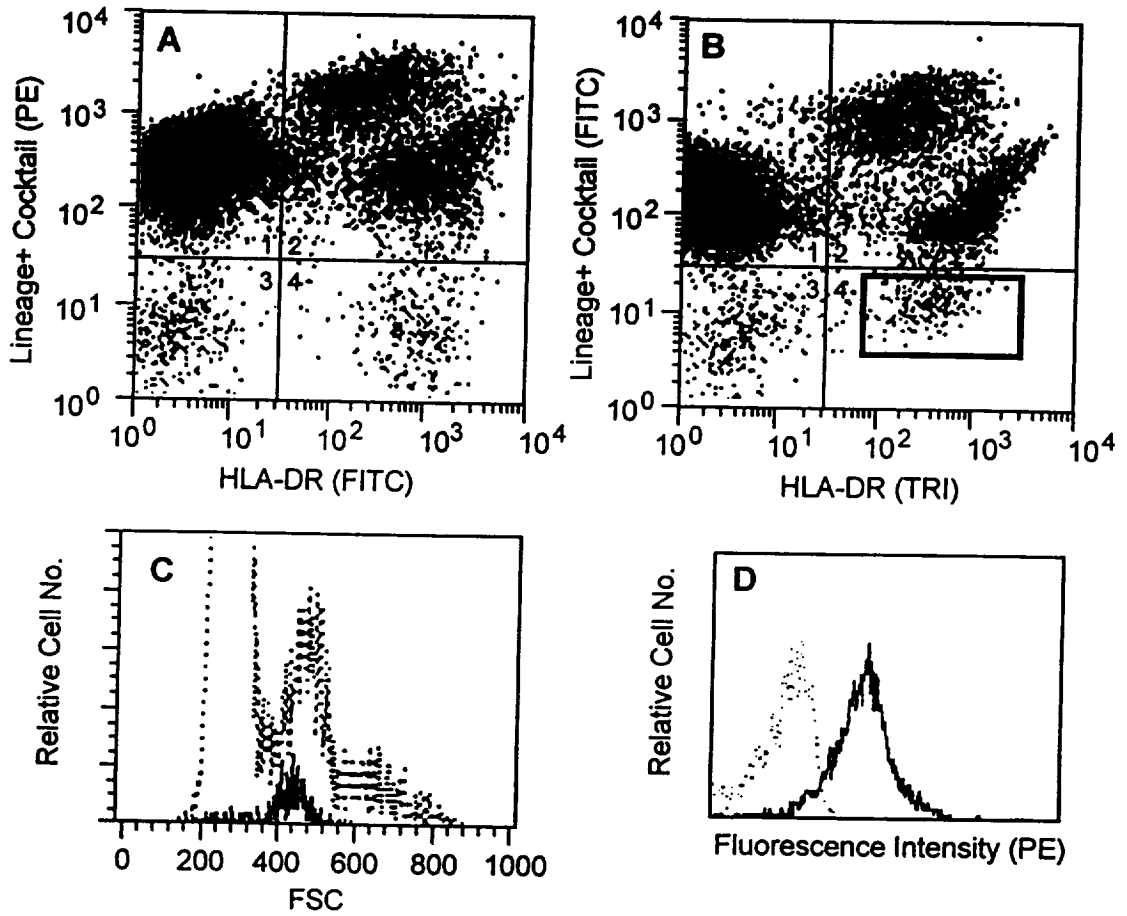


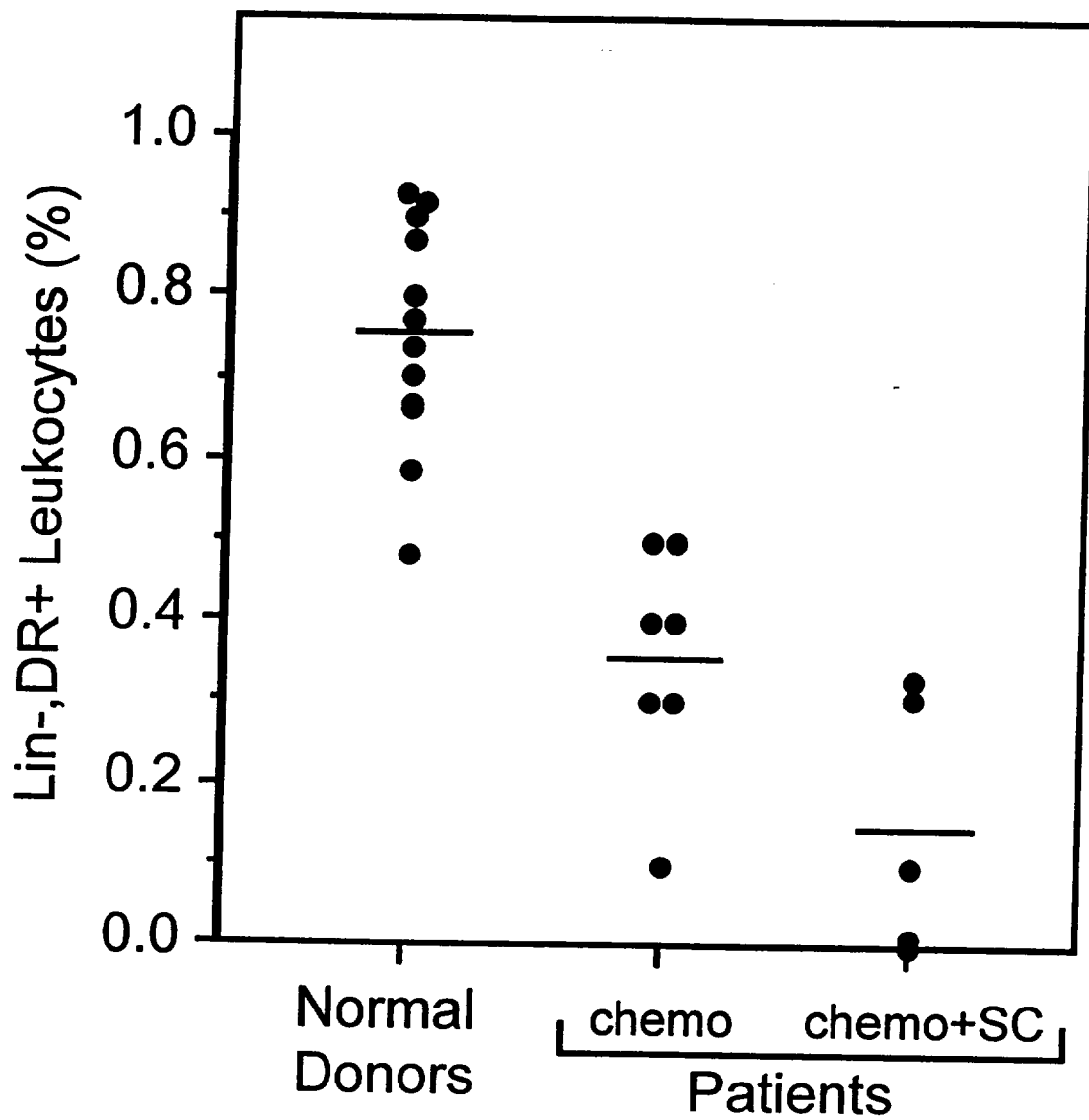
Figure 2



**Figure 3**



**Figure 4**



**Figure 5**

LINEAGE NEGATIVE HLA-DR<sup>+</sup> CELLS WITH THE PHENOTYPE  
OF UNDIFFERENTIATED DENDRITIC CELLS IN PATIENTS WITH  
CARCINOMA OF THE ABDOMEN AND PELVIS

Bohuslav MELICHAR<sup>1</sup>, Cherylyn A. SAVARY<sup>2</sup>, Andrzej P. KUDELKA<sup>3</sup>, Claire  
VERSCHRAEGEN<sup>1</sup>, John J. KAVANAGH<sup>3</sup>, Creighton L. EDWARDS<sup>1</sup>,  
Chris D. PLATSOUCAS<sup>1</sup>, Ralph S. FREEDMAN,<sup>1\*</sup>

<sup>1</sup>Departments of Gynecologic Oncology, <sup>2</sup>Surgical Oncology, and  
<sup>3</sup>Clinical Investigation and Section of Gynecologic Medical Oncology,  
The University of Texas M. D. Anderson Cancer Center,  
Houston, Texas, <sup>4</sup>Department of Microbiology and Immunology, Temple  
University School of Medicine, Philadelphia, Pennsylvania

\*Correspondence and reprint requests to:

Department of Gynecologic Oncology, The University of Texas  
M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 67  
Houston, Texas 77030

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### ABSTRACT

The characteristics of antigen-presenting cells in carcinomas that involve the abdominopelvic cavity are unknown. Dendritic cells, a population of antigen presenting cells have been identified as lineage-negative HLA-DR<sup>+</sup> leukocyte cells by two-color flow cytometry. We used this criterion to study the putative dendritic cells in ascites from 25 patients with peritoneal carcinomatosis. The mean proportion  $\pm$  standard deviation of lineage-negative HLA-DR<sup>+</sup> cells in ascites were  $3.1 \pm 4.6\%$  (range 0.05 - 17.3%). Most lineage-negative HLA-DR<sup>+</sup> cells expressed CD45RA or CD4 antigens. Dendritic cells had low proportions of CD80, CD11c, CD45RO and CD58, suggesting that they were of low maturity. The proportion of lineage-negative HLA-DR<sup>+</sup> cells in ascites was significantly higher compared with the proportion in peripheral blood from the same patients ( $4.5 \pm 5.7$  V  $0.5 \pm 0.4$ ;  $P < 0.05$ ). The proportion of lineage-negative HLA-DR<sup>+</sup> cells that coexpressed CD86 or CD58 was lower in ascites ( $56 \pm 19\%$ ) than in peripheral blood ( $91 \pm 10\%$ ,  $P < 0.05$ ), whereas a higher proportion of lineage-negative HLA-DR<sup>+</sup> cells in ascites expressed CD4. Relative fluorescence intensity of HLA-DR was also lower in dendritic cells from ascites and blood from patients with carcinomatosis than it was in blood from normal donors. As an indicator of macrophage activation, the concentration of neopterin in ascitic fluid correlated negatively with the numbers of lineage-negative HLA-DR<sup>+</sup> cells in ascites ( $r_s = -0.44$ ,  $P < 0.05$ ), and lineage-negative HLA-DR<sup>+</sup> CD4<sup>+</sup> cells ( $r_s = -0.63$ ,  $P < 0.005$ ), and correlated positively with the number of lineage-negative HLA-DR<sup>+</sup> CD80<sup>+</sup> cells ( $r_s = 0.43$ ,  $P < 0.05$ ). The proportion of lineage-negative HLA-DR<sup>+</sup> cells also correlated positively with the concentration of interleukin-10 in ascitic fluid ( $r_s = 0.40$ ,  $P =$

0.05). These findings suggest that certain factors associated with the tumor microenvironment might influence the number of these dendritic cells and their expression of function-associated markers.

## INTRODUCTION

Ovarian and other Müllerian carcinomas are the most frequent cause of peritoneal carcinomatosis, although other cancers, including those that originate in the gastrointestinal tract may spread to involve the serosal and peritoneal surfaces of the abdominopelvic cavity. Platinum-based chemotherapy and, more recently, taxanes have proven useful in the treatment of tumors of Müllerian origin. Most patients, however, will ultimately die of cancer-related complications. Tumors that originate in the gastrointestinal tract are generally less responsive to chemotherapeutic agents.

A number of clinical trials have been conducted on biotherapeutic agents administered intraperitoneally. These studies have utilized recombinant cytokines such as interferon- $\alpha$  (IFN- $\alpha$ ) (Willemsse *et al.*, 1990), interferon- $\gamma$  (IFN- $\gamma$ ) (Pujade-Lauraine *et al.*, 1996), and interleukin-2 (IL-2) alone (Freedman *et al.*, 1997), or with lymphokine activated killer cells (Steis *et al.*, 1990) or tumor-infiltrating lymphocytes (TILs) (Freedman *et al.*, 1994). Intraperitoneal treatments with some of these agents has produced complete responses, especially in patients who have a minimum of residual disease after prior chemotherapy. The outcome of intraperitoneal immunotherapy strategies in ovarian and other peritoneal carcinomas might possibly be improved when a



better understanding is acquired of the antigen-presenting cells associated with these tumors.

Tumor antigen peptides presented within the major histocompatibility complex (MHC) class I or II are specifically recognized by the T-cell receptor (Marrack and Kappler, 1987). The binding of the T-cell receptors to particular peptide MHC complexes produces a signal for T-cell activation. This first signal is transferred to structures within the cell by the monomorphic CD3 proteins that are in covalent association with the T-cell receptors. In addition, a need for a second signal has been established (Geppert *et al.*, 1990). This signal could be mediated by accessory molecules, such as the intracellular adhesion molecule-1 and its ligand (CD54) or late -function antigen-3 (CD58) (Geppert *et al.*, 1990; Singer, 1990). A most important, and probably indispensable signal involves the ligation of CD28 on lymphocytes by costimulatory molecules B7.1 (CD80) or B7.2 (CD86) on antigen-presenting cells or target cells (June *et al.*, 1994; Linsley and Ledbetter, 1993). The expression of both MHC and accessory molecules on tumor cells varies significantly (Hersey *et al.*, 1994; Vánky *et al.*, 1996). Moreover, we have found that (i) the presence of large numbers of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> infiltrating lymphocytes in solid tumor specimens from ovarian carcinomas *in vivo* and (ii) expansion of TIL-derived T-cell lines *in vitro* correlate positively with the expression of histocompatibility leukocyte class I antigens (HLAs) on these tumor cells (Kooi *et al.*, 1996).

CD80 or CD86 are usually absent on tumor cells (Vánky *et al.*, 1996). In contrast, professional antigen-presenting cells constitutively express MHC antigens as well as costimulatory molecules. In fact, MHC class II antigens and CD28 ligands are constitutively expressed only on macrophages, B lymphocytes and dendritic cells (Azuma *et al.*, 1993; Lenschow *et al.*, 1996; Young *et al.*, 1992). Dendritic cells are considered to be as effective as, or perhaps superior, in their antigen-presenting ability compared with either macrophages or B lymphocytes (Betjes *et al.*, 1993; Thomas *et al.*, 1993). Recently dendritic cells have been shown to influence the direction of an immune response towards either active immunity or anergy (Ridge *et al.*, 1996). Moreover, dendritic cells in both animals and humans have been shown to be involved in the control of tumor growth (Celluzzi *et al.*, 1996; Gabrilovich *et al.*, 1996a; Gabrilovich *et al.*, 1996b; Hsu *et al.*, 1996; Zitvogel *et al.*, 1996).

Dendritic cells were originally defined on the basis of their morphologic appearance, but the relationship of their precursors to the hemopoietic cell precursors is not fully defined (Peters *et al.*, 1996; Steinman, 1991). Because dendritic cells are present in small numbers and have no specific surface markers, they are difficult to study *in situ*. Recently dendritic cells were characterized by the absence of lineage-specific markers for other leukocytes and by their expression of HLA-DR and, in most cases, CD4 antigens (Ferbas *et al.*, 1994; O'Doherty *et al.*, 1994). In the present study we report: (i) the frequency of lineage negative HLA-DR<sup>+</sup> leukocytes in the malignant ascites and peripheral blood of patients with ovarian and other carcinomas that involve the abdominal cavity, (ii) the expression of costimulation molecules and

certain other surface markers on these cells consistent with that of dendritic cells, and (iii) correlations among the characteristics of dendritic cells in ascites according to the levels of cytokines and neopterin in the peritoneal cavity. These studies show for the first time the characteristics of dendritic cells associated with ovarian and other abdominal carcinomas. Dendritic cells may also be important in the therapeutic induction of active immunity.

## **MATERIAL AND METHODS**

### *Patients and normal donors*

Specimens of malignant ascites were obtained during diagnostic and/or therapeutic paracentesis from 25 patients (Patients 1-25 in Table I). These 25 patients included 20 patients who had epithelial ovarian cancer and one each who had granulosa cell tumor, endometrial cancer, gastric cancer, colon cancer and adenocarcinoma of unknown origin. Peritoneal washings were obtained during abdominal surgery from 6 additional patients, including 4 who had epithelial ovarian cancer, and 2 who had endometrial cancer (Patients 26-31 in Table I). Specimens of the peripheral blood were obtained from some of these patients and from another 3 patients who had gynecologic malignancies (Patients 32-34 in Table I). The mean age of the patients was  $57 \pm 12$  years (range 35 - 80 years). Peripheral blood specimens were also obtained from 5 normal women donors, aged  $35 \pm 7$  years.

### *Specimen preparation*

Fluid from ascites and peritoneal washings were collected into sterile bottles and heparin was added. Peripheral blood was collected into heparinized vacutainer tubes. All samples were processed within 2 hr of collection. Peritoneal exudate cells were sedimented from the ascites or washings by centrifugation at  $900 \times g$  for 10 min. The supernatant of ascites was collected for further analysis and stored at  $-70^{\circ}\text{C}$ . The cells were then resuspended in calcium/magnesium-free phosphate buffered saline (PBS), layered over a Histopaque 1077 density cushion (Sigma, St. Louis, MO) and centrifuged for 30

min at 800 x g. The layer of mononuclear leukocytes was then collected, and the cells were counted in a hemacytometer and washed with PBS.

The peripheral blood specimens were diluted with 1 part of PBS, layered over Histopaque 1077 and centrifuged at 800 x g for 30 min. After collection, the mononuclear leukocytes were counted and then washed with PBS.

### *Flow cytometry*

Phenotyping for the cell-surface antigens was determined by two- and three-color immunofluorescence using monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), or Tri-color. Antibodies were obtained from Caltag (Burlingame, CA), Becton-Dickinson (San Jose, CA), Calbiochem (La Jolla, CA), Olympus (Lake Success, NY) and Biosource International (Camarillo, CA). The specificity and source of these monoclonal antibodies are listed in Table II.

For two-color flow cytometric detection of lineage-negative HLA-DR<sup>+</sup> cells in preparations of mononuclear leukocytes, 10<sup>6</sup> cells were incubated for 30 min at 4°C with FITC-conjugated anti-HLA-DR, with a cocktail of PE-conjugated monoclonal antibodies directed against the following leukocyte antigens: CD3, CD11b, CD14, CD16, CD19, CD20, CD54, CD56 and CD58. The latter lineage cocktail was titrated to label all natural killer cells, T- cells, B- cells, monocyte/macrophages and granulocytes simultaneously. Cells labeled with PE- and FITC-conjugated isotype control monoclonal antibodies that were nonreactive to human cells served as controls. The labeled cells were washed with PBS, fixed in 1% paraformaldehyde and analyzed using a FACScan flow

cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a single 488-nm argon laser and 3 fluorescence detectors with filter settings for FITC (530 nm), PE (585 nm) and Tri-color (> 650 nm). A total of 20,000 to 50,000 events were collected in list mode and analyzed using FACScan Research Software (Becton Dickinson).

For three-color flow cytometric analysis,  $10^6$  cells were labeled simultaneously with a cocktail of FITC-conjugated lineage-specific antibodies, Tri-color-conjugated anti-HLA-DR, and PE-conjugated monoclonal antibodies that recognize other surface determinants. The cells were washed with PBS and fixed with 1% paraformaldehyde. For flow cytometric analysis, a live gate was set on the lineage-negative HLA-DR<sup>+</sup> cell population, and these cells were then analyzed for coexpression of a third marker identified as positive events (above background of isotype control) within the PE- channel. For some antigens, the expression was evaluated by relative fluorescence intensity as the fluorescence intensity of the antigen divided by the fluorescence intensity of the isotype control. Figure 1 is a representative plot that shows the detection of a lineage-negative HLA-DR<sup>+</sup> cell population. Histograms in Figure 2 show representative results for surface costimulatory and adhesion molecules and activation markers.

#### *Enzyme-linked immunosorbent assay*

Specimens of ascites were centrifuged at 900 x g. Supernatants were stored at -80°C until testing could be done. Concentrations of interleukin-4

(IL-4), interleukin-10 (IL-10), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN- $\gamma$  were determined in the ascitic fluid using commercially available enzyme-linked immunoabsorbant assay (ELISA) kits (BioSource International). The ELISA kit for neopterin was supplied by IBL (Hamburg, Germany). Testing was performed according to the manufacturer's instructions. The following standards were used as the lower limits of detectability: for IL-4 and TNF- $\alpha$ , 2 pg/ml, for IL-10, 6 pg/ml, for GM-CSF 8 pg/ml, for IFN- $\gamma$  4 pg/ml, and 1.35 nmol/l for neopterins.

#### *Statistical analysis*

Statistical significance of differences between unpaired observations was studied using the Mann-Whitney U test. The Wilcoxon paired test using StatView software (Abacus Concepts, Berkeley, CA was used for paired observations.). Correlations were studied by the Spearman rank coefficient, and correction for ties was performed with StatView software. Significance was determined as  $P < 0.05$ .

## Results

### *Lineage-negative HLA-DR<sup>+</sup> cells in malignant ascites and peripheral blood of patients with malignancies of the abdominopelvic cavity*

The proportion of lineage-negative HLA-DR<sup>+</sup> mononuclear leukocytes in malignant ascites are shown in Table III. The mean proportion of these cells  $\pm$  standard deviation was  $3.1 \pm 4.6\%$  and ranged from 0.05 to 17.3%. The mean number of lineage-negative HLA-DR<sup>+</sup> cells was  $8.0 \pm 9.5 \times 10^6$  cells/L, (range  $0.1-31.6 \times 10^6$  cells/L). A trend that favored higher numbers of lineage-negative HLA-DR<sup>+</sup> cells was observed in the malignant ascites from patients without a history of prior chemotherapy compared with the number seen in malignant ascites from previously treated patients  $11.3 \pm 10.2 \times 10^6$  cells/L, (range  $1.1-27.0 \times 10^6$  cells/L) V  $6.2 \pm 9.2 \times 10^6$  cells/L, (range  $0.1-31.6 \times 10^6$  cells/L), but this difference was not statistically significant. There was also no significant difference in the proportion of lineage-negative HLA-DR<sup>+</sup> cells in untreated and treated patients ( $3.0 \pm 2.8\%$ , (range 0.3-7.7%) V  $3.1 \pm 5.2\%$ , (range 0.05-17.3%), respectively). In contrast to the findings in malignant ascites, lineage-negative HLA-DR<sup>+</sup> cells were present in smaller proportions in peritoneal washings of 6 patients who had various abdominal cancers ( $0.15 \pm 0.1$  V  $3.1 \pm 4.6$ ;  $P < 0.005$ ).

Paired specimens of ascites and peripheral blood were obtained from 7 patients. The proportions of lineage-negative HLA-DR<sup>+</sup> cells was significantly



higher in the malignant ascites than in the peripheral blood ( $4.5 \pm 5.7\%$  V  $0.5 \pm 0.4\%$ ;  $P < 0.05$ ) (Fig. 3A).

Peripheral blood specimens of 17 patients who had abdominal cancer (ascites in 8 patients and tumor without ascites in 9 patients) contained  $0.4 \pm 0.3\%$  of lineage-negative HLA-DR<sup>+</sup> cells (Table IV). The proportion of lineage-negative HLA-DR<sup>+</sup> cells in peripheral blood specimens from patients with peritoneal or ovarian malignancies was significantly lower than the proportion in peripheral blood specimens from normal donors ( $0.4 \pm 0.3\%$  V  $0.9 \pm 0.3\%$ ,  $P < 0.02$ ).

*Cell surface differentiation and activation markers on lineage-negative HLA-DR<sup>+</sup> cells in ascites and peripheral blood of patients with carcinomas of the abdominopelvic cavity*

Three-color flow cytometric analysis of lineage-negative HLA-DR<sup>+</sup> cells in ascites revealed the following cell proportions: CD4<sup>+</sup>:  $92 \pm 9\%$  (range 63-100%) and CD45RA<sup>+</sup>:  $94 \pm 12\%$  (range 54-100%) (Tables III and IV, Fig. 3B). The proportions of lineage-negative HLA-DR<sup>+</sup> cells that coexpressed CD4 antigen was significantly higher in malignant ascites compared with peripheral blood specimens obtained from the same patients ( $95 \pm 4\%$  V  $63 \pm 17\%$ ;  $P < 0.05$ , (Fig. 3B).

The proportions of lineage-negative HLA-DR<sup>+</sup> cells in malignant ascites that expressed the costimulatory molecules CD80 and CD86 varied among specimens from individual patients (Table III). The mean proportion of lineage-

negative HLA-DR<sup>+</sup> cells from ascites that coexpressed CD80 was  $5 \pm 14\%$  (range 0-63%) (Table III), whereas the mean proportion in peripheral blood was  $4 \pm 8\%$  (range 0-12%) (Table IV). In 1 specimen of malignant ascites from 1 patient, CD80<sup>+</sup> lineage-negative HLA-DR<sup>+</sup> cells were detected at a very high frequency, 63%. The mean proportion of lineage-negative HLA-DR<sup>+</sup> cells in ascites that coexpressed CD86 was  $47 \pm 30\%$  (range 0-88%). The mean proportion of lineage-negative HLA-DR<sup>+</sup> cells in the peripheral blood that coexpressed CD86 was  $82 \pm 25\%$  (range 4-100%) When the proportions of CD86<sup>+</sup> lineage-negative HLA-DR<sup>+</sup> cells were compared in paired samples of ascites and peripheral blood from the same patients, the frequency of CD86<sup>+</sup> cells was significantly lower in lineage-negative HLA-DR<sup>+</sup> cells from ascites than peripheral blood ( $56 \pm 19\% \text{ V } 91 \pm 10\%$ ;  $P < 0.05$ ) (Fig. 3c).

The mean proportion of ascitic lineage-negative HLA-DR<sup>+</sup> cells that also expressed CD54 was  $98 \pm 2\%$  (range 93-100%) (Table III). In contrast, coexpression of CD58 in peripheral blood specimens ranged from 1 to 48% (mean  $13 \pm 14\%$ ), and the proportion of CD58<sup>+</sup> cells was significantly lower on lineage-negative HLA-DR<sup>+</sup> cells from ascites than those from peripheral blood in 6 paired specimens ( $11 \pm 18\% \text{ V } 68 \pm 22\%$ ;  $P < 0.05$ ) (Fig. 3d).

CD11c, which is a marker of maturation, was infrequently detected on lineage-negative HLA-DR<sup>+</sup> cells from ascitic fluid (mean  $4 \pm 8\%$ , range 0-34%) (Table III). The proportion of lineage-negative HLA-DR<sup>+</sup> cells that also expressed the CD45RO antigen correlated positively with lineage-negative HLA-

DR<sup>+</sup> cells that expressed either CD11c ( $rs = 0.763$ ;  $P < 0.02$ , Fig. 4a), or CD80 ( $rs = 0.598$ ,  $p < 0.05$ , Fig. 4b). The mean proportions of lineage-negative HLA-DR<sup>+</sup> cells in ascites that were CD45RO<sup>+</sup> appeared to be lower compared with that in peripheral blood ( $7 \pm 12\%$  V  $19 \pm 18\%$ ), whereas the proportion of lineage-negative HLA-DR<sup>+</sup> cells that were CD45RA<sup>+</sup> appeared to be lower in peripheral blood than in ascites ( $70 \pm 13\%$  V  $94 \pm 12\%$ ), but these results were not statistically significant.

Because cells of the monocyte/macrophage lineage may represent a population of antigen presenting cells in the peritoneal cavity that is different at the functional level from a population of dendritic cells, we compared certain maturation/activation-associated markers on CD14<sup>+</sup> macrophages and lineage-negative HLA-DR<sup>+</sup> cells. Figure 5 is an example from an analysis of one specimen which shows macrophages from peritoneal fluid expressing higher proportions of CD86, CD58 and CD11c compared with the expressions seen on lineage-negative HLA-DR<sup>+</sup> cells: CD14<sup>+</sup> cells were mostly CD45RO<sup>+</sup>.

As shown in Figure 6, the relative fluorescence intensity of HLA-DR on lineage-negative HLA-DR<sup>+</sup> cells was significantly lower in peripheral blood ( $21.8 \pm 13.6$ ;  $P < 0.05$ ) and ascites ( $15.0 \pm 8.1$ ;  $P < 0.01$ ) of patients compared with those seen in peripheral blood from normal donors ( $51.2 \pm 25.1$ ).

*Lineage-negative HLA-DR<sup>+</sup> cells and production of cytokines and neopterin in ascitic fluid*

Because the number and function of dendritic cells may be influenced by the cytokine microenvironment (Buelens *et al.*, 1995; Everson *et al.*, 1996; Mitra *et al.*, 1995), we set out to determine whether the numbers of dendritic cells correlated with (i) the ascitic fluid concentrations of certain cytokines involved with T-cell activation and (ii) with neopterin concentration as an indicator of macrophage activation. Detection of cytokines and neopterin was performed by ELISA as described in the Materials and Methods. IL-4 was not detected in specimens from 8 patients, and GM-CSF was detected in only 2 of 9 ascites. IFN- $\gamma$  and TNF- $\alpha$  were not detected in any of the specimens from 25 patients. In contrast, IL-10 was detected in most of the specimens examined. The mean concentration of IL-10 was  $71.4 \pm 82.5$  pg/ml (range 0-287 pg/ml) and the concentration of IL-10 detected correlated positively with the proportions of lineage-negative HLA-DR<sup>+</sup> cells detected in ascites ( $r_s = 0.40$ ;  $P = 0.05$ , Fig. 7a). Neopterin was detected in all but 1 of the ascites (mean  $19.1 \pm 17.5$ , range 0-76 nmol/l). The concentrations of neopterin correlated negatively with the numbers of lineage-negative HLA-DR<sup>+</sup> cells ( $r_s = -0.44$ ;  $P < 0.05$ , Fig. 7b), but correlated positively with the percentages of lineage-negative HLA-DR<sup>+</sup> cells that coexpressed CD4 ( $r_s = -0.63$ ;  $P < 0.005$ , Fig. 7c), or CD80 ( $r_s = 0.43$ ,  $P < 0.05$ , Fig. 7d).

### *Discussion*

Dendritic cells are antigen presenting cells that appear to have an important role in the activation of specific immune responses *in vivo* (Ridge *et al.*, 1996; Steinman, 1991). Because specific markers for human dendritic cells

have not been identified we utilized an alternative method that is based on defining the dendritic cell population by determining the absence of leukocyte-lineage markers and positivity for HLA-DR (Ferbass *et al.*, 1994; O'Doherty *et al.*, 1993). The use of two-color flow cytometry in the present study enabled quantitation of dendritic cells through the detection of lineage-negative HLA-DR<sup>+</sup> cells. Malignant ascites will usually provide large numbers of cells that can be recovered without extensive manipulations, e.g., enzymatic treatments that might cause significant alterations in a minor cell population such as dendritic cells (Abuzakouk *et al.*, 1996). Our study included patients who had received prior chemotherapy and who had not. We attempted to determine whether the variations in the numbers of lineage-negative HLA-DR<sup>+</sup> cells in ascites were related to prior chemotherapy. Significant differences could not be detected in either the percentages or number of lineage-negative HLA-DR<sup>+</sup> cells from previously treated or untreated patients. A larger sample size may be required to demonstrate differences between these two patient subgroups.

Dendritic cells may be detected at most anatomic sites (Steinman, 1991). Using different methods of detection from that used here, 2% (Kubicka *et al.*, 1985) to 6% (Betjes *et al.*, 1993) of peritoneal dendritic cells were detected in the peritoneal cavity of patients without a diagnosis of malignant disease. Dendritic cells have also been observed in solid tumor sections of certain nongynecologic tumors by immunohistochemical analysis, and a dense dendritic cell infiltrate has sometimes been seen (Furuhata *et al.*, 1992; Inoue *et al.*, 1993). These studies did not include an analysis of differentiation antigens or antigens associated with activation.

In agreement with previous reports, lineage-negative HLA-DR<sup>+</sup> cells were found to constitute a minor population in peripheral blood (O'Doherty *et al.*, 1994). The proportions of lineage-negative HLA-DR<sup>+</sup> cells was substantially lower in the peripheral blood of patients with abdominal or pelvic carcinomas compared with the proportion seen in normal donors. This difference could possibly be related either to prior systemic treatments that patients had received or to the disease process itself.

The number and proportion of dendritic cells that express antigens of differentiation and activation could be affected by the presence of cytokines in the microenvironment. IL-10 is a cytokine that is frequently detected in the ascites of patients with peritoneal carcinomatosis. We compared the production of IL-10 with the proportions of dendritic cells, and found that the proportions of lineage-negative HLA-DR<sup>+</sup> cells correlated positively with the concentration of IL-10 in the ascitic fluid. This could be a direct effect of IL-10. IL-10 may be produced by dendritic cells or other IL-10-producing leukocytes in the peritoneal cavity such as macrophages, which are present in large numbers in patients who have ovarian or peritoneal carcinomatosis. Because neopterin is a product of activated macrophages we measured the concentrations of neopterin in ascites to determine whether there was an association with the number or proportions of dendritic cells. Neopterin was detected in most of the ascites, but its concentration in ascites correlated negatively with the numbers of lineage-negative HLA-DR<sup>+</sup> cells. These results could be related to a previous association that has been shown between

neopterin production and depressed immunologic responses (Melichar *et al.*, 1996). Moreover, others have found an association between neopterin levels and poor prognosis in patients with certain cancers (Reibnegger *et al.*, 1986; Weiss *et al.*, 1993).

The antigen-presenting function of dendritic cells depends on the coexpression of MHC class II antigens and costimulatory molecules (June *et al.*, 1994; Lenschow *et al.*, 1996; Linsley and Ledbetter, 1993). The intensity of HLA-DR expression was detected at significantly lower levels on dendritic cells of peripheral blood and ascites from patients compared with levels found on peripheral blood cells from normal donors. Expression of CD80 on lineage-negative HLA-DR<sup>+</sup> cells was low or absent in most of the specimens studied. In contrast, CD86 was usually present but at significantly lower levels on lineage-negative HLA-DR<sup>+</sup> cells from ascites compared with the levels found on peripheral blood. This finding suggests that certain as yet undefined factors in the tumor microenvironment could have down-regulatory effects on CD86 expression on lineage-negative HLA-DR<sup>+</sup> cells, or could otherwise influence the maturity of dendritic cells in the peritoneal cavity. IL-10 has been shown to decrease the expression of CD86 antigen on human dendritic cells (Buelens *et al.*, 1995). In the current study, IL-10 was detected in most ascites but no relationship was detected between the expression of CD86 on lineage-negative HLA-DR<sup>+</sup> cells and the concentration of IL-10 in ascitic fluid. However, this does not necessarily imply that IL-10 is not an important contributing factor to the reduced proportion of dendritic cells that express either CD80 or CD56 *in vivo*.

The finding of a low expression of MHC class II antigens and costimulatory molecules on lineage-negative HLA-DR<sup>+</sup> cells in ascites from patients with peritoneal carcinomatosis suggests that dendritic cells may not function adequately as antigen-presenting cells in the tumor microenvironment of these patients, and this aspect deserves further study. Low expression of MHC class II antigens could be responsible for suboptimal stimulation through the T-cell receptor, which then increases the opportunity for an anergic response (Krummel and Allison, 1995; Lenschow *et al.*, 1996; Linsley and Ledbetter, 1993). Decreased expression of costimulatory factors on the lineage-negative HLA-DR<sup>+</sup> cells could have a similar result.

In a study of accessory molecules on lineage-negative HLA-DR<sup>+</sup> cells, we observed high expression of CD54. CD58 expression, however, was variable and significantly lower in ascites than in peripheral blood. CD45RO and CD11c have been reported to identify a subset of dendritic cells that have undergone activation and differentiation (O'Doherty *et al.*, 1994). Our studies show that the proportion of dendritic cells in ascites that express these markers is lower than the proportion of dendritic cells in peripheral blood. Dendritic cells have been reported to show a decrease in CD4<sup>+</sup> expression during the differentiation process (O'Doherty *et al.*, 1993). The lower proportion of CD4<sup>+</sup> lineage-negative HLA-DR<sup>+</sup> cells in the peripheral blood compared with that in ascites may be an indication of less mature functional characteristics of lineage-negative HLA-DR<sup>+</sup> leukocytes in malignant ascites. The same



conclusion could be reached from the expression of the CD45 isoform on lineage-negative HLA-DR<sup>+</sup> cells in peripheral blood and ascites.

In summary, we have detected lineage-negative HLA-DR<sup>+</sup> leukocytes in the malignant ascites associated with peritoneal carcinomatosis. These cells show a pattern of surface antigens that are characteristic of less mature dendritic cells. Decreases in the expression of MHC class II antigens and costimulatory or accessory molecules on dendritic cells associated with peritoneal carcinoma could be of importance in enabling the tumor to evade recognition by the host immune system.

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### **Figure legends**

#### **FIGURE 1-- Flow cytometric analysis of lineage-negative HLA DR<sup>+</sup> cells**

Specimens were processed as described in Material and Methods and stained with a cocktail of PE-conjugated (a and b), or FITC-conjugated (c and d) monoclonal antibodies against leukocyte lineage antigens, and FITC-conjugated (b), or Tri-color-conjugated (d) antibody against HLA-DR, or the appropriate isotype control antibody (a and c). Cells were then analyzed by flow cytometry, and the numbers of cells negative for leukocyte lineage antigens and positive for HLA-DR were determined. The figure shows results of a specimen from a representative (Patient 21). Lineage-negative HLA-DR<sup>+</sup> cells represented 5.1% of all mononuclear cells in this specimen. A live gate was placed on the lineage-negative HLA-DR<sup>+</sup> cell population (e), to analyze the expression of other surface antigens (see Fig. 2).

#### **FIGURE 2-- Expression of differentiation and activation markers on lineage-negative HLA-DR<sup>+</sup> cells**

An ascites specimen from Patient 21 was processed and stained as in Figure 1. In addition to the cocktail of FITC-conjugated antibodies against leukocyte lineage markers and Tri-color-conjugated anti-HLA-DR, the cells were labeled with a panel of PE-conjugated antibodies with reactivity to antigens directed to differentiation and activation associated antigens. A live gate was placed on the lineage-negative HLA-DR<sup>+</sup> cell population (Fig. 1e), and 5000 events were collected. The figure shows the following histograms: (A) IgG1 isotype control antibody, (b) CD86, (c) CD80, (d) CD11c; (e) IgG2 isotype control, (f) CD54, (g) CD58, (h) CD4, (i) CD45RA and (j) CD45RO.

**FIGURE 3**— Comparison in peripheral blood and malignant ascites of the proportions of lineage-negative HLA-DR<sup>+</sup> cells and the coexpression of CD4, CD86 and CD58 surface markers on these cells

Specimens of ascites (solid bars) and peripheral blood (hatched bars) obtained from the same patients were processed as described in Materials and Methods and analyzed (a) for proportions of lineage-negative HLA-DR<sup>+</sup> cells, expression of (b) CD4, (c) CD86 and (d) CD58. Values are shown for 7 patients except for (d) which shows values for 6 patients. Differences between findings in peripheral blood and ascites were statistically significant (Wilcoxon paired test,  $p < 0.05$ ).

**FIGURE 4**— Correlation between the number of lineage-negative HLA-DR<sup>+</sup> cells, IL-10 concentrations, and the phenotypic markers

The percentage of CD45RO<sup>+</sup> lineage-negative HLA-DR<sup>+</sup> cells in ascites was positively correlated with the proportion of cells that were (a) CD11c<sup>+</sup>, and (b) CD80<sup>+</sup>. Spearman correlation coefficients ( $r_s$ ) and significance levels are indicated on the figure.

**FIGURE 5--** Comparison of cell surface markers on lineage-negative HLA-DR<sup>+</sup> cells and CD14<sup>+</sup> cells in malignant ascites

Cells from Patient 11 were stained as described in Figures 1 and 2 using FITC-conjugated anti-CD14 and a panel of PE-conjugated antibodies with specificities for activation and differentiation antigens. Proportions of lineage-negative HLA-DR<sup>+</sup> or CD14<sup>+</sup> cells coexpressing each antigen indicated on the vertical axis. LN<sup>-</sup>DR<sup>+</sup>, Lineage-negative HLA-DR<sup>+</sup> cells.

**FIGURE 6--** Relative expression of HLA-DR on LN<sup>-</sup>CD4<sup>+</sup> cells

Cells from peripheral blood of normal donors and patients and malignant ascites were stained with FITC-conjugated leukocyte lineage cocktail, PE-conjugated anti-CD4, and Tri-color-conjugated anti-HLA-DR or Tri-color isotype control. A live gate was placed on the lineage-negative CD4<sup>+</sup> population (not shown). Relative fluorescence intensity of HLA-DR expression was determined by dividing the mean fluorescence intensity of HLA-DR stained cells by the mean fluorescence intensity of the isotype control as described in Materials and Methods. The relative fluorescence intensity of HLA-DR in the lineage-negative HLA-DR<sup>+</sup> CD4<sup>+</sup> cell population was significantly higher in normal donors than in peripheral blood (Mann-Whitney U test,  $P < 0.05$ ) or ascites ( $P < 0.01$ ) from patients.

**FIGURE 7--** Proportions of lineage-negative HLA-DR<sup>+</sup> cells that express surface antigens associated with differentiation and activation correlated with the concentrations of IL-10 and neopterin in ascitic fluids.

The proportion of lineage-negative HLA-DR<sup>+</sup> cells and those coexpressing CD4 or CD80 in ascites were determined by two- and three- color flow cytometry as described for Figures 1 and 2. Concentration levels of IL-10 and neopterin in ascitic fluid were measured by ELISA.

(a) The concentrations of IL-10 correlated positively with the percentage of lineage-negative HLA-DR<sup>+</sup> cells. (b) Concentrations of neopterin correlated negatively with the number of lineage-negative HLA-DR<sup>+</sup> cells and (c) with the expression of CD4 on these cells (d) Positive correlation was observed between the concentration of neopterin and the expression of CD80 on lineage-negative HLA-DR<sup>+</sup> cells.

TABLE I

## Clinical and pathological characteristics of patients

Patient	Age (yrs)	Disease stage	Tumor histology	Grade	Prior treatment
1	66	Recurrent	Mixed-- serous, endometrioid, undifferentiated ovarian carcinoma	PD	Yes
2	63	Recurrent	Adenocarcinoma of unknown origin	NA	Yes
3	41	IIIc	Serous ovarian carcinoma	PD	No
4	64	Recurrent	Mixed-- clear cell, undifferentiated ovarian carcinoma	PD	Yes
5	71	Recurrent	clear cell ovarian carcinoma	PD	Yes
6	54	IIIc	Serous ovarian carcinoma	PD	No
7	73	Recurrent	Serous ovarian carcinoma	NS	Yes
8	57	Recurrent	Mixed Müllerian tumor, serous carcinoma, spindle cell sarcoma	PD	Yes
9	49	Recurrent	Serous ovarian carcinoma	PD	Yes
10	52	Recurrent	Serous ovarian carcinoma	PD	Yes
11	62	IIIc	Mixed-- serous, undiff ovarian carcinoma	PD	No
12	58	Recurrent	Serous ovarian carcinoma	PD	Yes
13	50	Recurrent	Serous ovarian carcinoma	PD	Yes
14	70	IIIc	Mixed Müllerian tumor	PD	No
15	62	Recurrent	Undifferentiated ovarian carcinoma	PD	Yes
16	37	Recurrent	Clear cell ovarian carcinoma	NS	Yes
17	35	IIIc	Undifferentiated ovarian carcinoma	PD	No
18	48	IIIc	Serous ovarian carcinoma	PD	No
19	59	Recurrent	Colon adenocarcinoma	WD	Yes
20	77	Recurrent	Serous ovarian carcinoma	PD	Yes
21	40	Recurrent	Serous ovarian carcinoma	PD	Yes
22	45	Ic	Granulosa cell tumor	WD	No
23	45	IV	Gastric cancer; signet ring cell	PD	Yes
24	80	Recurrent	Endometrial carcinoma; mixed-- serous, endometrioid	MD	Yes
25	63	Recurrent	Mixed-- serous, transitional cell ovarian carcinoma	PD	Yes
26	42	I	Endometrial carcinoma, endometrioid; breast carcinoma	MD	Yes
27	67	Recurrent	Serous ovarian carcinoma	PD	Yes
28	71	Recurrent	Serous ovarian carcinoma	PD	Yes
29	58	Recurrent	Serous ovarian carcinoma	PD	Yes
30	78	I	Endometrial carcinoma; endometrioid	MD	No
32	47	I	Serous ovarian carcinoma	PD	No
33	65	Recurrent	Serous ovarian carcinoma	PD	Yes
34	52	Recurrent	Mixed, undifferentiated ovarian carcinoma	PD	Yes
35	46	Recurrent	Granulosa cell tumor	WD	Yes

NA, not available; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; NS, not stated

**TABLE II**  
**MURINE MONOCLONAL ANTIBODIES USED FOR CELL SURFACE CHARACTERIZATION**  
**OF DENDRITIC CELLS**

Specificity	Clone	Isotype	Working dilution	Label	Source
CD1a	VIT 6B	IgG1	1:50	R-PE	Caltag
CD3	S4.1	IgG2a	1:50	FTTC, R-PE	Caltag
CD4	S3.5	IgG2a	1:50	R-PE	Caltag
CD11b	CR3 (Bear-1)	IgG1	1:50	FTTC, R-PE	Caltag
CD11c	3.9	IgG1	1:50	R-PE	Caltag
CD14	TUk4	IgG2a	1:50	FTTC, R-PE	Caltag
CD16	3G8	IgG1	1:50	FTTC, R-PE	Caltag
CD19	SJ25-C1	IgG1	1:50	FTTC, R-PE	Caltag
CD20	HI47	IgG3	1:50	FTTC, R-PE	Caltag
CD45RO	UCHL1	IgG2a	1:50	R-PE	Caltag
CD45RA	MEM 56	IgG2b	1:50	R-PE	Caltag
CD54	MEM-111	IgG2a	1:50	R-PE	Caltag
CD56	ERIC-1	IgG1	1:50	R-PE	Caltag
CD56	MY31	IgG1	1:30	FTTC	Olympus
CD58	L306	IgG2a	1:30	R-PE	Becton-Dickinson
CD80	L307	IgG1	1:30	R-PE	Becton-Dickinson
CD86	BU 63	IgG1	1:80	R-PE	Becton-Dickinson
HLA-DR	TO36	IgG2b	1:50	FTTC, TC	Calbiochem

R-PE, R-phycoerythrin; FTTC fluorescein isothiocyanate; TC Tri-color.

TABLE III

PROPORTIONS AND ABSOLUTE NUMBERS AND CELL SURFACE PHENOTYPE OF LINEAGE-NEGATIVE HLA-DR POSITIVE LEUKOCYTES IN MALIGNANT ASCITES

Patient	Proportion of MNL	Absolute numbers (10 <sup>6</sup> /l)	CD86	CD80	CD54	CD58	CD11c	CD4	CD45 RA	CD45 RO
1*	0.3	2.7	28	1	100	nt	nt	91	nt	nt
2*	0.3	0.2	45	1	97	nt	nt	63	nt	nt
3	5.4	27.0	8	1	100	nt	7	99	nt	nt
4*	0.3	0.5	81	63	98	nt	34	81	nt	nt
5*	2.8	0.6	4	0	97	nt	0	94	nt	nt
6	0.3	4.0	0	0	97	nt	2	95	nt	nt
7*	0.2	0.8	5	3	94	nt	1	89	nt	nt
8*	0.4	0.5	1	0	99	3	1	94	nt	nt
9*	0.05	0.6	nt	nt	nt	nt	nt	nt	nt	nt
10*	0.2	1.1	nt	nt	nt	nt	nt	nt	nt	nt
11	0.9	1.8	52	0	98	1	0	97	nt	nt
12*	1.2	0.1	83	0	100	42	0	98	99	98
13*	4	10.8	56	0	nt	11	6	99	99	99
14	1.0	1.1	80	0	100	6	1	92	98	98
15*	1.9	10.3	58	0	99	2	0	100	100	100
16*	0.5	7.4	nt	nt	nt	nt	nt	nt	nt	nt
17	4.2	19.2	74	2	100	7	7	99	99	96
18	7.7	7.7	38	0	98	2	0	98	100	100
19*	17.3	26	57	0	98	17	2	99	99	99
20*	3	2.5	88	15	97	19	2	79	92	92
21*	5.1	12.1	74	11	99	5	0	98	99	99
22	1.3	18.6	60	2	93	48	nt	91	80	80
23*	0.2	1.2	68	14	97	10	nt	80	94	94
24*	1.7	11.9	12	4	99	20	1	98	98	98
25*	16.1	31.6	61	0	nt	4	1	94	94	94
Number	25	25	22	22	20	15	17	22	14	14
Range	0.05 - 17.3	0.1 - 31.6	0 - 88	0 - 63	93 - 100	1 - 48	0 - 34	63 - 100	54 - 100	0 - 47
Mean ± SD	3.1 ± 4.6	8.0 ± 9.5	47 ± 30	5 ± 14	98 ± 2	13 ± 14	4 ± 8	92 ± 9	94 ± 12	7 ± 13

\* These patients had a history of prior chemotherapy. MNL mononuclear leukocytes; nt not tested..



TABLE IV

PROPORTIONS OF L<sup>v</sup>HLA-DR<sup>+</sup> CELLS AND CELL SURFACE PHENOTYPE  
IN PERIPHERAL BLOOD OF PATIENTS WITH CARCINOMAS OF THE  
ABDOMINOPELVIC CAVITY

Patient	%	CD86	CD80	CD54	CD58	CD11c	CD4	CD45RA	CD45RO
1*	0.3	71	1	85	nt	nt	70	nt	nt
11	0.1	89	0	40	40	0	73	67	10
14	0.6	98	0	84	79	8	65	79	1
16*	0.1	nt	nt	nt	nt	nt	nt	nt	nt
17	1.3	96	3	88	52	25	71	70	5
18	0.4	100	5	100	94	11	61	70	49
22	0.4	94	0	82	56	nt	76	83	9
25*	0.6	86	4	nt	88	nt	27	nt	nt
26*	0.1	4	1	92	43	8	83	50	38
27*	0.2	nt	nt	nt	nt	nt	nt	nt	nt
28*	0.8	78	0	92	45	nt	89	82	nt
29*	0.3	98	0	97	96	26	85	82	33
30	0.8	96	0	100	89	58	100	50	38
31	0.5	53	0	51	46	nt	48	51	4
32*	0.6	78	2	87	nt	nt	88	nt	nt
33*	0.2	97	12	62	39	2	54	73	0
34*	0.1	96	2	49	46	19	71	70	5
N	17	15	12	14	12	9	15	12	11
Mean±SD	0.4±0.3	82±25	2±3	79±20	63±24	17±18	71±18	69±12	17±18
Range	0.1 - 1.3	4 - 100	0 - 12	40 - 100	39 - 96	0 - 58	27 - 100	50 - 83	0 - 49

\* These patients had a history of prior chemotherapy; nt not tested.

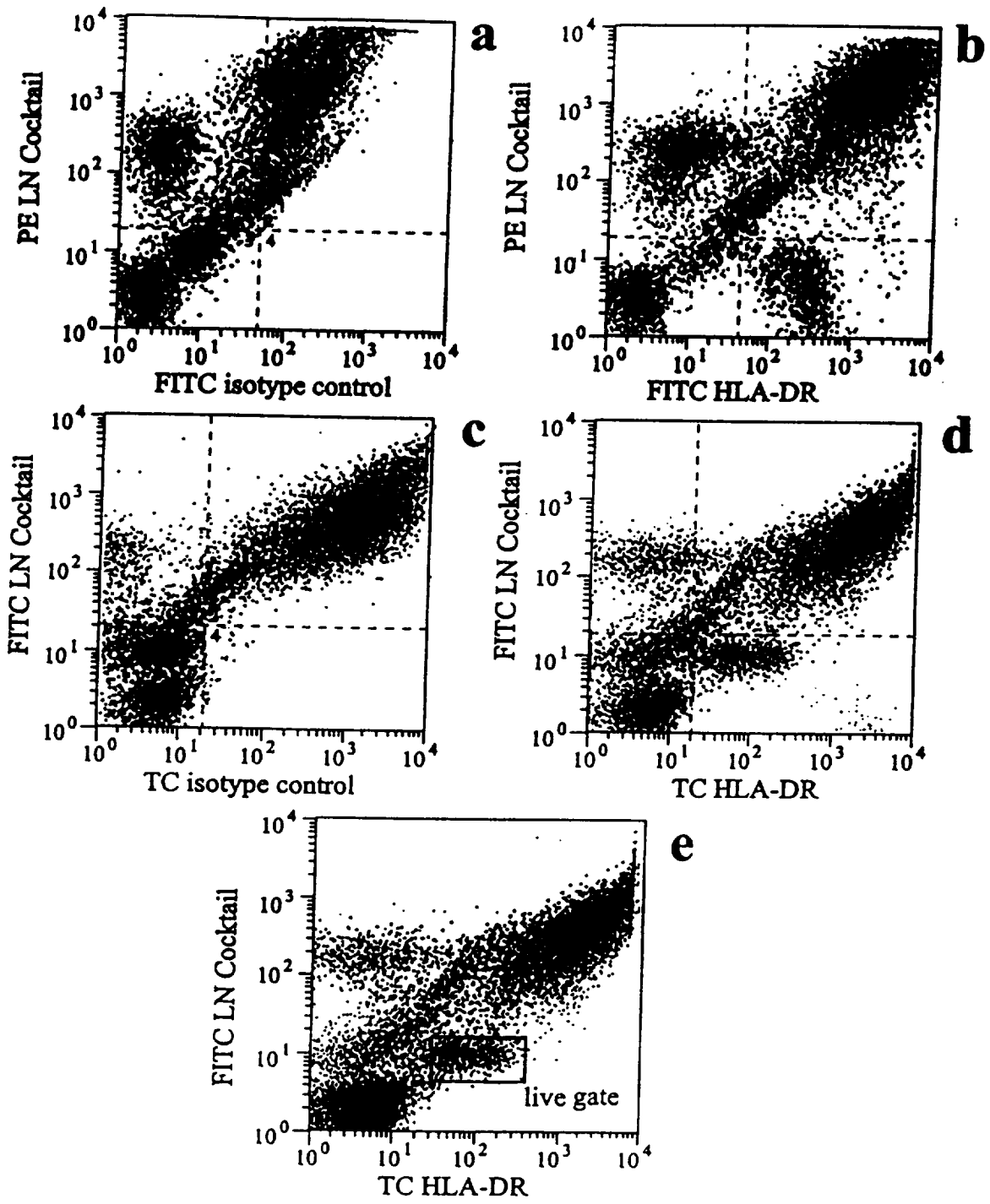


Fig 1

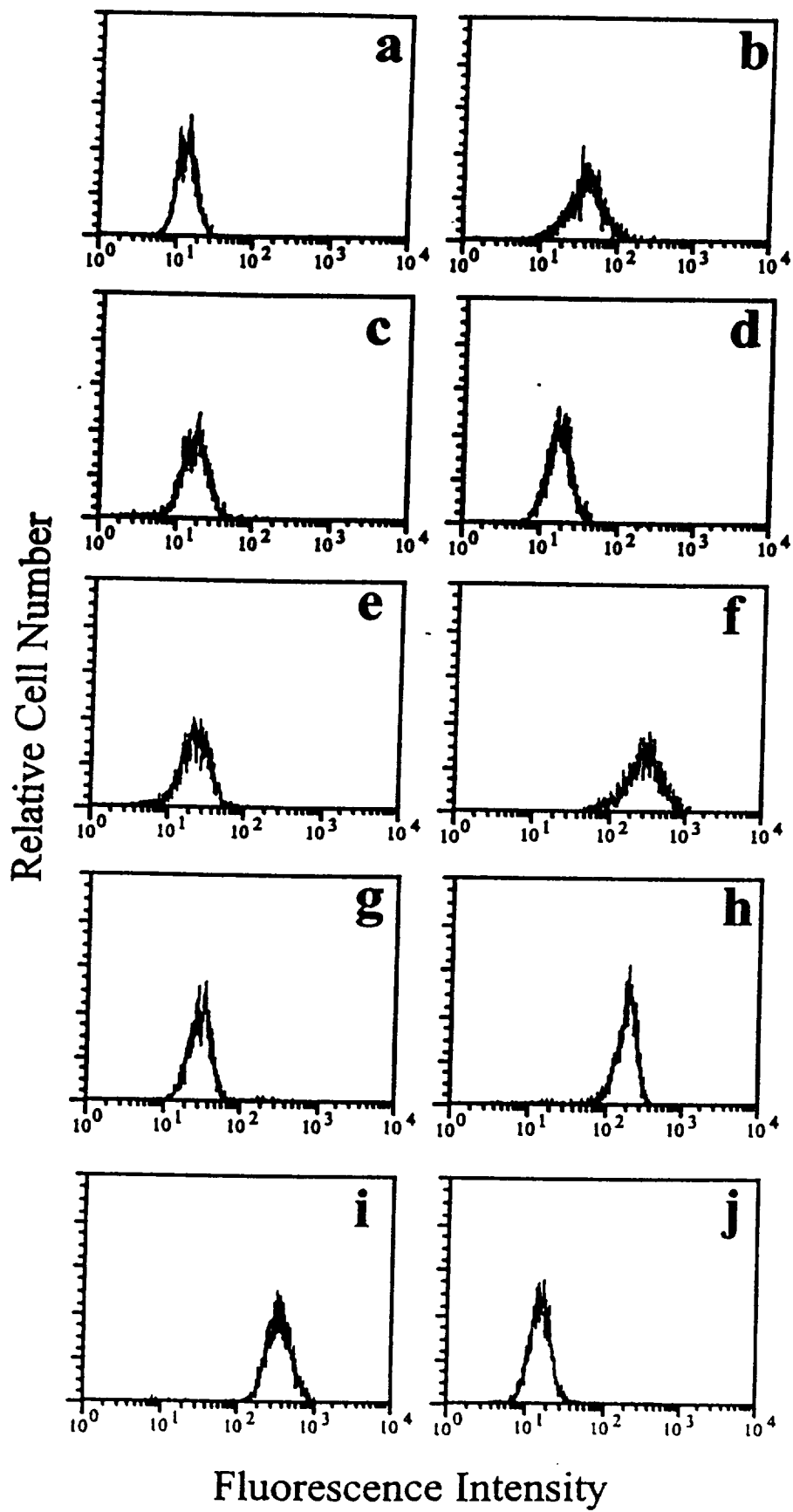


Fig 2

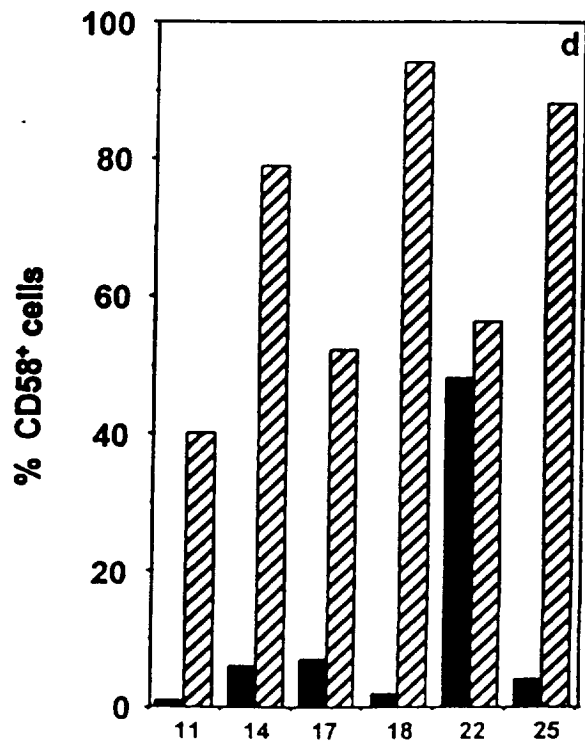
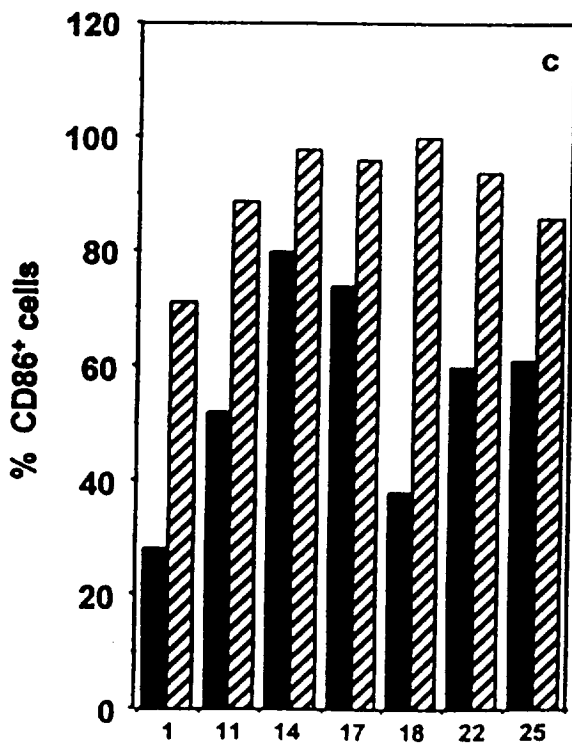
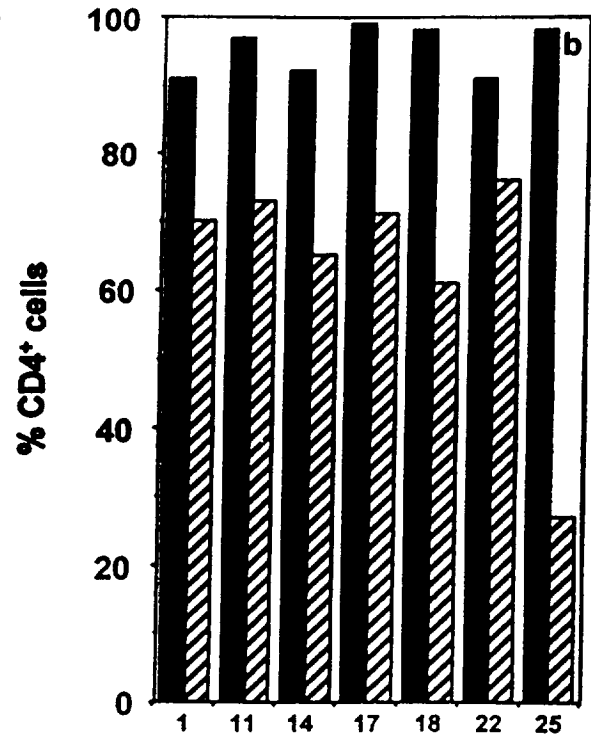
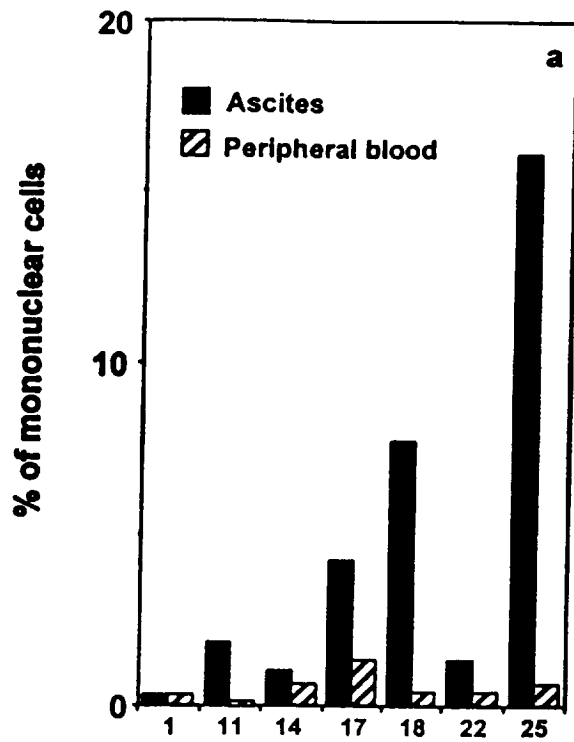


Fig 3

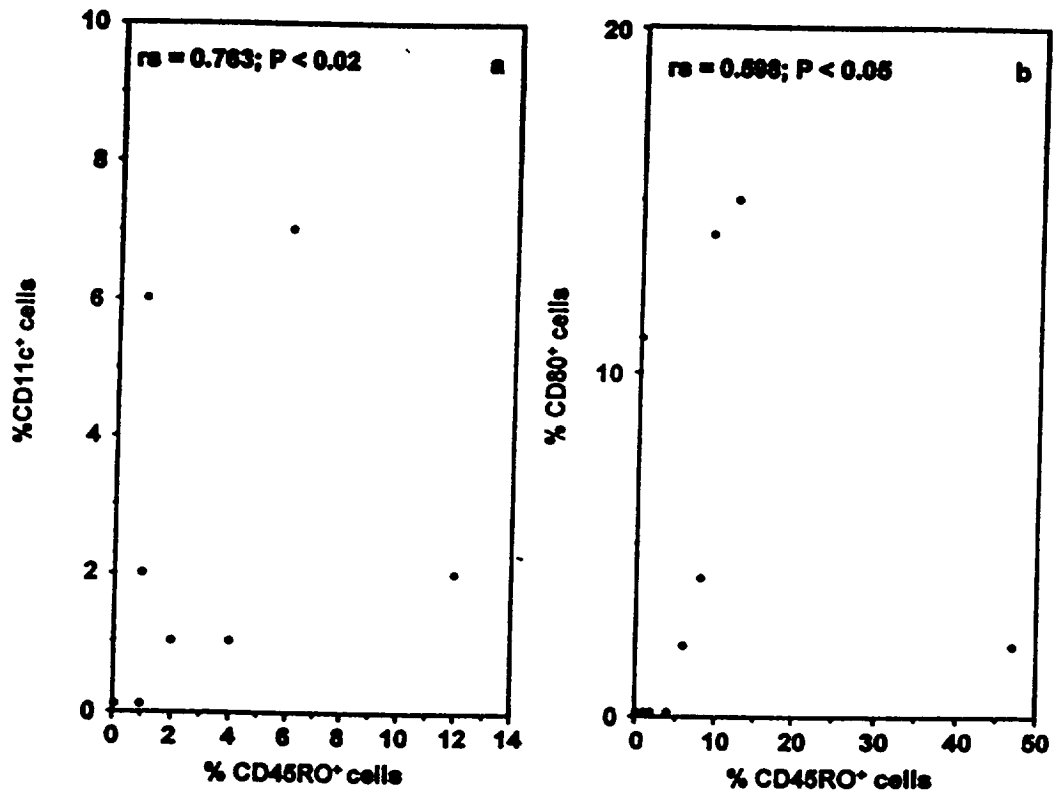


Fig 4

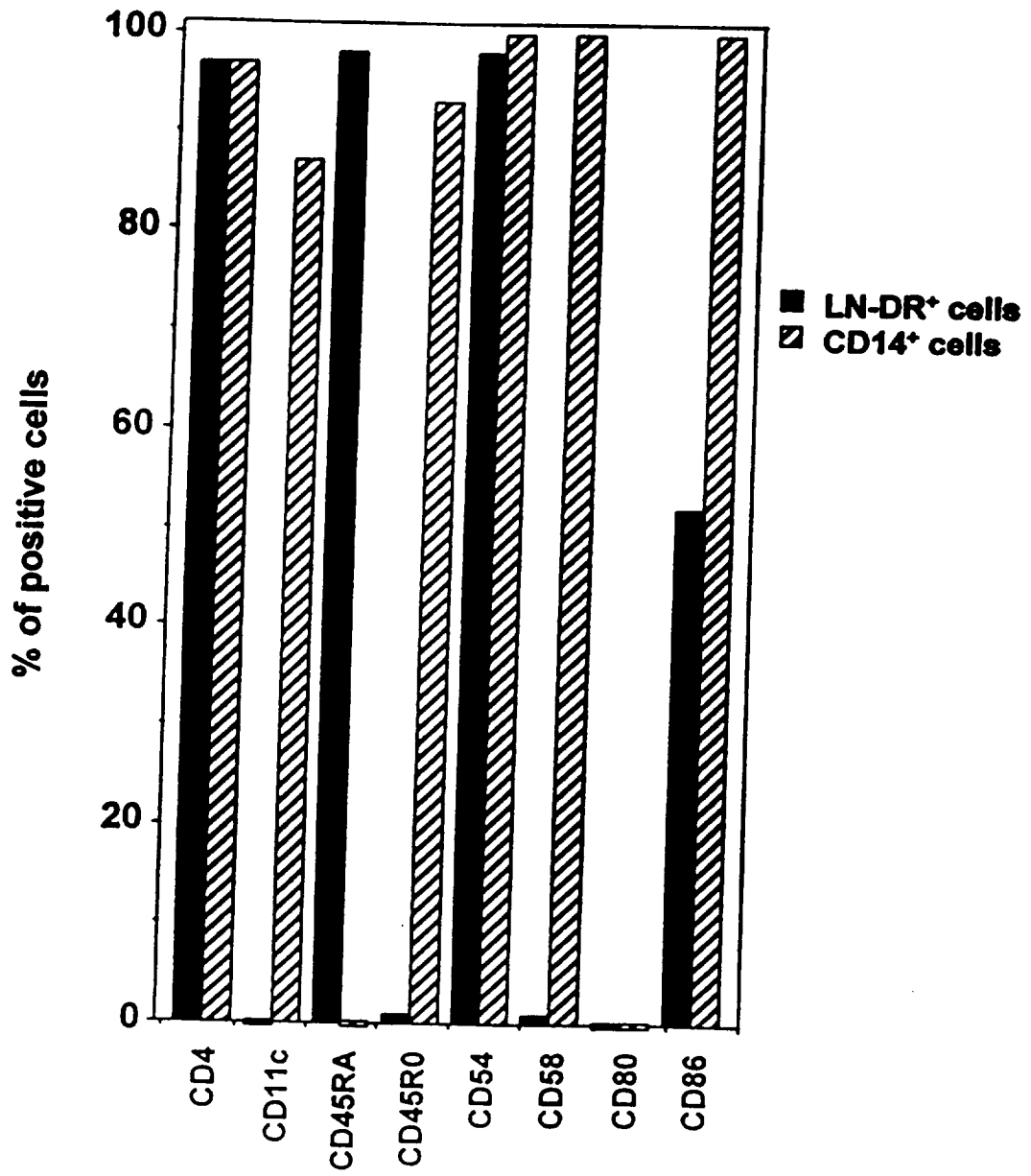


Fig 5

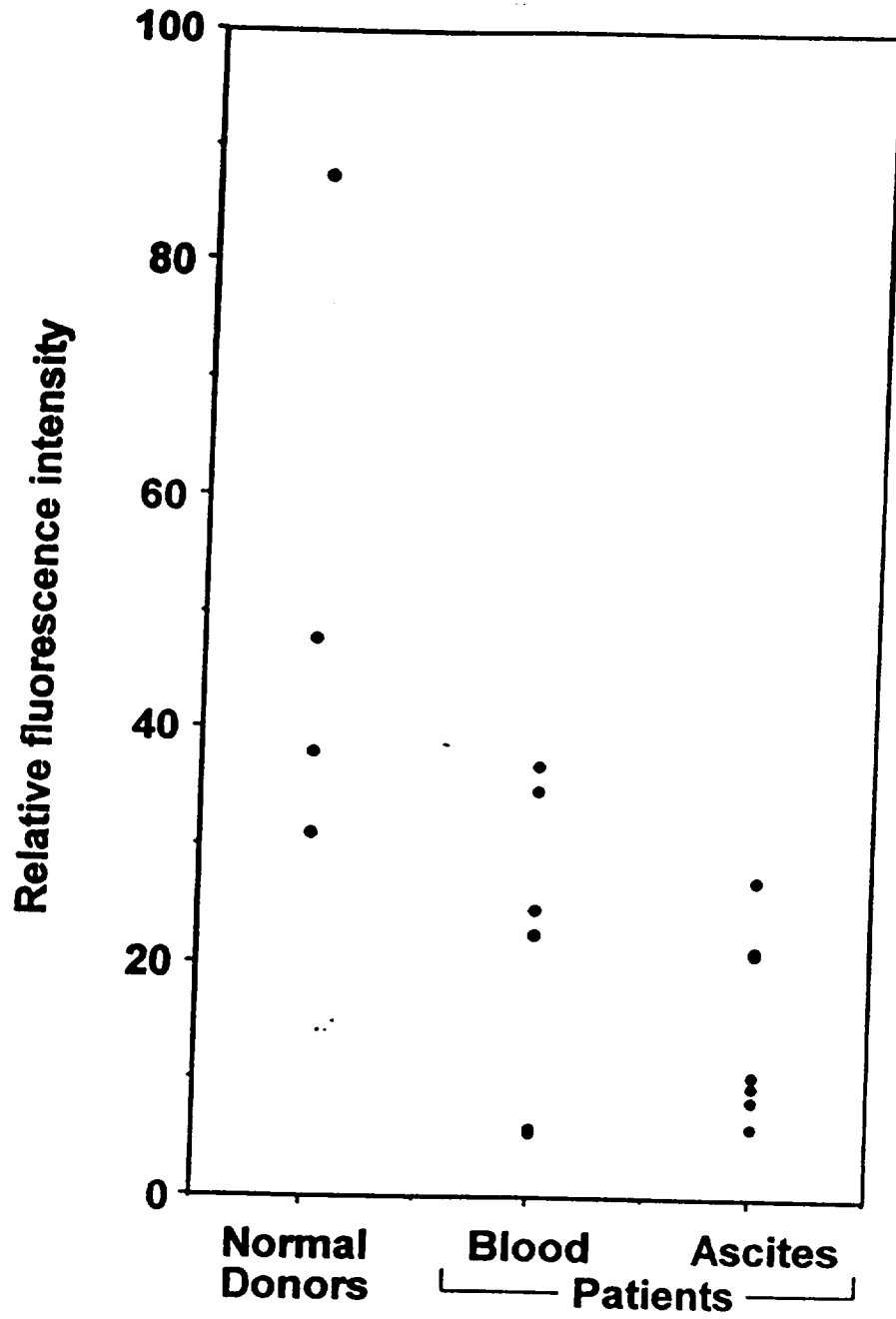


Fig 6

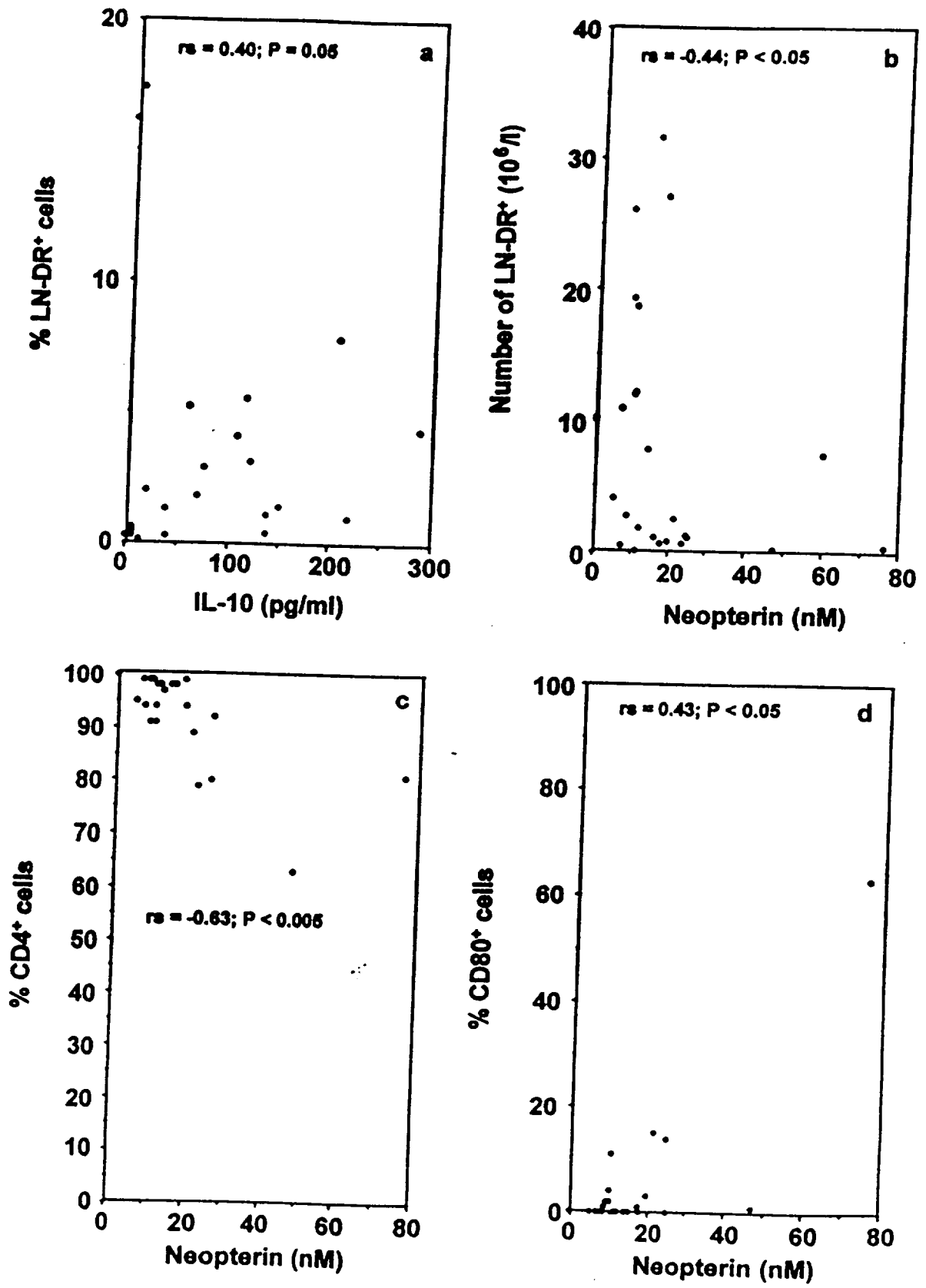


Fig 7



**ILLINOIS MATHEMATICS AND SCIENCE ACADEMY**

A Pioneering  
Educational Community

December 4, 1996

Dr. Cherylyn Savary  
Dept. of Surgical Oncology  
MD Anderson Cancer Center  
151 Holcolm Blvd., PO Box 18  
Houston, TX 77030

Dear Dr. Savary,

One of the goals of IMSA's Mentorship Program is to develop young scientists and scholars who not only understand and experience research, but are able to make a significant contribution to the evolving body of knowledge. This is an ideal, and although most never reach his level as high school students, a few do. Those few have extraordinary mentors who are able to guide the students' development of specific research skills and concepts, immerse them in realities of the process and at the same time instill excitement for research, structure the experience to stretch and accommodate the student's talents, and be an advocate and friend in the often tangled and uncertain process.

You are one of these rare mentors; please know how much this is appreciated. Manu Goyal is one of eleven Illinois students selected to represent the state at this year's AJAS/AAAS (American Junior Academy of Science/American Association for the Advancement of Science) annual conference in Seattle in February. Manu will present his paper, Consequences of Heat-Shocked Proteins and the Dendritic Cell-Tumor Interaction in the Immune Response Against Breast Cancer, which was done under your guidance. I hope you will be able to attend AAAS and hear the presentation. Students will give both oral and poster presentations Friday, February 14; poster presentations from 10 AM -12:30 in Hall C4 of the Seattle Convention Center, oral from 2-5:15 PM (in six concurrent sessions) in rooms 615-620 of the center. Students will also have the opportunity to visit with scientists, tour research facilities, and attend AAAS conference sessions. This is such an incredible opportunity, and could not have happened without your generous and exceptional guidance.

Again, thank you so much for everything you have done for Manu. Hope to see you in Seattle.

Enclosed are the students' abstracts: I thought you might find them interesting!

Sincerely,

A handwritten signature in cursive script that reads "Dr. Peggy Connolly".

Dr. Peggy Connolly  
Mentorship Coordinator

cc: Dr. Raphael Pollock

**American Association for the Advancement of Science**  
**1997 AAAS Annual Meeting and Science Innovation Exposition - AMSIE '97**  
***American Junior Academy of Science***  
***Poster Abstract Form - Deadline: November 1, 1996***

Category (Circle One): *Education Life Physical Social*

Consequences of Heat-Shocked Proteins and the Dendritic Cell-Tumor Interaction in the Immune Response Against Breast Cancer. MANU S. GOYAL (Illinois Mathematics and Science Academy, 1500 West Sullivan Rd., Aurora, IL 60506-1000), DR. CHERYLYN A. SAVARY (Dept. of Surgical Oncology, MD Anderson Cancer Center, 1515 Holcolm Blvd., PO Box 18, Houston, TX 77030)

Dendritic Cells (DC) are professional antigen-presenting cells that have been shown to effectively immunize lymphocytes against tumors. This study investigated DC ability to kill, bind, and respond to breast tumor cells. Some of the tumor cells were heat-shocked prior to incubation in order to induce Heat-Shocked Proteins (Hsp) and investigate their effect on DC. Hsp have been attributed as targets to the immune system as well as protective aids to breast tumor cells. No significant tumor cytotoxicity by DC was detected as tested in a  $^{51}\text{Cr}$  release assay. We found isolated DC to bind to tumor cells as measured using flow cytometry analysis, but when B-cells and T-cells were included, they seemingly did not. This suggests that B-Cell, T-Cell, and DC ratios may affect the immune response to breast cancer. Hsp made no significant difference in this test. The expression of several DC surface markers, measured by flow cytometry analysis, increased when incubated with breast tumor cells. Hsp lowered this expression significantly. This suggests that Hsp may protect breast tumors from the immune system. These results indicate a novel role for both Hsp and DC, and may potentially lead to a new form of immunotherapy for breast cancer.

Presenter's Name: Manu S. Goyal  
 Mailing Address: 228 Whispering Hills Rd.  
 City/State/Zip: Naperville, IL 60540  
 Phone: (630) 420-8880 (weekends)  
 - (630) 907-5250 (weekdays)

School Presenter Attends: Illinois Mathematics  
 and Science Academy (IMSA)  
 Current Grade Level: 12th  
 Academy You Represent: Illinois  
 Junior Academy of Science  
 Funding Sponsor: IMSA Fund for  
 Advancement of Education

Research Advisor: Dr. Cherylyn A. Savary  
 Affiliation: MD Anderson Cancer Center  
 Daytime Phone: (713)792-3134

Name of School Sponsoring Research:  
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 Academy Sponsor: Dr. Peggy Connolly  
 Daytime Phone: 630-907-5985