CHARACTERIZATION OF DENDRITIC CELLS FOR IN VITRO INDUCTION OF T CELL TOLERANCE

by

Subhashini Mahto

A thesis submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Master of Science

in

Medical Laboratory Science

Department of Pathology

The University of Utah

December 2002

THE UNIVERSITY OF UTAH GRADUATE SCHOOL

SUPERVISORY COMMITTEE APPROVAL

of a thesis submitted by

Subhashini Mahto

This thesis has been read by each member of the following supe visory committee and by majority vote has been found to be satisfactory.

Spang ude

THE UNIVERSITY OF UTAH GRADUATE SCHOOL

FINAL READING APPROVAL

To the Graduate Council of the University of Utah:

I have read the thesis of Subhashini Mahto in its final form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the supervisory committee and is ready for submission to The Graduate School.

Linda L. Kelley
Chair: Supervisory Committee

Approved for the Major Department

Chair

Approved for the Graduate Council

David S.
Dean of The Graduate School

ABSTRACT

A major barrier to effective allogeneic bone marrow transplantation is Graft versus host disease (GVHD). Donor T cells are a recognized prerequisite for generation of GVHD. Dendritic cells (DCs) are the most powerful antigen-presenting cell (APC) in the immune system. The ability of DCs to stimulate primary T lymphocytes and T cell dependant immune responses may provide opportunities for therapeutic intervention in bone marrow transplantation. According to recent reports, existing myeloid and plasmacytoid DC subsets, both under specific in vitro culture conditions are capable of secreting specific cytokines responsible for the polarization of T cells to a T helper 2 (Th2 response). Generation of Th2 cells in turn results in donor specific T cell tolerance to recipient DC presented antigen. DCs have great potential in preventing GVHD. The first goal of this study was to isolate DC subsets from human peripheral blood or apheresis product using newly developed markers for blood dendritic cell antigens (Miltenyi Biotech) and characterize them. The second goal was to determine the culture conditions that would support the maintenance and/or development of specific recipient DC subsets capable of inducing donor T cell tolerance. DCs were isolated by immunomagnetic cell sorting method from a population of mononuclear cells (MNCs). MNCs were labeled with recently developed antiBDCA-1 (blood dendritic cell antigen-1) and BDCA-4 immunomagnetic beads to isolate myeloid (mDCs) and plasmacytoid dendritic cells (pDCs) respectively, on a magnetic separation column. Positively selected dendritic cells were characterized by evaluating the presence or absence of specific

markers by flow cytometry with simultaneous three-color staining and a two-step acquisition procedure. A total of 12 isolations for BDCA-1 positive cells and three for BDCA-4 positive cells were done. Staining with fluorochrome-labeled antibodies to CD11c, IL-3Rα (CD123), and HLA-DR confirmed the identity of mDC ($CD11c^+$, IL-$3R\alpha^{low}$/$HLA\text{-}DR^+$/$Lineage^-$) and pDC ($CD11c^-$/IL-$3R\alpha^{high}$/$HLA\text{-}DR^+$/$Lineage^-$) subsets. A simultaneous staining with antilineage cocktail which contained a mixture of monoclonal antibodies (CD3, CD14, CD16, CD19, CD20 and CD56 monoclonal antibodies) specific for individual lineage markers, was done to exclude lineage positive cells during flow cytometry analysis. Flow cytometry data confirmed successful isolation of both myeloid and plasmacytoid dendritic cell subsets. Dendritic cells are otherwise generated by culturing MNCs and $CD34^+$ cells in the presence of cytokines such as GM-CSF and IL-4. It is concluded that recently developed antibodies to BDCA can be used for the isolation of dendritic cells directly from peripheral blood, instead of culturing MNCs or $CD34^+$ cells over prolonged period for the generation of dendritic cells.

TABLE OF CONTENTS

ABSTRACT.....iv

LIST OF FIGURES..... viii

LIST OF ABBREVIATIONS.....ix

ACKNOWLEDGEMENTS.....xi

INTRODUCTION..... 1

Background.....1
Objectives..... 6

BLOOD DENDRITIC CELL ISOLATION METHOD..... 8

Magnetic Cell Sorting.....8
General Principles.....8
MACS System..... 10
Detection of Human Blood Dendritic Cells.....12
Isolation of BDCs..... 14

IDENTIFICATION AND CHARACTERIZATION OF BLOOD DENDRITIC CELLS..... 17

General Principles of Flow Cytometer..... 17
Flow Cytometry Assay Development.....19
Antibody Panel Creation.....20
Staining..... 22
Data Acquisition..... 23
Data Analysis.....23
Morphology of BDCA-1^{+}Cells..... 28
Costimulatory Molecules and Activation Markers.....35
Culture of Dendritic Cells.....36

DISCUSSION..... 43

Immunophenotypic Characterization.....43
Frequency of BDCs in Peripheral Blood.....43
Purity of Isolated BDCs.....43
DC Activation and Maturation.....44

REFERENCES 47

LIST OF FIGURES

Figure		Page
1.	Dendritic cell (DC) lineage	5
2.	Cell separation by MACS technology	9
3.	MACS system with MS column in mini MACS separator	11
4.	Positive selection of BDCA-1$^+$ cells	16
5.	Schematic diagram of a flow cytometer analyzer	18
6.	FCM data of MNCs before BDCA-1 isolation	24
7.	FCM data of BDCA-1 enriched sample	25
8.	Immunophenotypic characterization of BDCA-1$^+$ cells	27
9.	Staining of MNCs for CD19	29
10.	FCM data of CD19 depleted samples	30
11.	FCM data of MNCs before BDCA-4 isolation	31
12.	FCM data of BDCA-4 enriched sample	32
13.	Immunophenotypic characterization of BDCA-4$^+$ cells	33
14.	Morphology of isolated BDCA-1$^+$cells	34
15.	Staining of BDCA-1$^+$ cells for activation markers	37
16.	Staining of isolated BDCA-4$^+$cells for activation markers	38
17.	Culture of BDCA-1$^+$ DCs without cytokine supplementation	39
18.	Optimization of culture conditions	42

LIST OF ABBREVIATIONS

Antigen presenting cell.....APC

Blood dendritic cell antigen.....BDCA

CD40 ligand.....CD40L

Cytotoxic T lymphocyte.....CTLA4

Dendritic cell.....DC

Flow cytometry.....FCM

Fluoroscein isothiocynate.....FITC

Granulocyte macrophage colony stimulating factor.....GMCSF

Human leukocyte antigen.....HLA

Interleukin.....IL

Interferon gamma.....IFNγ

Graft versus host disease.....GVHD

Major histocompatibility complex.....MHC

Magnetic cell separation.....MACS

Monoclonal antibodies.....MoAbs

Mononuclear cells.....MNCS

Myeloid dendritic cell.....mDC

Plasmacytoid dendritic cell.....pDC

Phycoerythrin.....PE

Peridinin chlorophyll protein.....PerCP

T helper type 1.....Th1

T helper type 2...Th2

ACKNOWLEDGEMENTS

Grateful acknowledgment is given to all who have supported and contributed to this project. I especially wish to thank Dr. Linda Kelley, committee chair, for her many hours of patient instruction, guidance and discussions. I would like to thank Dr. Wayne Green for his invaluable contribution to the flow cytometry aspect of the project. I would also like to thank committee member Dr. Gerald Spangrude for his insight and guidance. My sincere thanks to Ms. Fen Fen Hsieh, Ms. Annie Kline and Mr. Hawk Oh for their technical support. I thank all the members of the hematopoietic stem cell lab for their generous support and making my stay a pleasant one. Finally a special thanks to my husband, Dr. Ram Mahato, for his constant support and encouragement.

INTRODUCTION

Background

Allogeneic hematopoietic stem cell transplantation is effective in the treatment of a variety of hematological and nonhematological malignancies as well as nonmalignant disorders. Graft versus host disease (GVHD) is a major barrier to effective allogeneic bone marrow transplantation. Forty percent of recipients of major histocompatibility complex (MHC) matched sibling donor transplants and 70% of recipients of MHC matched unrelated donor transplants develop GVHD despite the use of intensive immunosuppression.[1] The differences in MHC class I and class II molecules render a unique immunological identity to each individual. GVH reaction is initiated when immunologically competent cells (donor T cells) contained in the graft recognize recipient MHC antigen as nonself.[2] The signal mediated by the T cell receptor/CD3 complex (TCR/CD3) on T lymphocytes and peptide bearing MHC on antigen presenting cells (APCs) is the primary event in the initiation of GVHD.[3] The initiation of this alloimmune response requires specific antigen recognition by T cells, stable adhesion of T cells to the APCs, and transduction of activating signals to the T cells. Each of these events is mediated by distinct sets of costimulatory molecules on T cells.[3] Upregulation of CD80 (B7-1)/CD86 (B7-2) on APCs leads to an interaction between B7 and low affinity CD28 receptor on T cells.[4] A third interaction involves CD40L (CD154) on T cells and CD40 on APCs.[5] After appropriate costimulation, T cells up-regulate IL-2 cytokine and IL-2 receptor expression, and expand as antigen-specific T cell clones

capable of mediating GVHD. GVHD results from differences in highly polymorphic minor histocompatibility antigens in the case of MHC compatible stem cell transplantation.[6] Experiments with heterologous antithymocyte globulin and specific T cell subset depletions have demonstrated that donor T cells are a prerequisite for GVHD.[7] A substantial amount of work has focused on T cell depletion of the donor stem cell graft as a method of preventing GVHD.[8] Although elimination or suppression of donor T cells decreases the incidence of GVHD, it results in undesirable suppression of immunity to viral and tumor antigens, increased graft failure and relapse with no improvement in overall survival.[9] Donor T cells help to eliminate or inactivate residual host lymphocytes, which have survived the transplant preparative regimen and are therefore able to cause graft rejection.[10] After depletion, very few $CD4^+$ and $CD8^+$ T cells develop from the transplanted stem cells over the first 3 months following the transplant and therefore T cell immunity during this initial period is dependant on donor T cells transplanted with stem cells.[11] Donor T cells thus have a dual role in transplantation mechanism. On the one end they are required for successful tissue engraftment, and on the other end they complicate the transplant by causing GVHD.

Suitable therapies that can overcome GVHD need to be developed. At the same time, there is a growing need to increase the donor pool by developing safe therapies, which will allow transplantation among MHC-mismatched donors and recipients. T cell costimulatory molecule blocking strategies have been employed to induce T cell tolerance exclusively to recipient MHC antigen peptide complexes, while retaining the desirable antiviral and antitumor properties of donor T cells. These studies have shown varying degrees of success in human and animal models. Blockade of B7/CD28

interaction following TCR/CD3 signaling results in T cells, which are unresponsive to secondary encounter with the same antigen, but not to naïve antigens.[12] This state is called anergy. Induction of anergy in donor T cells can be used to prevent these cells from responding to alloantigens in recipient tissue. Blockade of B7/CD28 pathway has been achieved by using monoclonal antibodies to B7,[13] antiCD28 Fab,[14] or a soluble form of CTLA4 (CTLA4-Ig). The recombinant fusion protein CTLA4-Ig, contains the extracellular domain of human CTLA4 fused to hinge region of human immunoglobulin heavy chain and specifically binds the CD28 ligand B7 with high affinity. It acts as a competitive inhibitor by blocking CD28-B7 interactions and subsequently inhibits the proliferation of T cells.[15] CTLA4-Ig blocked rejection of human pancreatic islet cells in mice and induced long term, donor specific tolerance.[16] In vitro co-culture in the presence of CTLA4-Ig of recipient peripheral blood mononuclear cells (PBMCs) with donor bone marrow mismatched for one MHC haplotype resulted in reduced GVHD in patients transplanted for malignant diseases.[17] In summary, these and other studies support the hypothesis that blockade of B7/CD28 interaction is effective in generating antigen-specific tolerance and minimizing GVHD. Since CTLA4-Ig appears to be dependant on TCR engagement for its effectiveness, the specificity of the T cell response can be exploited rather than depending on pan T cell suppression.

In addition, certain subtypes of blood derived dendritic cells (DCs) may themselves induce T cell tolerance and this property may be applied for the prevention of GVHD.[18] DCs are the only APCs that can prime naive T cells to a new antigen.[19] DCs are a rare subpopulation in the blood representing 0.1 to 1% of circulating leukocytes.[20] DCs constitute a complex system of cells which, under different microenvironmental

conditions, can induce such contrasting states as immunity and tolerance. DC heterogeneity is reflected in types of DC subsets, developmental stage, function and anatomical localization and the final outcome of immune response. The four stages of development include bone marrow progenitors, precursors, immature and mature DCs Precursor DCs patrol through blood and lymphatics as well as lymphoid tissues, and, upon pathogen recognition, release large amounts of cytokines, e.g., IFN-α thereby limiting the spread of infection. Tissue residing immature DCs possess high phagocytic and endocytic capacity, pick up and process antigen, and subsequently migrate to T cell dependant areas of secondary lymphoid organs. During this process, they loose antigen-capturing capacity and become mature immunostimulatory DCs that express high levels of costimulatory molecules permitting antigen presentation to naïve T cells.[21] The two discrete subsets of DCs identified in human blood are myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Both subsets are negative for a variety of lineage-specific antigens (CD19, CD3, CD16, and CD14) and positive for HLA-DR, CD4 and CD33. The $CD11c^{bright}$, $CDIL\text{-}3R\alpha^{low}$ (mDCs) differentiate directly from $CD34^{pos}$ cells or indirectly from $CD14^{pos}$ monocytes.[22] Initially myeloid $CD34^+$ progenitors differentiate into $CD11c^+CD14^+$ monocytes and $CD11c^+$ $CD14^-$ DC precursors (Fig.1). Both $CD11c^+CD14^+$ and $CD11c^+CD14^-$ DC precursors in the presence of GM-CSF and IL-4 yield immature DCs. The immature DCs, in the presence of LPS or CD40L, differentiate into mature mDCs. mDCs elaborate a variety of cytokines including IL-6 and IL-12.[23] $CD11c^-IL\text{-}3R\alpha^+CD14^-$ pDC precursor are thought to originate from lymphoid $CD34^+$ progenitors. The immature pDCs depend on IL-3 and CD40 L for their survival and differentiation into mature pDC.[24, 25] As described by Rissoan et al., mDCs polarize naive

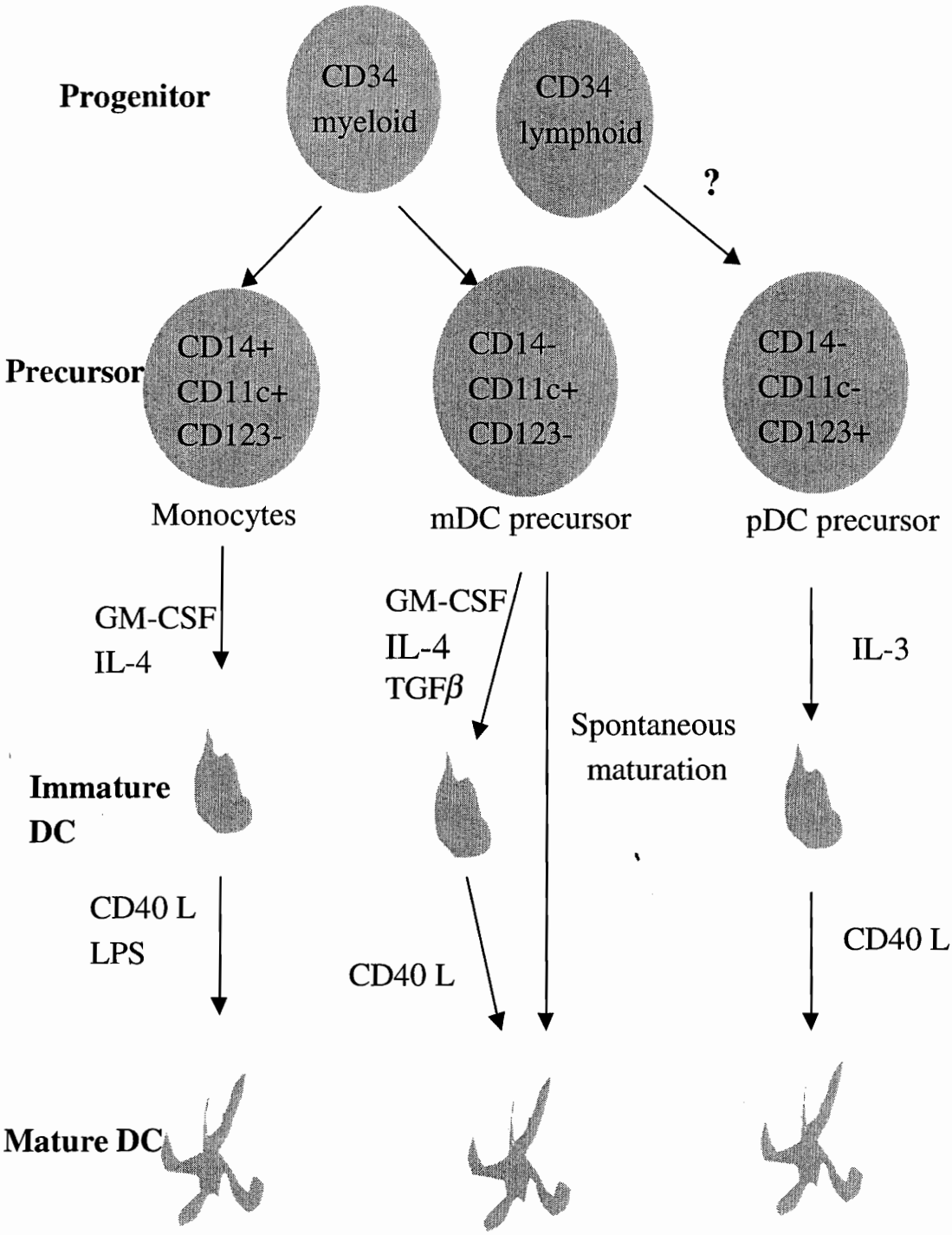


Fig. 1. Dendritic cell (DC) lineage.

T cells predominantly towards a T helper type 1 (Th1) profile, whereas the pDC subset induces T cells to predominantly produce T helper type 2 (Th2) cytokines.[24] Amplification of Th1 cells plays an important role in the alloreactive immune response. Generation of Th2 cells is thought to result in tolerance to pDC presented antigen.[26] However, recent studies challenge the hypothesis that distinct types of DC regulate distinct types of T cell responses and suggest that both mDC[21] and pDC[27] can prime naïve T cells for either a Th1 or Th2 response depending on the in vitro maturation conditions. DC maturation is recognized as a key event in the induction of the immune response.[19] Langenkamp et al.[22] demonstrated that a short pulse with lipopolysaccharide (LPS) resulted in IL-12 producing "active" mDCs that primed T cells for a Th1 response. However, on prolonged incubation with LPS, the mature mDCs, termed "exhausted" mDCs, ceased production of cytokines and preferentially primed Th2 and Th0 (memory) cells. Similarly, depending on the presence or absence of CD40L in culture, pDCs differed in IL-12 production and primed either Th1 or Th2 responses.[22] These studies suggest that Th2 inducing DCs can be generated in vitro from both the mDC and pDC subsets and these may tolerize allogeneic donor T cells to recipient alloantigen prior to stem cell transplant. It is hypothesized that, if Th2 inducing DCs are treated in vitro with CTLA4-Ig to block co-stimulation via B7/CD28 interaction, these DCs should provide the maximum amount of tolerance induction to allogeneic donor T lymphocytes.

Objectives

The first purpose of this study was to verify the efficiency of newly developed BDC isolation kits by isolating blood dendritic cells using recently developed BDCA antibodies and characterize the isolated BDCs. The second goal of this study was to

determine the culture conditions that will support the maintenance and/or development of recipient DC subsets capable of inducing donor T cell tolerance for MHC-matched and mismatched cases.

BLOOD DENDRITIC CELL ISOLATION METHOD

Magnetic Cell Sorting

Dendritic cells were isolated by magnetic cell sorting (MACS) system developed by Miltenyi Biotech.

General Principles

Magnetic separations work due to an affinity group on the surface of the magnetic particles.[28] Cells are incubated with an antibody specific for a particular cell that is covalently linked to a magnetic bead. After this incubation period, bound cells are placed in a magnetic field to immobilize the magnetic particles and their trapped analytes. There are two main strategies for isolating cells: positive selection or enrichment, and negative selection or depletion. With positive selection, the target cells are labeled with the magnetic bead and are retained in the column (Fig. 2). In negative selection the non-target cells are labeled, and the target cells elute with the flow-through. The method of choice depends on antibodies available and the subsequent use of the cells. A combined procedure of depletion followed by positive selection is useful for isolation of extremely rare cells. It is also useful, if unwanted cells express the same antigen used for the positive selection of the target cells.

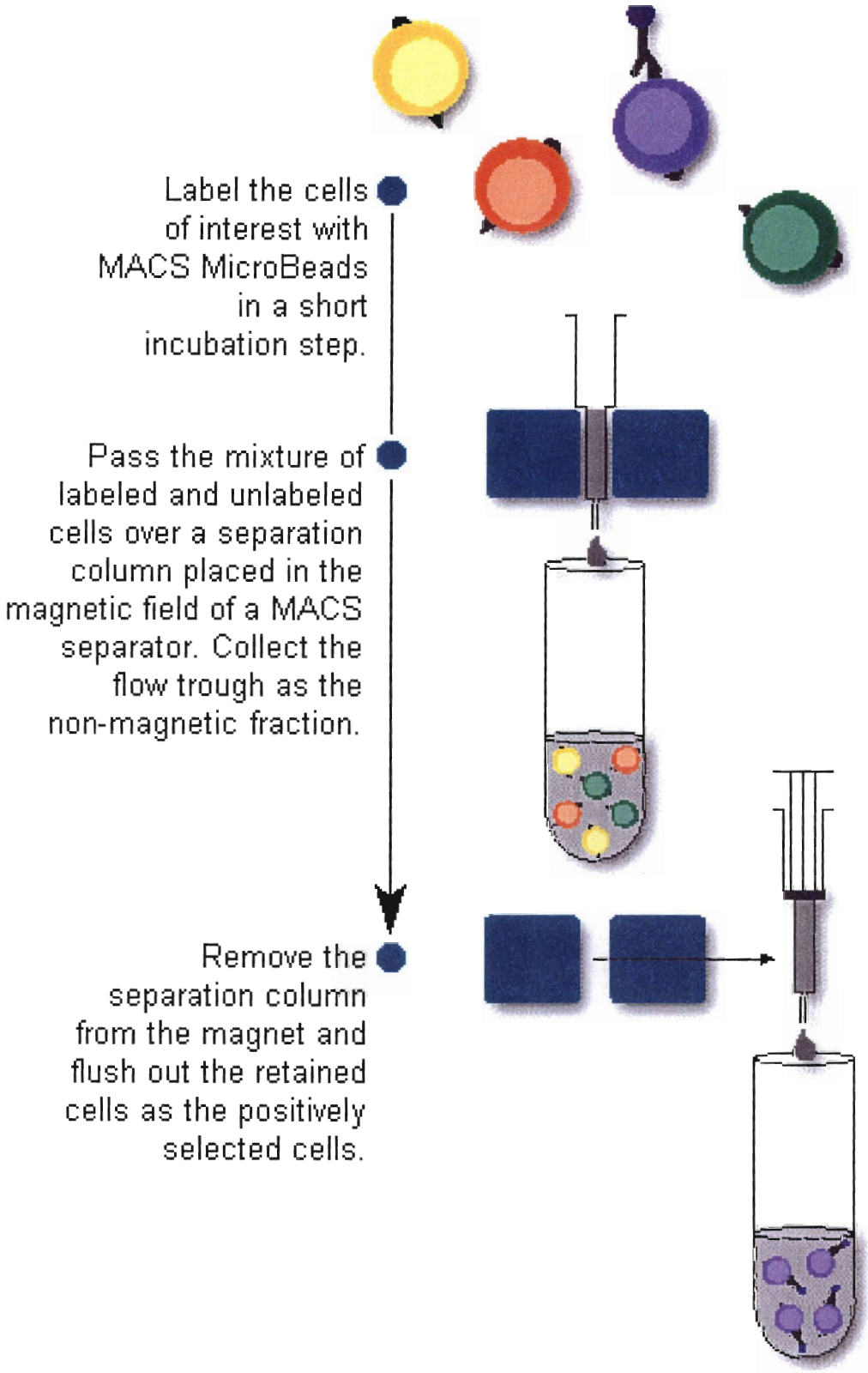


Fig 2. Cell separation by MACS technology (reproduced from http://www.miltenyibiotech.com with permission)

MACS System

For the purpose of this project Magnetic activated Cell Sorting (MACS) system developed by Miltenyi Biotech (Berish Gladbach, Germany) was used (Fig. 3). MACS system comprises the following components.

MACS microbeads

MACS super-paramagnetic beads are an average of 50 nm in diameter and are made of iron oxide and polysaccharide. The beads form a colloidal suspension and do not sediment or aggregate in magnetic fields. Their size and composition make the microbeads biodegradable and typically do not activate cells or influence function and viability. Microbeads do not retain residual magnetism when removed from the magnetic field, so positively selected cells can be used immediately after separation for cell culture or for further study by flow cytometry, PCR, fluorescent microscopy, or fluorescent in situ hybridization.

MACS columns

With MACS, cells are labeled using positive selection for 15 minutes with the microbeads. The labeled cell suspension is loaded on a separation column, which is placed in a strong permanent magnet. The column is packed with a matrix of ferromagnetic spheres coated with a biopolymer.[29] The column matrix serves to create a high-gradient magnetic field. The magnetically labeled cells are retained in the column while nonlabeled cells pass through. After removal of the column from the magnetic field, the magnetically retained and positively selected cells are eluted. The flow through

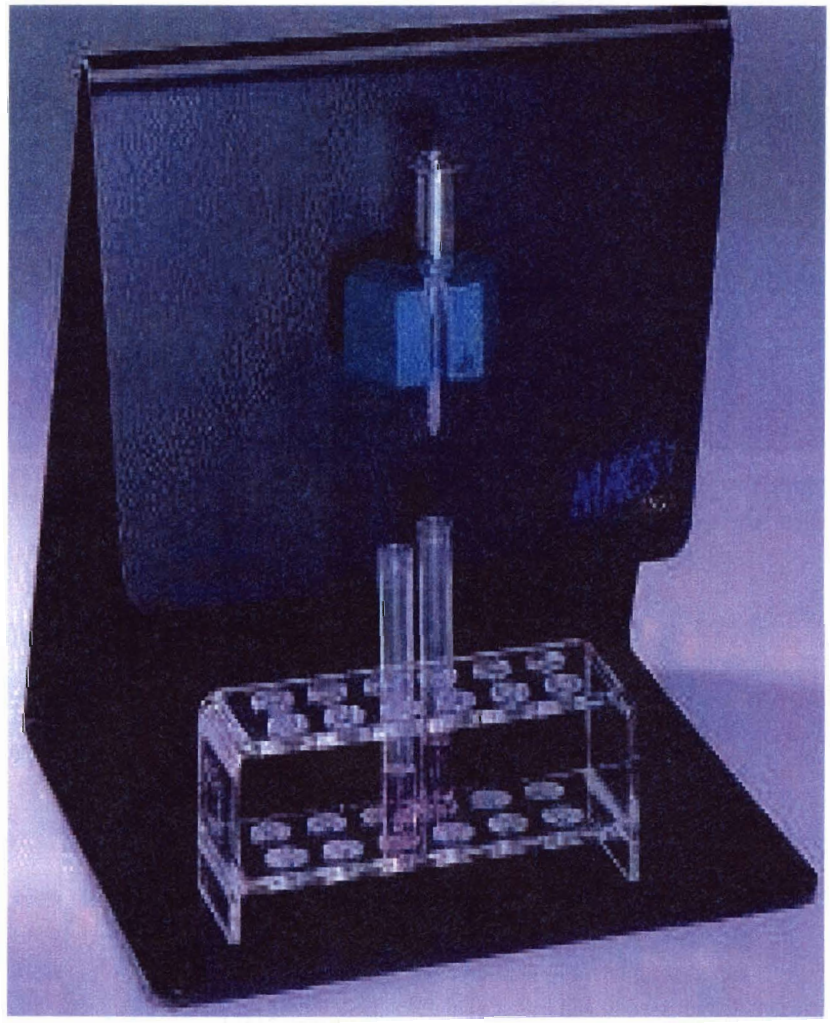


Fig 3. MACS system with MS column in mini MACS separator (reproduced from http://www.miltenyibiotech.com with permission)

contains the negative fraction. MACS technology can separate 10^5-10^{11} cells using the same technique with different size columns (Table 1).

MACS separators

MACS separator includes the magnet to hold the column. There is a choice of MACS separators for a particular type of column being used (Table 1).

Blood dendritic cell isolation kits

Isolation kits methods may involve direct or indirect labeling strategies. With direct labeling (antibodies are coupled to beads), selection occurs in one step. With indirect labeling, cells are first incubated with a primary unconjugated, biotinylated, fluoroscein, or phycoerythrin-conjugated antibody followed by magnetic labeling using an antiimmunoglobulin, streptavidin, antibiotin, antiFITC or antiPE microbeads, respectively.

Detection of Human Blood Dendritic Cells

Recently four markers have been developed by Miltenyi Biotech for distinct subset analysis of dendritic cells. AntiBDCA-1, BDCA-2, BDCA-3, and BDCA-4 monoclonal antibodies identify novel human dendritic cell antigens BDCA-1, BDCA-2, BDCA-3, and BDCA-4 respectively.[30]

BDCA-1 and BDCA-3

In fresh human blood, BDCA-1 is expressed on a major population of myeloid dendriticcell subset in blood.[30] In blood, apart from myeloid dendritic cells, a subset of B cells also express BDCA-1. BDCA-3 antigen is expressed at high levels on a minor

Table 1. Choice of MACS columns and MACS separators for a range for a total number of cells and total number of marker positive cells.

B DCA+ Cells	Total Cells	MACS Column	MACS Separator
$\leq 10^7$	$\leq 2x10^8$	MS Column	Mini MACS
10^7- 10^8	$\leq 2x10^9$	LS Column	Midi MACS
10^8- 10^9	$\leq 2x10^{10}$	XS Column	Super MACS
$\leq 2x10^8$	$\leq 4x10^9$	auotMACS Column	autoMACS

subpopulation of myeloid dendritic cells in human blood. Both BDCA-1$^+$ and BDCA-3$^+$ dendritic cells are CD4$^+$, lineage$^-$, express myeloid markers such as CD13 and CD33 and show a monocytoid morphology. Unlike BDCA-3, BDCA-1 expression is also found on dendritic cells generated in vitro from monocytes or hematopoietic precursor cells (CD34$^+$ cells).

BDCA-2 and BDCA-4

In blood both BDCA-2 and BDCA-4 antigens are expressed on plasmacytoid dendritic cells. These cells possess plasmacytoid morphology, express CD45RA, pre-TCR α-chain and depend on IL-3 for their survival and differentiation into mature dendritic cells. They do not express the myeloid lineage markers such s CD13 and CD33. BDC-4, but not BDCA-2 is expressed on CD1a$^+$ dendritic cells generated in vivo from monocytes or CD34$^+$cells.[30]

Isolation of BDCs

BDCs were isolated from a population of Mononuclear cells (MNCs) using magnetic beads according to the isolation kit protocol. MNCs were separated from whole blood or apheresis product by ficoll hypaque density gradient method (Pharmacia Biotech). Whole blood samples were obtained from healthy adult donors. Apheresis products were obtained both from normal bone marrow donors as well as from autologous bone marrow transplant recipients. The sample type depended on availability.

Isolation of BDCA-1$^+$ cells

The isolation of BDCA-1$^+$ cells was performed by a two step indirect magnetic labeling methods (Fig. 4). In the first step, MNCs were incubated with antiCD19

microbeads, biotin conjugated antiBDCA-1 antibody, and FcR blocking reagent. The purpose of the use of FcR blocking reagent is to block the unwanted bindings of MACS antibody microbeads to nonspecific human cells and increase the specificity of the selection. Fc receptor expressing cells such as monocytes and macrophages, may bind MACS antibody microbeads nonspecifically, and thereby become magnetically labeled and decrease the specificity of the isolation. The CD19 microbead labeled BDCA-1 expressing B cells were subsequently depleted by separation over a MACS LD column placed in a magnetic field of a midi MACS separator. In the second step, BDCA-1^+ cells in the B cell depleted, flow through fraction were incubated with antibiotin microbeads. Upon separation, the labeled BDCA-1^+ cells were retained on the MS column placed in a mini MACS separator and were eluted after removing the column from the magnet.

Isolation of BDCA-4^+ cells

The isolation of BDCA-4^+ cells was performed by a one step direct magnetic labeling method (Fig. 4). MNCs were incubated with antiBDCA-4 microbeads and FcR blocking reagent. Labeled and unlabeled cells were subsequently separated on a MS column placed in the magnetic field of a mini MACS separator. The magnetically labeled BDCA-4^+ cells were retained on the column and were eluted by the removal of the column from the magnet.

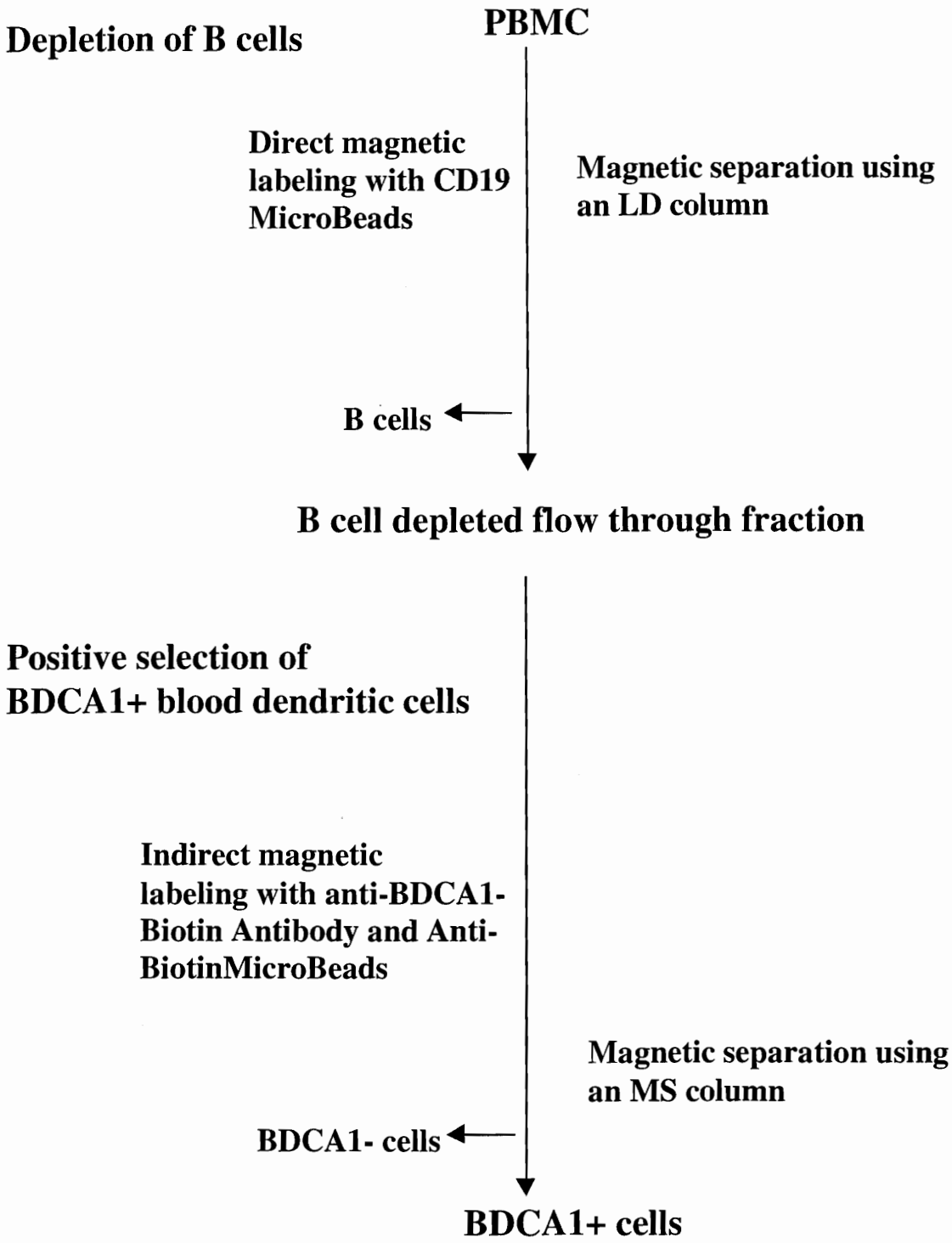


Fig 4. Positive selection of BDCA-1^{+} cells. Depletion of B cells was followed by positive selection of BDCA-1+ cells.

IDENTIFICATION AND CHARACTERIZATION OF BLOOD DENDRITIC CELLS

Following isolation, the cells were analyzed by flow cytometry for the presence or absence of specific dendritic cell subset markers.

General Principles of Flow Cytometer

Flow cytometry is a means of measuring certain physical and chemical characteristics of cells or particles as they travel one by one in a fluid stream aspirated into a flow channel through which focused laser beams are directed to create light scatter and fluorescent signals.[31] The flow cytometer instrument comprises of a light source, sample chamber, optical assembly and electronic signal processors (Fig. 5). The sample chamber and optical assembly is where the sample is introduced into the flowing sheath fluid stream. The sample is hydrodynamically focused in the center of the sheath stream before it encounters the light source. Most flow cytometers use two lasers as light source for two-or three-color fluorescence analysis. These are usually gas ion lasers. When the stained cells injected into the sheath stream pass through the laser beam, light is deflected through the bandpass filters and scattered in all directions. Laser or fluorescent light is then collected and detected at an orthogonal angle by appropriate photodetectors. The cytometer then converts this light into an electronic signal, which is processed to generate an image of the specimen. The light scatter and fluorescent signals are amplified by photomultipliers and stored in computer databases for analysis. Quantitative

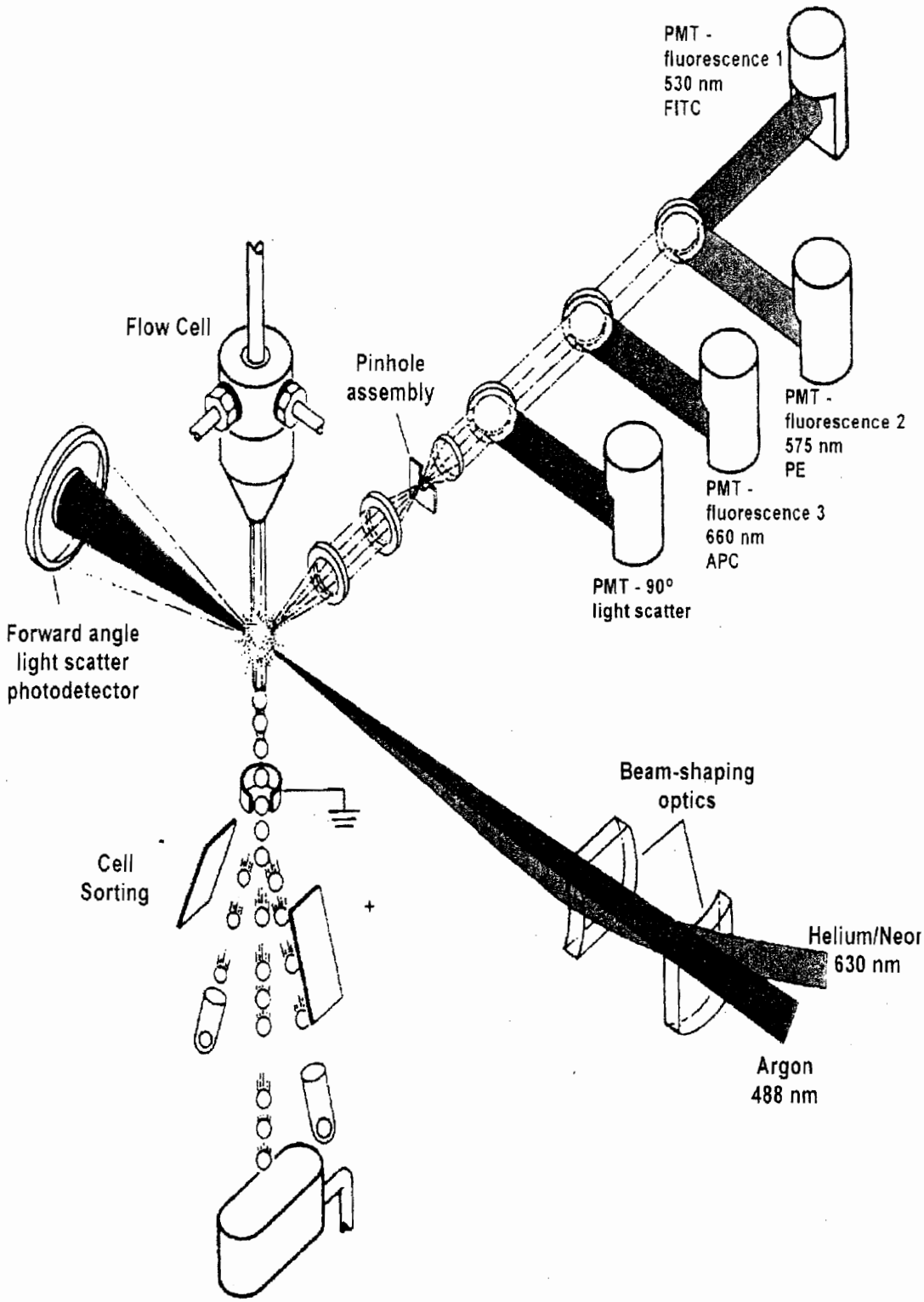


Fig. 5. Schematic diagram of a flow cytometer analyzer.

measurements of light are presented as analog or voltage values. The analog signal is converted to a digital value and amplified using a linear or log scale.

The cells of interest are first identified by their morphology. The scattered light signals detected in a forward direction are indicative of cell size and viability, whereas the signals detected at an orthogonal, or right angle, are indicative of cell granularity and surface structure. Other unique features must be marked or probed, to be evaluated by the cytometer. The probes are generally fluorochromes. Attachment or association of a fluorochrome to the cells is typically accomplished by using antibodies (usually MoAbs) specific for cellular constituents (membrane, cytoplasm, and nucleus). There are many fluorochromes and each is capable of absorbing light energy at one wavelength (excitation), raising the molecule to a higher (excited) energy state and later releasing that energy while emitting a photon at a longer wavelength. Cells are measured as they move in a fluid sheath stream. Physical and chemical properties such as antigens, surface sugars, cytoskeletal organizations, chromatin organizations, DNA content, RNA content, protein and several cell functions are evaluated as the cells pass through the light source and through the stationary sensor or photometer. As a sample is being evaluated by the cytometer, several parameters are measured on each cell (event) and collected in a correlated manner. This fluorescent detection provides statistical accuracy, reproducibility, and sensitivity, and allows the simultaneous measurement of multiple parameters on a cell to cell basis.

Flow Cytometry Assay Development

Difficulties in studying DCs mainly relate to their low frequency in blood. In addition, DCs do not represent a homogenous cell population, but different cell subsets

exist, which further increase the difficulties related to their phenotypic identification and characterization. Flow cytometry has been shown to be a well-suited technology for the analysis of single cell suspensions, even when cells are present at very low frequencies, as are the different DC subsets in normal human peripheral blood. Good applications of flow cytometry for the analysis of cells present at low frequencies are the enumeration of $CD34^+$ hematopoietic progenitor cells, the identification and characterization of mast cells, and minimal residual disease monitoring in patients with hematological malignancies who achieved morphologically complete remission. Previous reports have shown that the use of a two-step acquisition procedure (i.e., acquisition of cells through a live gate, which increases the number of cells understudy) represents an optimal approach for the analysis of rare cells. This double-step acquisition procedure allows the unequivocal and sensitive detection of both malignant and normal hematopoietic cells, even when their frequency is as low as one malignant cell in 10^6 normal cells.[32] By applying a similar strategy, it has been shown that the use of multiple stainings analyzed by flow cytometry using appropriate multiple gating strategies allow accurate identification and analysis of a large number of DCs.

Antibody Panel Creation

A three-color flow cytometry assay was designed for detection and quantitation of two distinct DC subsets. The isolated BDCA-1^+ DCs (mDCs) were identified by staining the postbead (postisolation) BDCs with PE conjugated streptavidin. The isolated BDCA-4^+ DCs (pDCs) were detected by staining with antiBDCA-2 monoclonal antibody. The monoclonal antibodies used for the isolation of particular DC subsets were not used for

FCM staining purpose, as the associated epitopes would be already bound to antibodies used for isolation purpose, resulting in a less bright staining with the same antibody.

To confirm the purity and identity of isolated mDC and pDC subsets, their further phenotypic characterization was done. A panel of monoclonal antibodies was created to detect the presence or absence of multiple marker expression. The antibody panel included FITC conjugated antilineage cocktail (BD Biosciences), PE conjugated anti-CD11c (BD Biosciences), antiCD123 (BD Biosciences), antiBDCAs (Miltenyi Biotech), streptavidin (BD Biosciences), and respective isotype control antibodies, and PerCP conjugated antiHLA-DR (BD Biosciences).

Antilineage cocktail

The antilineage cocktail included a mixture of MoAbs to individual lineage markers CD3, CD56, CD19, CD20, CD14, and CD16. CD3 is mainly expressed on T cells. CD19 and CD20 are mainly expressed on B cells. CD56 is expressed on natural killer cells. CD14 and CD16 are mainly but not exclusively expressed on monocytes and granulocytes, respectively.

CD11c

CD11c is expressed on mDCs. This antigen is also present on monocytes and in low density on granulocytes and natural killer lymphocytes in peripheral blood.[33]

CD123 (AntiIL-3Rα)

CD123 binds to the α-chain of the interleukin-3 receptor (IL-3α).[34] It is expressed on pDC subsets of peripheral blood. It is also expressed on a subset of progenitor cells, monocytes, eosinophils and basophils.

AntiHLA-DR

AntiHLA-DR recognizes a human class II major histocompatibility complex antigen. HLA-DR is expressed on dendritic cells, B lymphocytes, monocytes, macrophages, activated T lymphocytes, activated natural killer cells and human progenitor cells. The negative controls for the analysis included isotype-matched, PE labeled unrelated antibodies.

Staining

Analysis of prebead (preisolation) and postbead (postisolation) samples were done by direct immunofluorescence using three-color staining with MoAbs directly conjugated with FITC, PE and PerCP. In all of the tests, antiLIN FITC cocktail, and anti HLA-DR PerCP MoAbs were used. The PE conjugated MoAb for each test varied depending on the particular BDC being isolated. It was antiBDCA-1PE and antiBDCA-4 PE for the identification of prebead BDCA-1^+ and BDCA-4^+ cells respectively. For the identification of postbead BDCA-1^+ and BDCA-4^+ cells, PE conjugated streptavidin and antiBDCA-2 were used respectively. For further characterization purpose, PE conjugated CD11c and CD123 were used in all postbead tests. In case of BDCA-1 isolation, after the CD19 depletion step, MNCs were stained with PE conjugated anti-CD19 MoAb (BD Biosciences) in order to confirm the depletion of B cells. The control samples for the corresponding test samples were stained with FITC conjugated antiLIN cocktail, PerCP conjugated antiHLA-DR and the isotype mouse immunoglobulin for the PE conjugated MoAbs.

Data Acquisition

Stained cell suspensions were acquired on the FACScan (Becton Dickinson). The flow cytometer was equipped with argon and helium-neon lasers, four fluorescence detectors, and appropriate filters sets for detection of FITC, PE and PerCP. Forward scatter (FS), side scatter (SC), and fluorescence (FL) windows of analysis were positioned.

Instrument setting was first established first by acquiring unstained leukocytes. Color compensation (electronic correction for spectral overlap) was adjusted by acquiring cells stained with each fluorochrome conjugated MoAbs. Three data files were acquired for each test and corresponding isotype sample aliquot. The first file R1 included total events (Fig. 6). The second file R2 was a gated file in which only LIN FITC negative and HLA-DR PerCP positive events were stored (Fig. 7). Five thousand events were gained in R2 while acquiring prebead MNC sample, and 10,000 events were gained in case of post bead BDC sample. The number of total events stored in R1 depended on the number of lineage negative events to be gained in R2. The third file R3 displayed PE conjugated monoclonal antibody reactivity versus HLA-DR PerCP reactivity (Fig. 8). The controls provided markers for the plots.

Data Analysis

Gating strategy

CellQuest software program (Becton Dickinson) was used for data analysis. A side scatter versus forward scatter (SSC/FSC) dot plot was created. Region R1 was drawn to

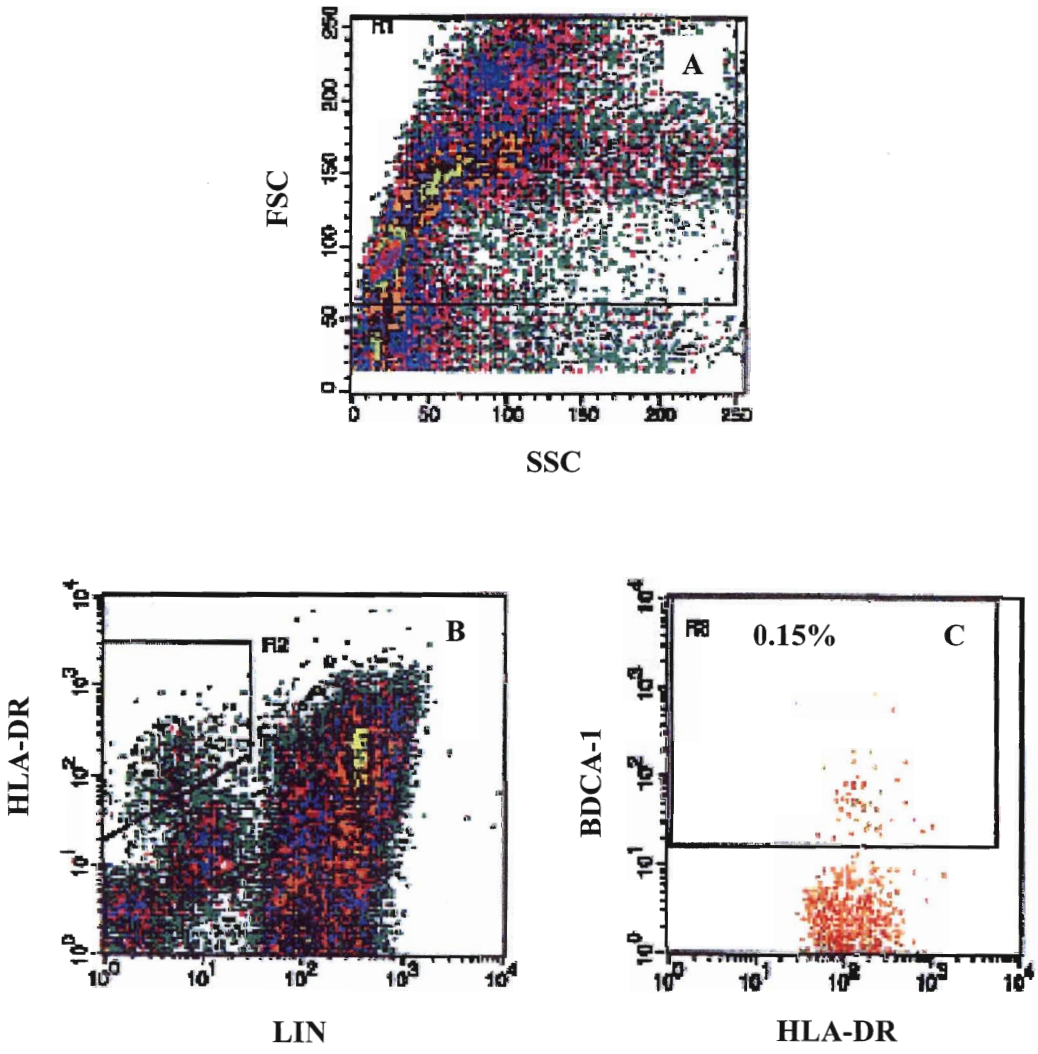


Fig. 6. FCM data of MNCs before BDCA-1 isolation. Light scatter plot of mononuclear cells (A). Three color staining of mononuclear cells (B and C) for lineage markers, HLA-DR and BDCA-1.

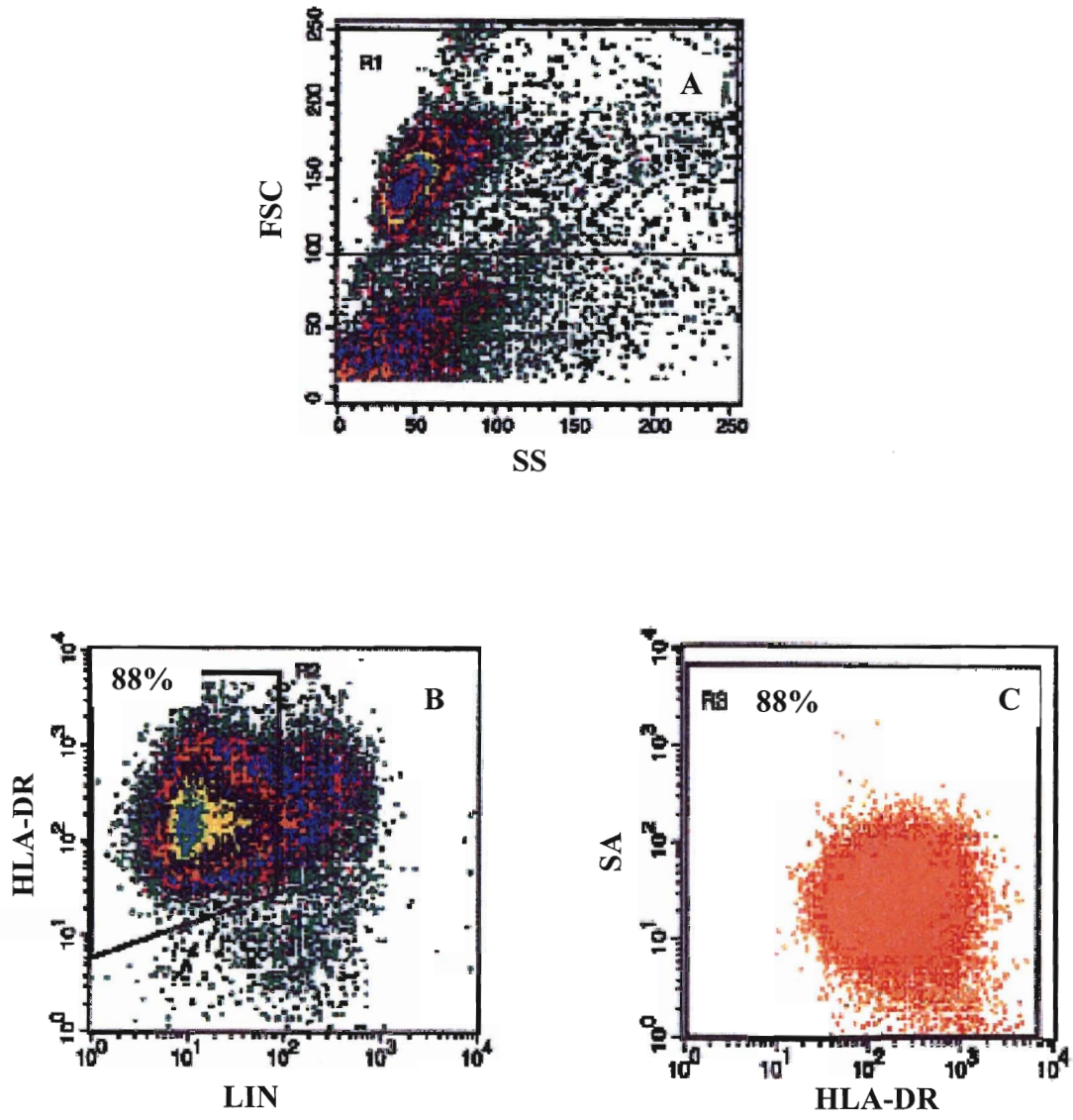


Fig. 7. FCM data of BDCA-1 enriched sample. Light scatter plot of postbead cells (A). Three color staining (B and C) of BDCA-1 enriched population for lineage markers, streptavidin and HLA-DR.

exclude debris and dead cells. An antiHLA-DR versus antiLIN dot plot gated on region R1 was created. Region R2 was drawn to include lineage negative and HLADR$^+$ population. Region R3 was drawn to include antiHLA-DR versus antiBDCA, CD11c or CD123 PE dot plot. A similar analysis was performed on the isotype control and the result subtracted from the test to adjust for nonspecific staining and autofluorescence.

Data analysis of BDCA-1 isolation

Fig. 6 shows the flow data from the pre bead sample. The first file includes total events in R1 and forward versus side scatter. The heterogeneous cell population displaying characteristic light scatter properties of lymphocytes and monocytes, is evident. Debris and dead cells as distinguished by low light scatter property were excluded. R2 identifies the lineage negative and HLA-DR positive cells in the prebead sample. The very small population of lineage negative cells is evident in this pre-enrichment sample. R3 identifies the lineage negative population gated in R2 as BDCA-1$^+$ and HLA-DR$^+$. The percentage of BDCA-1$^+$ cells in peripheral blood before enrichment was 0.15%.

Fig. 7 shows the postbead FCM data. First file shows the total events in R1 excluding debris and dead cells. The single cell population is evident in this postbead forward versus side scatter dot plot. The light scatter profile of this population is distinct from that of lymphocytes and monocytes. The postbead lineage negative population as displayed in R2 was 88% of the total cells. R3 identified the BDCA-1$^+$ and HLA-DR$^+$ population as stained by streptavidin, the percentage purity of which was 88%, the same as the total percentage of lineage negative cells. In Fig. 8, R3 identifies the expression of

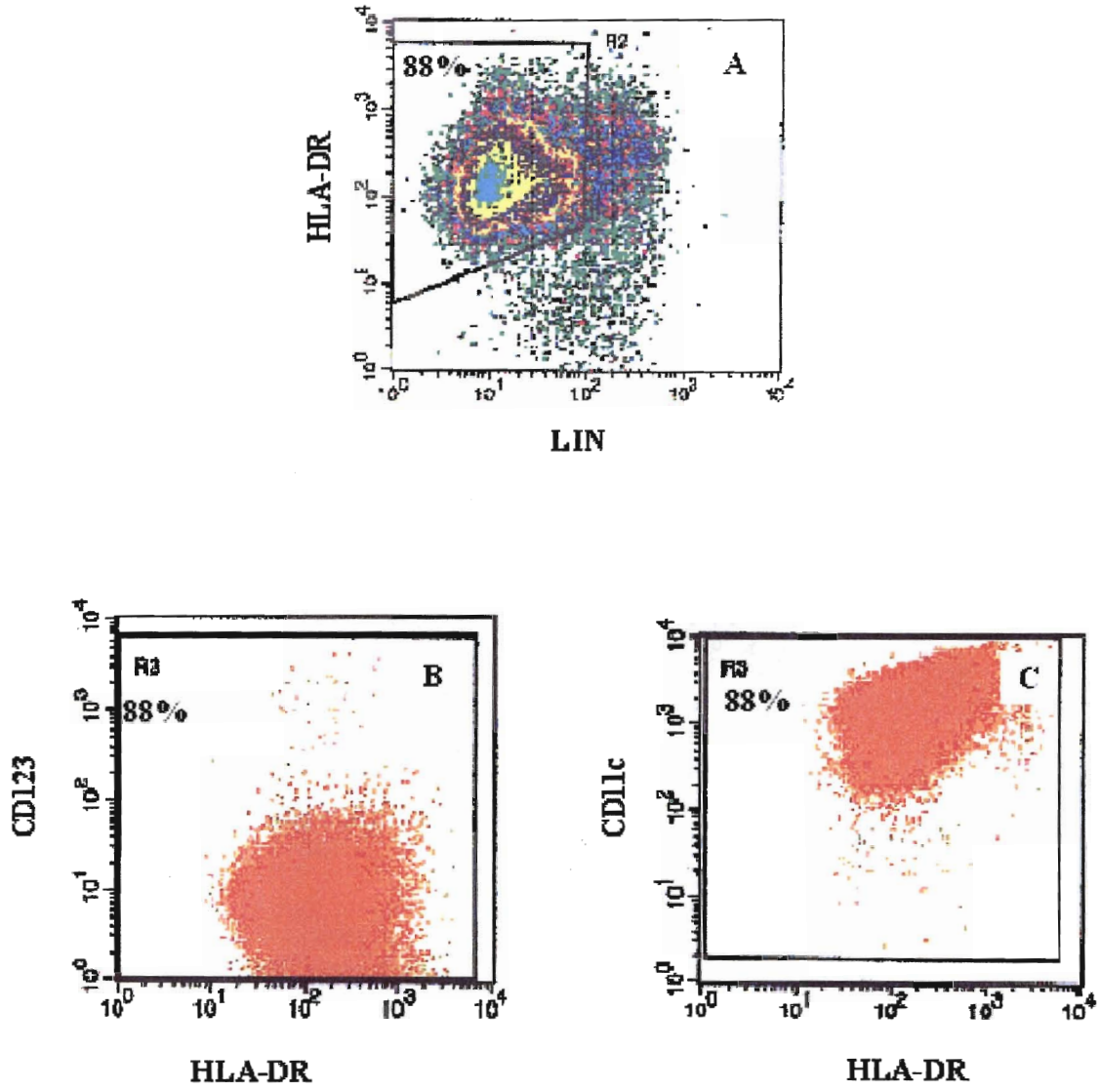


Fig. 8. Immunophenotypic characterization of BDCA-1^{+} cells.

CD11c and CD123 markers on the lineage negative population gated in R2. CD11c is brightly expressed on lineage negative population, the percentage of which was 88% of total cells. CD123 is expressed on the entire population of lineage negative cells, which are BDCA-1^+. However expression of CD123 is very dim. Fig. 9 shows $CD19^+$ cells prior to the CD19 depletion step. Fig. 10 shows the CD19 versus FL3 dot plot after the CD19 depletion step. 5.9% of the cells were $CD19^+$ before depletion. After depletion, the percentage of CD19 was only 0.18%.

Data analysis of BDCA-4 isolation

Fig. 11 shows the prebead FCM data. Fig. 12 and 13 show the postbead data for BDCA-4 isolation. The file format is same as that for BDCA-1 isolation. The percentage of BDCA-4^+ cells in peripheral blood before isolation, as indicated in R3 was 0.22%. In the postbead data, R3 identifies BDCA-4^+ and $HLADR^+$ cells as stained by antiBDCA-2 MoAb, the percentage purity of which was 65%, the same as the total percentage of lineage negative cells (Fig. 12). In Fig. 14, R3 identifies the expression of CD11c and CD123 markers on the lineage negative population gated in R2. CD123 is expressed on lineage negative population, the percentage of which is 65% of total cells. CD11c is expressed on a very minor population of lineage negative cells, the percentage of which was only 3% of the total cells.

Morphology of BDCA-1^+ Cells

The morphological characteristics of $HLADR^+/LIN^-$/BDCA-1^+ cells were assessed. For this purpose, the isolated cell fraction was centrifuged to prepare a cytospin slide. The cytospin slide (Fig. 14), when stained with Wright-Giemsa stain and observed under

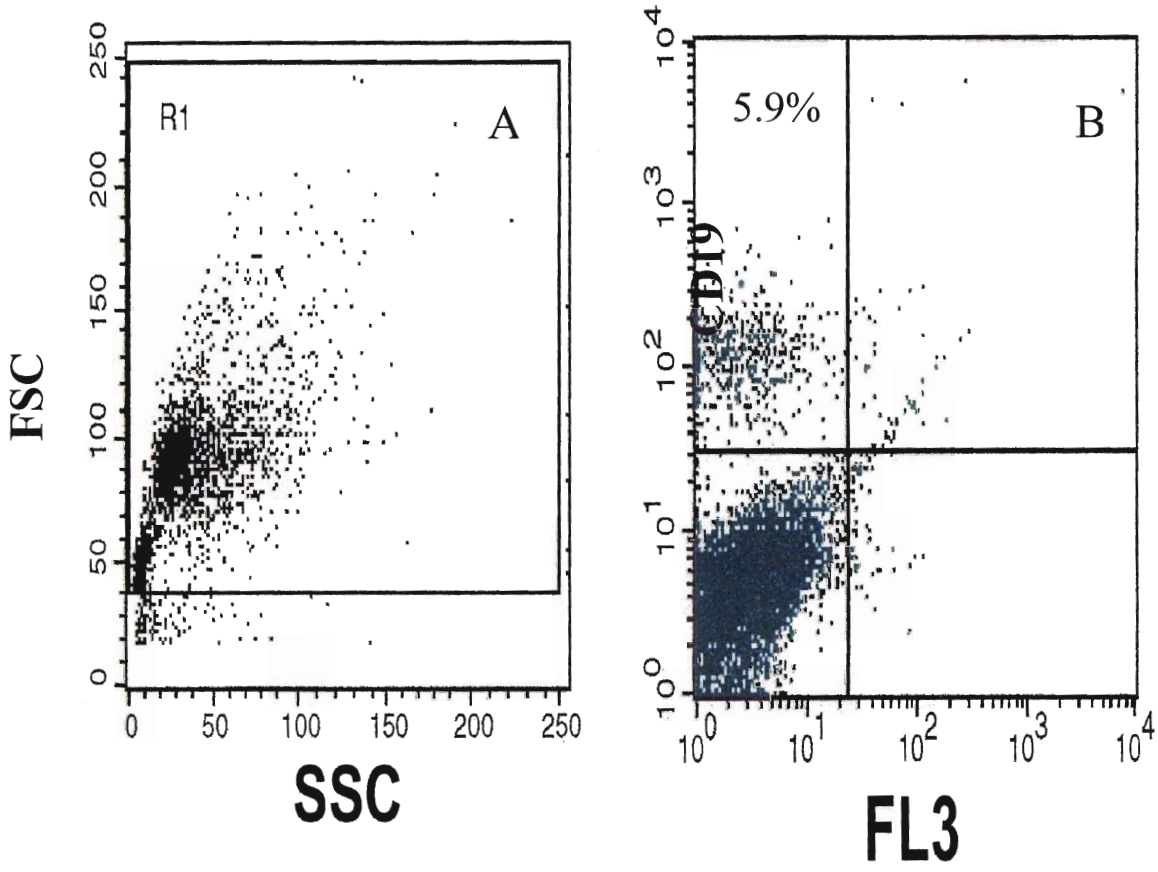


Fig. 9. Staining of MNCs for CD19. Light scatters of mononuclear cells (A) and their staining for CD19 (B). Total number of CD19 cells before depletion is 5.9%.

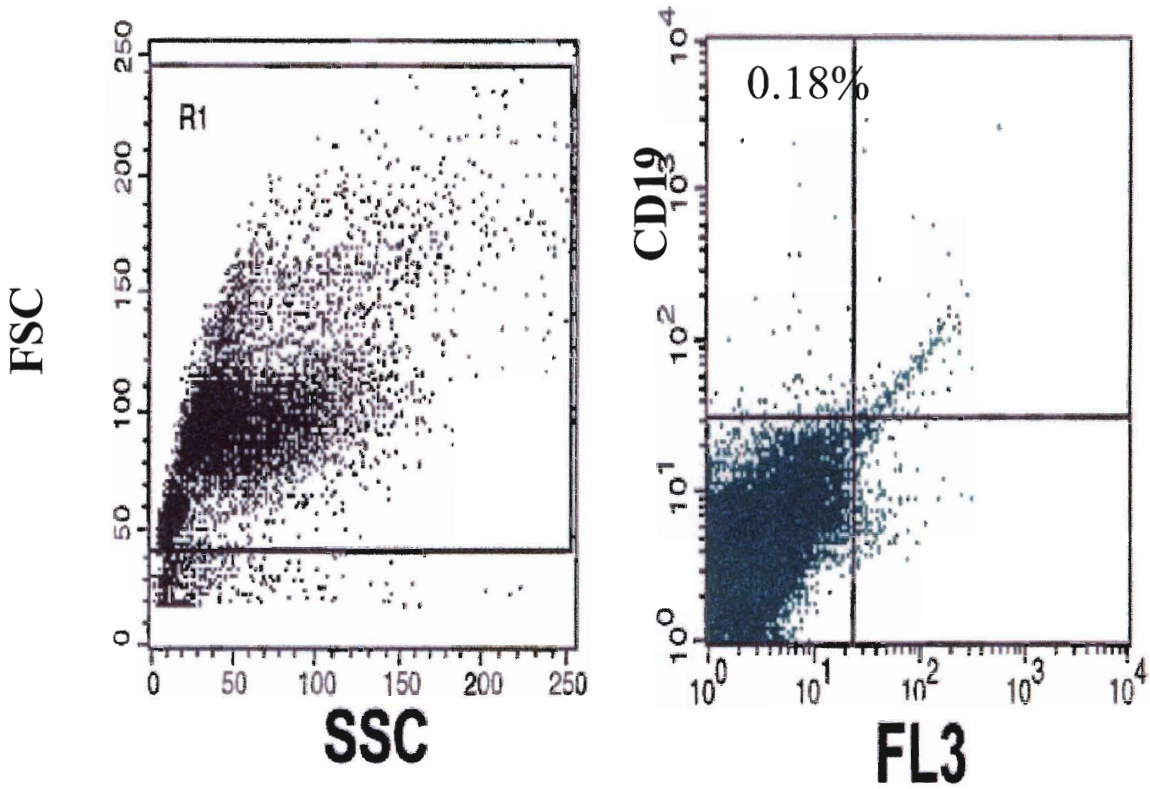


Fig. 10. FCM Data of CD19 depleted samples. Light scatters of mononuclear cells after CD19 depletion (A). Total number of CD19 cells after depletion is 0.18% (B).

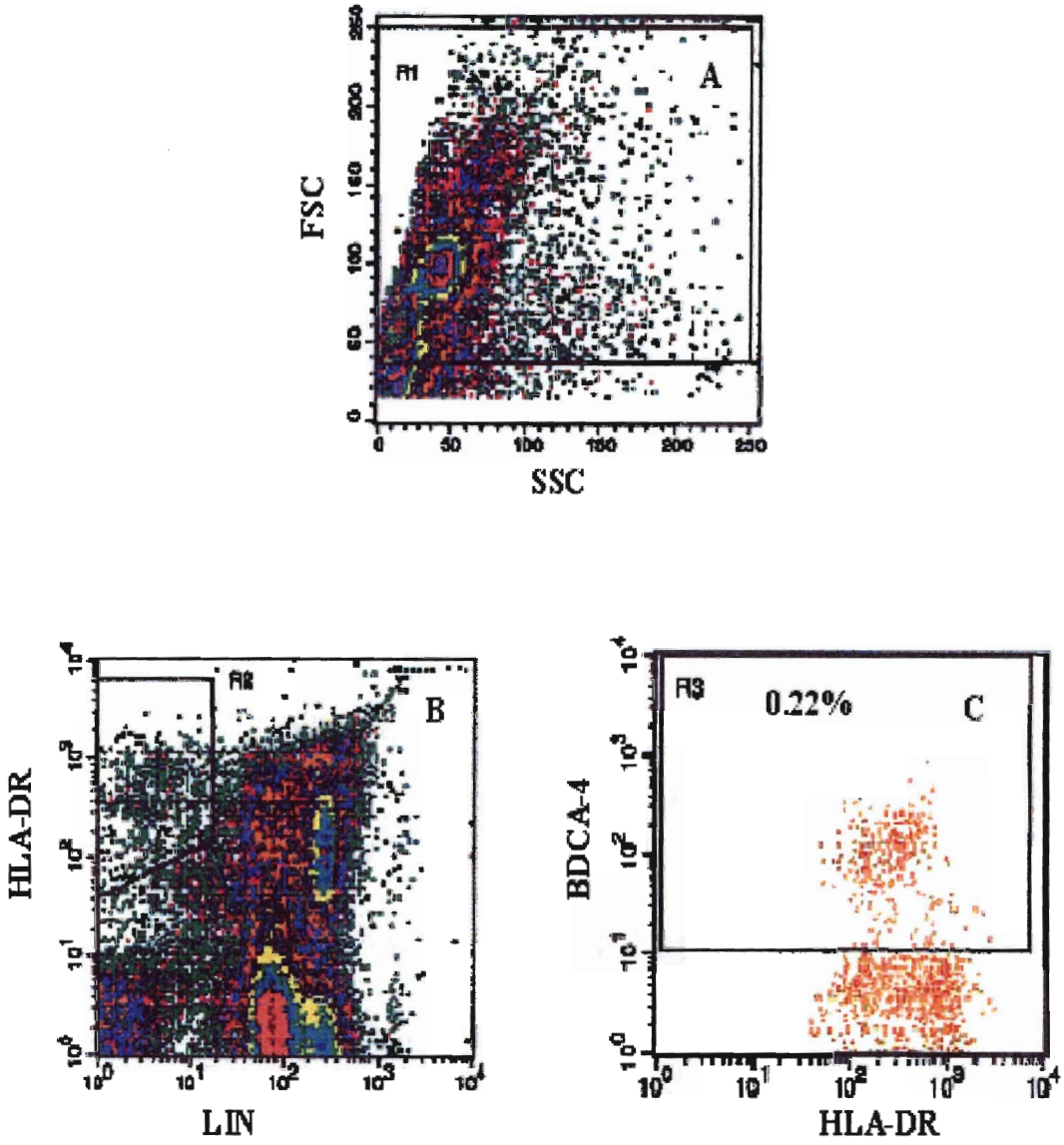


Fig. 11. FCM data of MNCs before BDCA-4 isolation. Light scatter plot of mononuclear cells (A). Three color staining of mononuclear cells (B and C) for lineage markers, HLA-DR and BDCA-4.

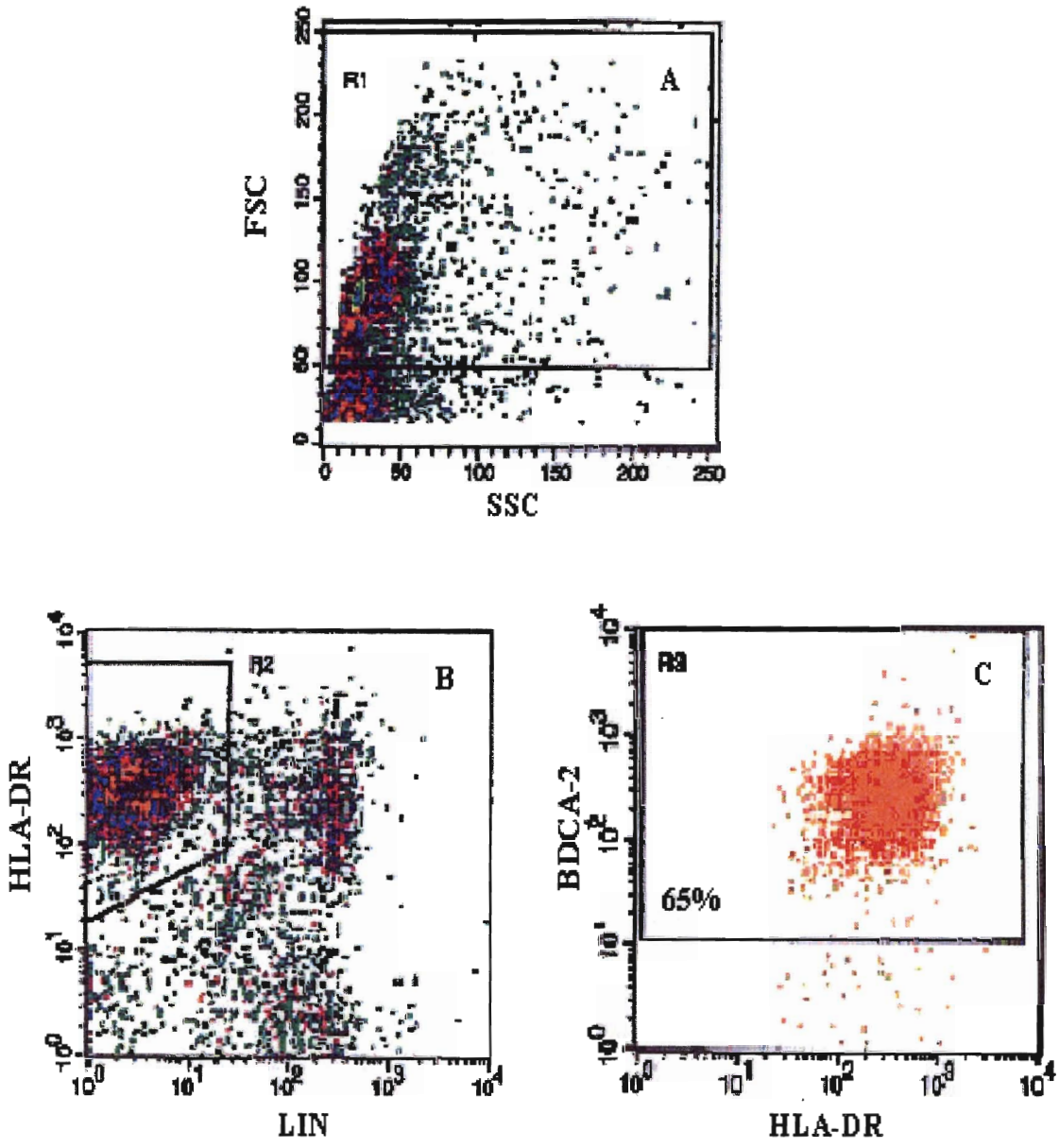


Fig. 12. FCM data of BDCA-4 enriched sample. Light scatter plot of postbead cells (A). Three color staining of BDCA-4 enriched population (B and C) for lineage markers, HLA-DR and BDCA-2.

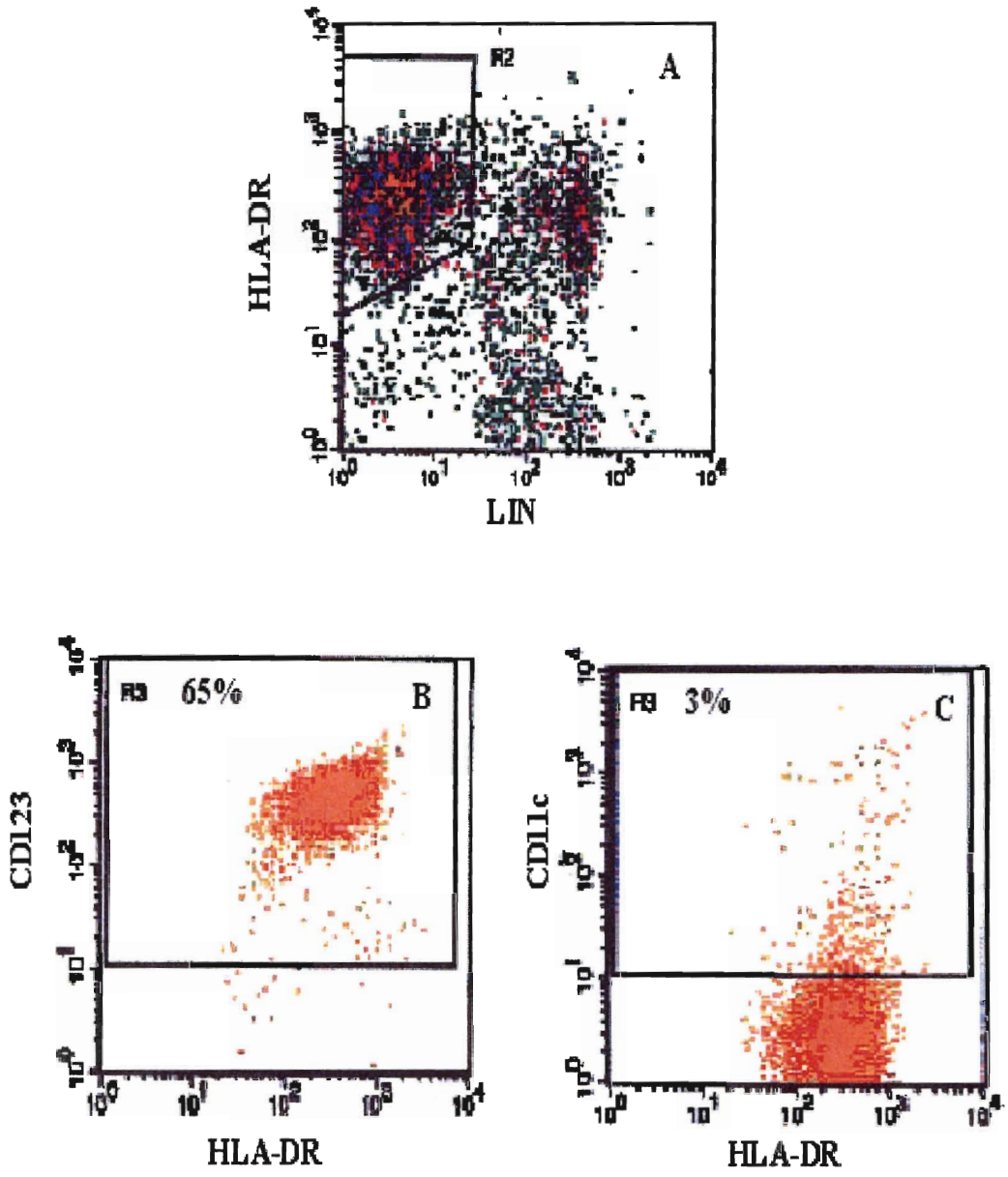


Fig. 13. Immunophenotypic characterization of BDCA-4^{+} cells.

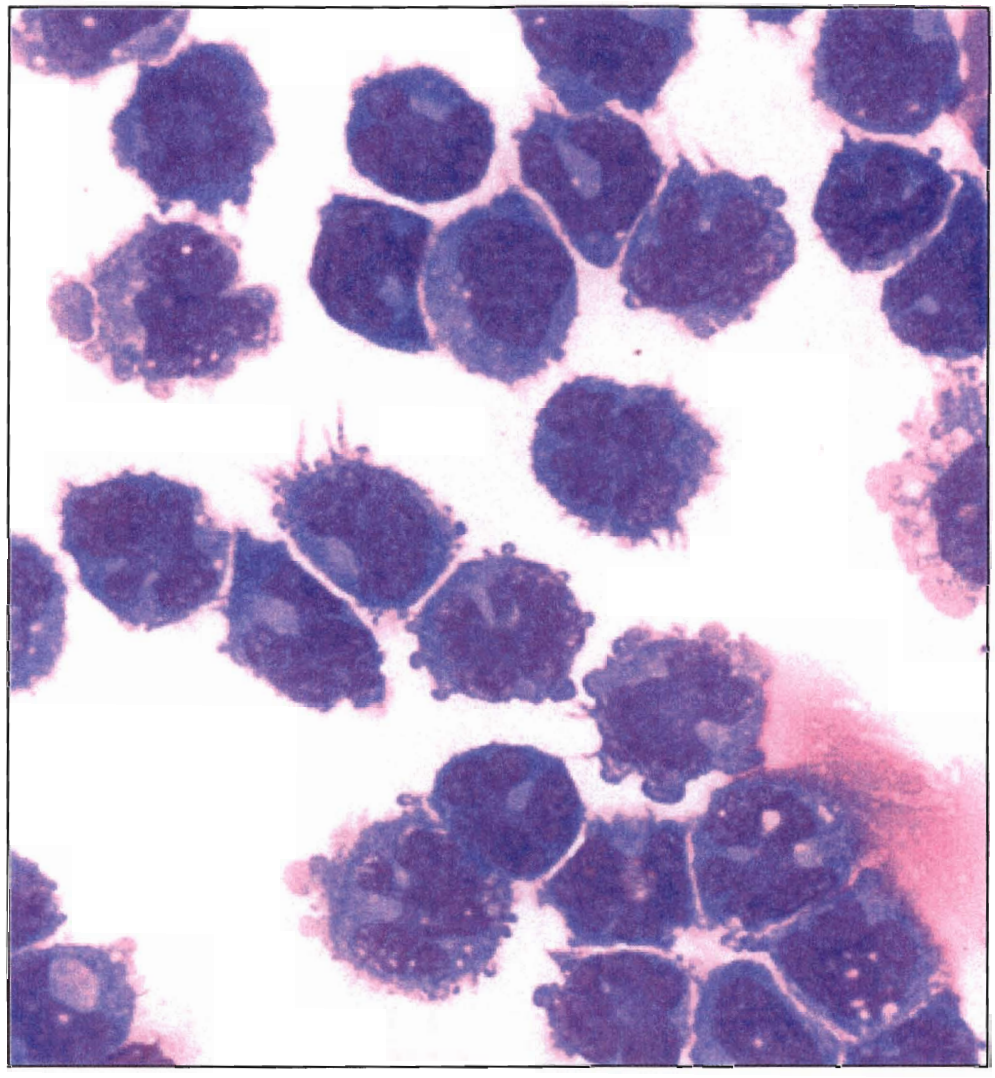


Fig. 14. Morphology of BDCA-1 cells.

light microscopy, showed a major population of large cells with large hyperlobulated nuclei. The entire cell population was not homogenous. Some of the cells resembled monocytes. Some cells had unlobulated round nuclei and some cells had large reniform nuclei. Most, of the cells with lobulated nuclei had small dendritic processes. However, these cytoplasmic projections covered the cell circumference partially only.

Costimulatory Molecules and Activation Markers

The expression of costimulatory molecules and activation markers on the isolated BDCA-1^+ and BDCA-4^+ cells were evaluated by staining with MoAbs against CD80 (B7-1) /CD 86 (B7-2) and CD83 respectively.

CD83

CD83 is an inducible glycoprotein expressed predominantly by dendritic cells and B lymphocytes. Expression of membrane CD83 is widely used as a marker of differentiated/activated DCs.[35, 36]

CD 80

CD 80 (B7-1) is expressed on activated B and T cells and macrophages. It is expressed at low levels on monocytes and dendritic cells. CD80 expression is increased upon activation of B and T cells, peripheral blood monocytes.

CD86

CD86 (B7-2) is expressed constitutively on interdigitating dendritic cells in T zones of secondary lymphoid organs and at a lower level on Langerhans cells and peripheral blood dendritic cells. It is also expressed on T cells, B cells and at very low levels on

monocytes. The postbead cells obtained from BDCA-1 and BDCA-2 isolations were simultaneously stained with FITC conjugated antiLIN cocktail, PE conjugated individual activation markers and PerCP conjugated antiHLA-DR. Fig. 15 and 16 show the flow data from this experiment. Ninety nine percent of BDCA-1^+ and 49.3% of BDCA-4^+ cells expressed CD86. The expression of CD83 and CD80 was insignificant for both subsets. Only 1.3% of BDCA-1^+ cells and only 0.37% of BDCA-4^+ cells expressed CD80. The expression of CD83 on BDCA-1^+ cells BDCA-4^+ cells was only on 1.20% and 1.23% respectively of the total BDCA$^+$ population.

Culture of Dendritic Cells

The in vitro maintenance of the isolated dendritic cells was checked under two conditions. They were cultured in tissue culture Medium (TCM) specific for dendritic cells in the presence and absence of cytokines. The TCM used was RPMI supplemented with 10% fetal calf serum, kanamycin (50µl/ml) and glutamine (2mM).[37] First the BDCA-1^+ cells were cultured in the presence of GM-CSF (50ng/ml) and IL-4 (1000U/ml) for 4 days. The initial number of BDCA-1^+ cells put in culture was $5x10^6$. However on day 4, cell count dropped to $2.8x10^5$. In the next experiment, the BDCA-1^+ cells were cultured in the absence of cytokines using the same TCM, and cell morphology was monitored by preparing cytospins at different time points (Fig. 17). On day 0, freshly isolated BDCA-1^+ cells showed normal morphology. On day 1, 50% of the cells showed signs of progressive death. Cells appeared vacuolated and many of them had pyknotic nuclei. On day 2, only cell debris and isolated nuclei could be visualized. Since cell survival and maintenance was poor in both conditions, it was concluded that

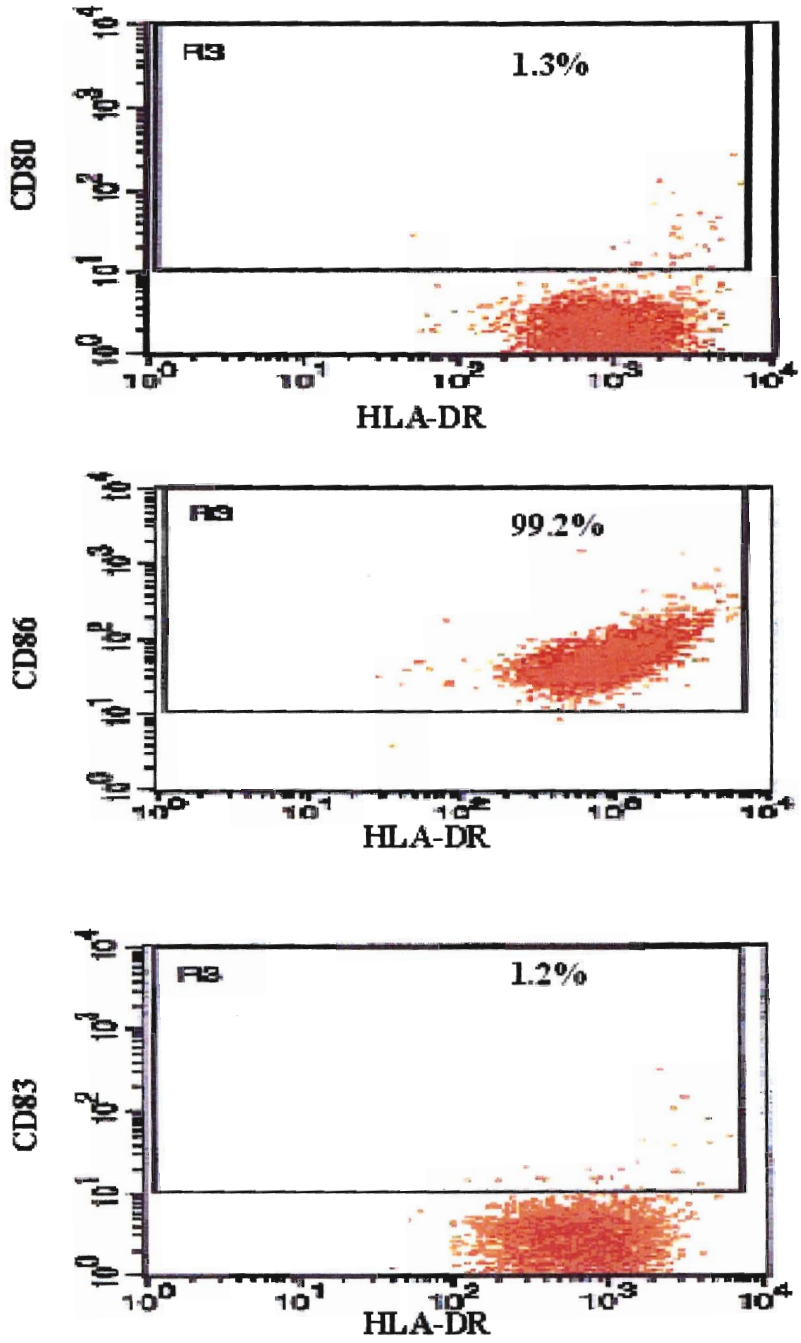


Fig. 15. Staining of BDCA-1^{+} cells for activation markers.

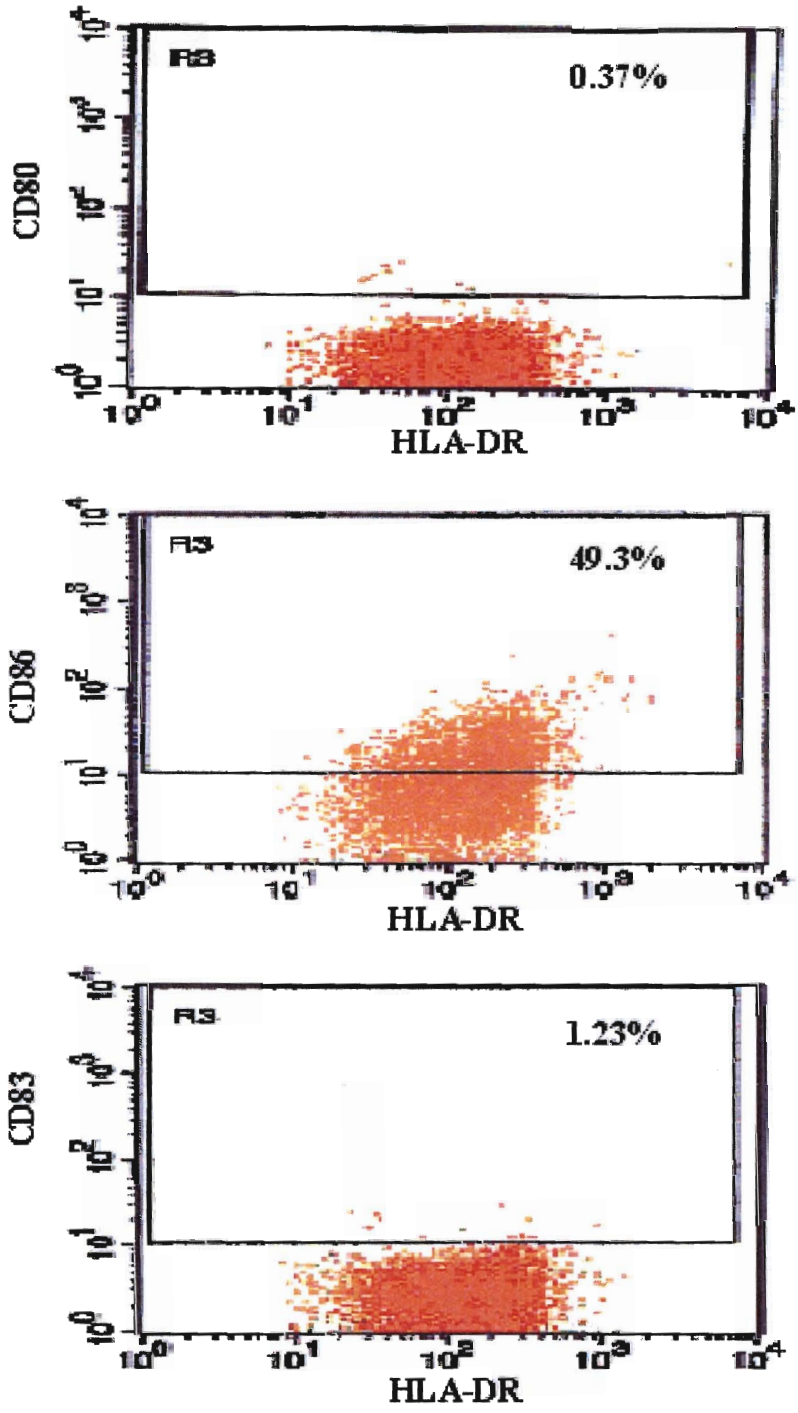
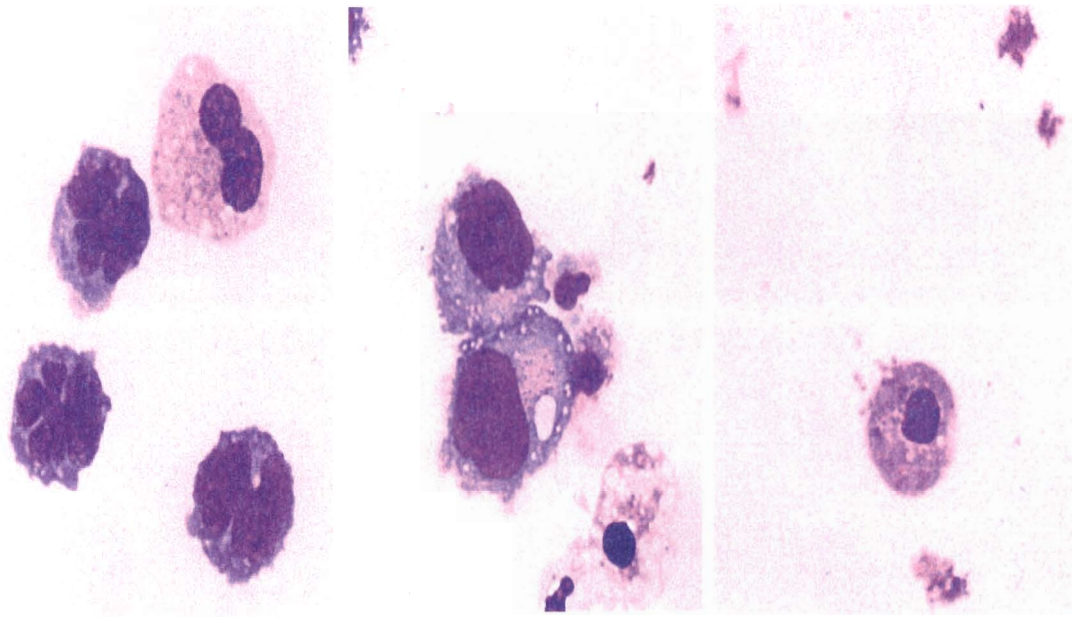


Fig. 16. Staining of BDCA-4^{+} cells for activation markers.



Day 0 Day 1 Day 2

Fig. 17. Culture of BDCA-1 DCs without cytokine supplementation.

the TCM being used was not optimal or a different cytokine condition was essential.

TCM optimization

TCM optimization was done by evaluating six different culture conditions. The TCM used in all six conditions was RPMI. However they differed in the type and the percentage of the serum, antibiotics, and other supplements such as glutamine, nonessential amino acids, sodium pyruvate and vitamins as follows:

TCM# 1: 5% hAB serum

TCM# 2: 10% hAB serum + 100,00u/ml penicillin and streptomycin + 1% glutamine + 1% sodium pyruvate + 1% nonessential amino acids

TCM# 3: 5% hAB serum + 100,00u/ml penicillin and streptomycin + 1% glutamine + 1% sodium pyruvate + 1% nonessential amino acids

TCM# 4: 2% hAB serum + 100,00u/ml penicillin and streptomycin + 1% glutamine + 1% sodium pyruvate + 1% nonessential amino acids

TCM# 5: 10% fetal calf serum + 50ul/ml kanamycin + 2mM glutamine

TCM# 6: 10% pooled human serum (PHS) serum + 100,00 u/ml penicillin and streptomycin + 1% glutamine

An assay similar to mixed lymphocyte reaction was set with the above six TCMs in triplicates to determine the type, which provided optimum condition for cell recovery. The cells used in this experiment were mononuclear cells, isolated from whole blood by ficoll hypaque density centrifugation method. Phytohemagglutinin (PHA) was used in this assay to induce human lymphocyte proliferation. Later tritiated thymidine (^{3}H thymidine) was incorporated in the assay on day third to determine the degree of cell metabolism and growth, as radioactive thymidine is taken up by growing daughter DNA

strand during DNA replication. Cells were harvested 24 hr post-^{3}H thymidine incorporation, and radioactivity was counted on β scintillation counter. The data obtained from beta counter are shown in Fig. 18. The highest count recorded was 223724.0 for TCM # 3 (5% hAB serum + 100,00u/ml penicillin and streptomycin + 1% glutamine + 1% sodium pyruvate + 1% nonessential amino acids), suggestive of highest metabolism, cell proliferation and optimum culture condition. TCM # 1 (5% hAB serum + 100,00 u/ml penicillin and streptomycin) had the lowest count of 16664.0, suggestive of insignificant activity. It was concluded that RPMI supplemented with 5% hAB serum, 100,00u/ml penicillin and streptomycin, 1% glutamine, 1% sodium pyruvate and 1% nonessential amino acids provided the optimum culture condition. It was also concluded that human serum was superior to fetal calf serum and glutamine was an essential supplement.

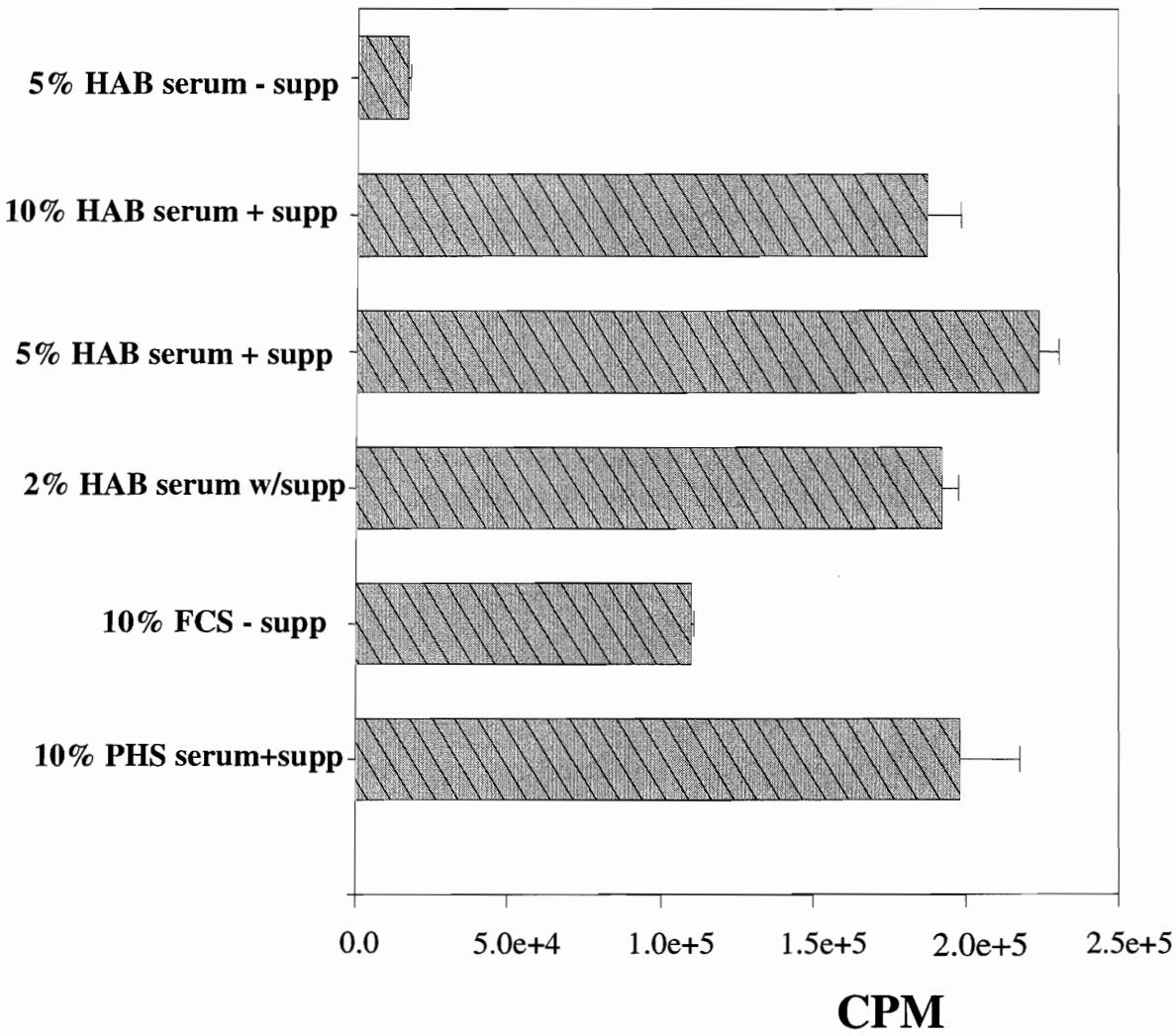


Fig. 18. Optimization of culture conditions.

DISCUSSION

Immunophenotypic Characterization

The post bead analysis of BDCA-1$^+$ cells identified a population of lineage negative cells, which was HLADR$^+$, CD11c bright and CD123 low, similar to the previously described mDC precursors. It was therefore concluded that BDCA-1 is a marker for mDC. Similarly, the post bead analysis of BDCA-4$^+$ cells identified a population of lineage negative cells, which was HLADR$^+$, CD11c$^-$ and CD123 bright and correspond to the previously described pDC precursors. It was therefore concluded that BDCA-4 is a marker for pDC.

Frequency of BDCs in Peripheral Blood

The mean percentage of BDCA-1$^+$ cells in a total population of MNCs, as obtained from 11 experiments was 0.39±0.24%. The mean percentage of BDCA-4$^+$ cells in a total population of MNCs as obtained from seven experiments was 0.39±0.18%.

Purity of Isolated BDCs

The purity of these isolated BDCs, however, was variable, as a significant percentage of the isolated cells were lineage positive. In a total of eight BDCA-1 isolations, the mean purity obtained was 61±15.09%. In a total of three BDCA-4 isolations, the mean purity was 74.16±8.32%. Interestingly when only the lineage$^+$ population was included in the gate, this population was also CD11c$^+$, CD123^{low}, HLA-DR$^+$, but BDCA-1$^-$. The lineage positive cells were further characterized by staining the

postbead cells with individual lineage markers such as CD14, CD16, CD19, CD20, CD56 and CD3. The lineage positive cells stained positive mainly for CD14 and partly for CD16 (data not shown) individually. As the cytospin of postBDCA-1 isolation cells did not exhibit a homogenous population, we concluded that this heterogenity may be contributed by the contaminating lineage positive cells. For the definitive characterization, the lineage positive cells should be sorted and analyzed individually. The lineage positive cells appearing in BDCA-4 isolation also need to be characterized in future experiments.

DC Activation and Maturation

Several molecules activate DCs and trigger their transition from immature antigen-capturing cells to mature antigen-presenting DCs. The interaction between co-stimulatory molecules (CD80/CD86) expressed by DCs and their ligands expressed by T cells, sustain T cell activation during DC-T cell interaction. These costimulatory molecules have been used as a marker of DC activation and maturation. An another molecule CD83 was shown to be a selective cell surface marker for mature human blood dendritic cells by Zhou et al.[38, 35] In their study, $CD83^+$ cells expressed extremely high levels of MHC class II molecules and were also the most potent stimulator cells in an allogeneic MLR when compared with other leukocytes. As described by Sallusto and Lanzavecchia, monocyte derived DCs generated by 7 days of culture of monocytes in recombinant GM-CSF and IL-4 gave rise to immature DC expressing high levels of HLA-DR and CD86 and low to no CD14 or CD83. Incubation of these cultured DCs with tumor necrosis factor-α or soluble CD40L for 24 hours resulted in increased expression of activation markers. Willmann et al. [39] have further reported that CD86 is

constitutively expressed on resting $CD11c^+$ DCs and is up-regulated in activated $CD11c^+$s, whereas CD80 is not expressed in resting and is up-regulated in activated samples. In our study, the expression of CD83 and CD80 was insignificant for both the mDC and pDC subsets. 99% of mDCs or BDCA-1^+ cells displayed positive staining for CD86. Only 49% of pDCs or BDCA-4^+ cells expressed CD86. We therefore concluded that freshly isolated BDCA-1 and BDCA-4^+ cells are immature DCs. Our DCs also lacked the extensive cytoplasmic projections or veils extending from all aspects of the cell body, as seen with previously described mature dendritic cells with high immunostimulatory capability. Our finding was therefore consistent with previous reports that freshly isolated peripheral BDCs have a resting phenotype characterized by low level of accessory molecules.[36, 40] The analysis of DC functionality and DC cytokine profile in our study was hampered by low recovery of freshly isolated BDCs in culture. Experiments need to be repeated in order to determine the culture condition or cytokine supplementation requirements for the in vitro maintenance and maturation of these cells.

Due to their low frequency, heterogeneity and lack of specific markers, isolation procedures (negative selection of DCs by using anti-lineage marker and anti-HLA-DR MoAbs) and in vitro generation of DC following culture of $CD34^+$ cells or monocytes have been a prerequisite for the collection of adequate number of DCs so far. However, such procedures for the enrichment of DC may induce the selection of particular cell subsets as well as changes in both the phenotype and functional characteristics of the initial subsets of DC present in the sample. We have verified that the recently developed markers recognize peripheral blood mDC and pDC subsets distinctly and can be used as a

standard method for the positive selection of DCs. The conflicting results regarding both the phenotype and functional characteristics of the different subsets of DC arising from the use of varying culture conditions, precursor cells or antibodies can thus be avoided.

REFERENCES

1. Appelbaum FR (2001) Hematopoietic cell transplantation as immunotherapy. *Nature* **411:** 385-389.

2. Billingham RE (1966-67) The biology of graft-versus-host reactions. *Harvey Lect.* **62:** 21-78.

3. Tanaka J, Asaka M and Imamura M (2000) T-cell co-signaling molecules in graft-versus host disease. *Ann. Hematol.* **79:** 283-290.

4. Harding F, McArthur J, Gross J, Raulet D and Allison J (1992) CD28-mediated signaling co-stimulates murine T cells and prevents induction of anergy in T cell clones. *Nature* **356:** 607-609.

5. Clark E and Ledbetter J (1994) How B and T cells talk to each other. *Nature* **367:** 425-428.

6. Parham P (1992) Deconstructing the MHC. *Nature* **360:** 300-301.

7. Kernan NA (1994) in *Bone Marrow Transplantation* (eds Forman SJ, Blume KG and Thomas ED). 124-135 (Blackwell Scientific Publications, Boston).

8. Vallera DA, Ash R and Zanjani ED (1983) Anti-T cell reagents for human bone marrow transplantation: Ricin linked to three monoclonal antibodies. *Science* **22 (4623):** 512-515.

9. Kernan N, Flomberg N, Dupont B, O'Reilly RJ (1987) Graft rejection in recipients of T cell depleted HLA-nonidentical marrow transplants for leukemia. *Transplantation.* **43:** 842-847.

10. Martin P (1990) The role of donor lymphoid cells in allogeneic marrow engraftment. *Bone Marrow Transplant.* **6:** 283-289.

11. Storek J and Storb R (2000) T-cell reconstitution after stem-cell transplantation-by which organ? *Lancet* **355:** 1843-1844.

12. Tan P, Anasetti C, Hansen JA, Metrose J, Brunvand M, Bradshaw, Ledbetter JA and Linsley PS (1993) Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J. Exp. Med.* **177:** 165-173.

13. Gribben JG, Guinan EC, Boussiotis VA, Ke X-Y, Linsely L, Sieff C, Gray GS, Freeman G J and Nadler LM (1996) Complete blockade of B7 family-mediated costimulation is necessary to induce human alloantigen-specific anergy: a method to ameliorate graft-versus-host disease and extend the donor pool. *Blood* **87:** 4887-4893.

14. Yu X-Z, Bidwell SJ, Martin PJ and Anasetti C (2000) CD28-specific antibody prevents graft-versus-host disease in mice. *J. Immunol.* **164:** 4564-4568.

15. Jenkins MK and Schwartz RH (1987) Antigen presentation by chemically modified splenocytes induces antigen-specific T-cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* **165**: 302-319.

16. Lenschow DJ, Zeng Y, Thistlethwaite JR, Montag A, Brady W, Gibbon MG, Linsey PS and Bluestone JA (1992) Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4-Ig. *Science* **257:** 789-792.

17. Guinan E, Boussiotis V, Neuberg D, Brennan L, Hirano N, Nadler L and Gribben J (1999) Transplantation of anergic histoincompatible bone marrow allografts. *N. Eng. J. Med.* **340:** 1704-1714.

18. Kirk AD, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH and Knechtle SJ (1997) CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc. Natl. Acad. Sci. USA* **94:** 8789-8794.

19. Bancheraeu J, Steinman R (1998) Dendritic cells and the control of immunity. *Nature* **392:** 245-252.

20. Freudenthal P, Steinman R (1990) The distinct surface of human blood dendritic cells, as improved after an improved isolation method. *Proc. Natl. Acad. Sci. USA* **87:** 7698-76702.

21. Austyn J.M. (1992) Antigen uptake and presentation by dendritic leukocytes. *Semin. Immunol.* **4:** 227-236.

22. Langenkamp A, Messi M, Lanzavecchia A and Sallusto F (2000) Kinetics of dendritic cell activation: impact on priming of Th1, Th2 and nonpolarized T cells. *Nature Immunol.* **1:** 311-316.

23. De Saint-Vis B, Fugier-Vivier I, Massacrier C, Galliard C, Vandervilet B, Ait-Yahia S, Banchereau J, Liu Y-J, Lebecque S, Caux C (1998) The cytokine profile expressed by human dendritic cells is dependant on cell subtype and mode of activation. *J. Immunol.* **160:** 1666-1676.

24. Rissoan M, Soumelis V, Kadowwaki N, Grouard G, Briere F, de Waal Malefyt R, Liu Y-J, (1999) Reciprocal control of T helper cell and dendritic cell differentiation. *Science* **283:** 1183-1186.

25. Grouard G, Rissoan M, Filgueira L, Durand I, Banchereau J, Liu Y-J (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin3 (IL-3) and CD40 ligand. *J. Exp. Med.* **185:** 1101-1111.

26. Liu Y-J and Blom B (2000) Th2 inducing DC2 for immunotherapy. *Blood* **95:** 2482-2483.

27. Cella M, Facchetti F, Lanzavecchia A and Colonna M (2000) Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent Th1 polarization. *Nature Immunol.* **1:** 305-310.

28. Lea T, Vartdal F, Nustad K, Funderud S, Berge A, Ellingsen T, Schmid R, Stenstad P and Ugelstad J (1988) Monosized, magnetic polymer particles: their use in separation of cells and subcellular components, and in the study of lymphocyte function in vitro. *J. Mol. Recognit.* **1:** 9-18.

29. Recktenwald et al. (1996) Magnetic cell sorting with colloidal supermagnetic particles in *Cell Separation Methods and Applications*, New York, Marcel Dekker Inc., 153-173.

30. Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, Buck DW, and Schmitz Jurgen (2000) BDCA-2, BDCA-3, and BDCA-4: Three markers for distinct subsets of dendritic cells in human peripheral blood. *J. Immunol.* **165:** 6037-6046.

31. Goetzman E (1993) Flow cytometry instrumentation in research and clinical laboratories. *Clin. Lab. Sci.* **6:** 167-173.

32. Almeida J, Orfao A, Ocqueteau M, Mateo G, Corral M, Caballero MD, Blade J, Moro MJ, Hernandez J and San Miguel JF (1999) High sensitive immunophenotyping and DNA ploidy studies for the investigation of minimal residual disease in multiple myeloma. *Br. J. Hematol.* **107:**121-131.

33. Brodsky FM (1984) A matrix approach to human class II histocompatibility antigens: reactions of four monoclonal antibodies with the products of nine haplotypes. *Immunogenetics.* **19:** 179-194.

34. Lampson LA (1980) Two populations of a Ia-like molecules on a human B cell line. *J. Immunol.* **125:** 293-299.

35. Zhou L and Tedder TF (1996) CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. *Proc. Natl. Acad. Sci. USA*. **93:** 2588-2592.

36. O'Doherty U, Peng M, Gezelter S, Swiggard WJ, Betzes M, Bhardwaj N and Steinman RM (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and other immature. *Immunology*. **82:** 487-493.

37. Sallusto F and Lanzavecchia A (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* **179:** 1109-1118.

38. Zhou L and Tedder TF (1995) Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J. Immunol.* **154:** 3821-3835.

39. Willmann K and Dunne JF (2000) A flow cytometric immune function assay for human peripheral blood dendritic cells. *J. Leukocyte Biol.* **67:** 536-544.

40. O'Doherty U, Steinman RM, Peng M, Cameron PU, Gezelter S, Kopeloff I, Swiggard WJ, Pope M and Bhardwaj N (1993) Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J. Exp. Med.* **178:** 1067-1078.