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Identification of a dendritic cell lineage of myeloid origin

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IDENTIFICATION OF A DENDRITIC CELL LINEAGE
OF MYELOID ORIGIN

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Andrew BitMansour

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ABSTRACT

IDENTIFICATION OF A HUMAN DENDRITIC CELL LINEAGE OF MYELOID ORIGIN

by Andrew BitMansour

Dendritic cells (DC) are the most potent antigen-presenting cells and function to stimulate antigen-specific T cell responses. The paucity of DC coupled with the lack of a DC-specific marker has made their isolation and subsequent characterization difficult. This report describes a subset of DC which expresses high levels of the IL-3 α chain receptors (IL-3R α) and represents a specific lineage among the heterogeneous family of DC. The IL-3R α^{hi} DC are of myeloid origin and their CD34⁺ bone marrow progenitor population terminally differentiates into IL-3R α^{hi} DC. This DC subset appears to migrate to lymphoid organs in the absence of inflammatory stimuli or foreign antigens. In the absence of such triggers they fail to up-regulate accessory molecules needed for T cell stimulation. This alternative migration pattern suggests that IL-3R α^{hi} DC may participate in the induction of T cell tolerance during steady state conditions.

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GENERAL INTRODUCTION

This report presents a study on the identification of a subset of human bone marrow-derived Dendritic cells (DC) which is generated along a separate lineage than presently described DC. This DC subset can be identified by expressing high levels of the interleukin-3 receptor alpha chain (IL-3R α). This report is presented in two chapters. Chapter 1 (Dendritic Cell ontogeny: A human dendritic cell lineage of myeloid origin) presents the results and analysis of experiments performed in characterizing the IL-3R α ^{hi} DC in human adult tonsils, fetal lymph nodes, human peripheral blood, and fetal bone marrow. Chapter 2 (The Dendritic Cell system: A discussion on dendritic cell development, characterization, and function) presents a review of recent DC research and examines the current understanding of DC biology such as the DC migration model. This chapter also addresses current questions on DC biology through the examination of the IL-3R α ^{hi} DC population and provides unique insight especially when compared to the currently described CD11c⁺ DC population. The characteristics IL-3R α ^{hi} DC population also suggest an alternative model of DC migration which is also addressed in chapter 2.

CHAPTER I

**Dendritic Cell ontogeny: A human
dendritic cell lineage of myeloid
origin**

INTRODUCTION

Dendritic cells (DC) in lymphoid organs are potent APC, which play an important role in the initiation of immune responses (1). Studies showing that epidermal Langerhans cells are precursors of DC, have suggested that the unique role of DC as "nature's adjuvant" is linked to their developmental pathway. Langerhans cells reside in the epidermis where the cells are capable of antigen uptake but have low ability for antigen presentation (2). In response to inflammatory signals, the cells migrate rapidly to lymphoid tissues and differentiate into mature, activated DC with potent ability for stimulation of T cells (3-6). Cells with characteristics of DC precursors have also been found in other tissues (7), and such cells also migrate in response to inflammatory mediators (6, 8). Thus, DC in lymphoid organs have been widely considered to represent the end stage of a stepwise differentiation and migration process, which is completed during inflammation and serves to initiate immune responses (9-12).

Presently, most of the knowledge about the developmental pathway of DC is based on results obtained by cell culture. Cells with characteristics of Langerhans cells and DC can be generated in vitro by culture of CD34⁺ cells in the presence of granulocyte-macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor alfa (TNF- α) (13-19). Results from studies of in vitro colony formation have further indicated that the GM-CSF/TNF- α -responsive progenitors represent a separate DC colony forming cell (15). These and other observations have supported the view that DC are a family of closely related cells that constitute a distinct "DC lineage". Progenitors committed to become DC have, however, not yet been identified directly in

bone marrow. The interpretation of results obtained by colony assays and cell culture is furthermore complicated by the fact that populations of lymphoid progenitors, granulo-monocytic progenitors and peripheral blood monocytes also assume characteristics of DC in vitro (14, 18-22).

Primitive hematopoietic progenitors and cells committed to become lymphocytes, monocytes, granulocytes and erythroid cells can be identified as discrete populations of freshly isolated CD34⁺ bone marrow cells using specific cell surface markers (23-29). Similar characterization of DC progenitors has been difficult due to the lack of selective markers that identify the cells at an early stage of differentiation. In the present study, however, we demonstrate that antibodies to the interleukin-3 receptor alpha chain (IL-3R α) selectively react with a large subset of DC in lymphoid organs, and identify their precursors in blood and bone marrow. The CD34⁺IL-3R α ^{hi} progenitors are of myeloid origin, but committed to become DC and distinct from those that give rise to Langerhans cells. Unlike Langerhans cells, IL-3R α ^{hi} DC home to lymphoid tissue independently of inflammation or stimulation with foreign antigens.

MATERIALS AND METHODS

Tissue.

Tissue from aborted fetuses of gestational age 19-21 weeks was obtained from Advanced Bioscience Resources (Alameda, CA) a non-profit organization which provides tissue in compliance with state and federal laws. Blood donor buffy coats were obtained from the Stanford Blood Bank (Stanford, CA). Tonsils and adult lymph nodes were obtained from the tissue acquisition

service and the clinical flow cytometry laboratory, Dept. of Pathology, University of Texas Southwestern Medical Center (Dallas, TX).

Cell preparation.

Mononuclear cell suspensions were obtained by Lymphoprep gradient centrifugation (Nycomed, Oslo, Norway). Antibody-free CD3⁺CD4⁺ T cells were isolated to 99% purity from peripheral blood mononuclear cells (PBMC) using CD4 Dynabeads in combination with Detachabead reagent (Dyna, Oslo, Norway) after depletion of myeloid cells (CD14⁺ and CD36⁺) by Dynabeads (Dyna). Fetal bone marrow CD34⁺ cells were isolated by a CD34⁺ cell MACS selection kit (Miltenyi Biotech, Auburn, CA), as described previously (29). Where noted, subsets of immunostained cells were sorted using a FACSVantage flow cytometer (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA).

Immunophenotypic analysis.

Multi-color immunofluorescence staining and analysis was performed by standard methods (see e.g. ref (29)). Primary or secondary antibodies were conjugated to biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll protein (PerCP), or allo-phycoerythrin (APC). Antibodies and streptavidin conjugates were from BDIS, except CD40, CD86 PE, anti-IL-3R α biotin and anti-IL-3R α PE (Pharmingen, San Diego, CA); CD64 (Meda Rex, West Lebanon, NH); anti-M-CSFR (Santa Cruz Biotech, Santa Cruz, CA); donkey anti-rat IgG PE, goat-anti-mouse IgG PE and goat-anti-human IgM FITC (Jackson ImmunoResearch, West Grove, PA). Cells were

analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems).

Media and cytokines.

Except where specifically noted, cells were cultured in Yssel's medium (30) supplemented with 10% heat-inactivated pooled human AB⁺ serum and 10% fetal bovine serum. Recombinant human cytokines were used as noted at the following concentrations: granulocyte macrophage-colony-stimulating factor (GM-CSF, 10 ng/ml), (IL-3, 10 ng/ml), IL-6 (500 U/ml), IL-7 (50 ng/ml) (all from Collaborative Biomedical Products, Bedford, MA), erythropoietin (Epo, 2.5 U/ml, CILAG AG, Schaffhausen, Switzerland), stem cell factor (SCF, 40 ng/ml, Peprotech, Rocky Hill, NJ), granulocyte-CSF (G-CSF, 50 ng/ml, Amgen, Thousand Oaks, CA), macrophage-CSF (M-CSF, 10 ng/ml, R&D Systems, Minneapolis, MN).

Cell cultures

IL-3R α^{hi} HLA-DR⁺ cells from tonsil and PBMC and CD14^{hi} monocytes from PBMC were sorted by FACS and cultured for 36h before mixing with T cells to allow maturation of DC precursors. GM-CSF and IL-3 were added to IL-3R α^{hi} HLA-DR⁺ cells to enhance survival. Stimulator cells were washed twice before co-culture with CD4⁺ T cells (10⁵ per well) in flat-bottomed 96-well plates for six days. Bromodeoxyuridine (BrdU, Sigma Chemicals, St. Louis, MO) (50 μ M) was added 12h before harvest and latex particles (10⁵ per well) immediately prior to harvest as a reference for cell counts (29). The cells were

stained with CD3 PE, followed by fixation, permeabilization, staining with anti-BrdU FITC and flow cytometric analysis, as described in detail in ref (29). Co-cultures with stimulator cells derived by culture of fetal bone marrow cells were pulsed with $1\mu\text{Ci } ^3\text{H-thymidine}$ for 8h before collecting and counting.

RESULTS AND DISCUSSION

IL-3R α is a selective marker for a large subset of DC in T-cell rich zones of human peripheral lymphoid organs.

Human tonsils contain DC that can be identified as cells that lack lineage markers for monocytes and lymphocytes (lin^-) and are positive for HLA-DR and CD4 ($\text{HLA-DR}^+\text{CD4}^+\text{lin}^-$) (Fig. 1A, B) (31, 32). In an attempt to identify DC-selective markers, we screened antibodies to leukocyte differentiation antigens for selective reactivity with this population. Antibodies to IL-3R α reacted strongly with more than 85% of $\text{HLA-DR}^+\text{CD4}^+\text{lin}^-$ cells (0.32-0.37% of tonsillar mononuclear cells, $n=3$), but weakly with most other cells ($n=3$) (Fig. 1C). The staining was sufficiently specific to allow a 200-fold enrichment of $\text{HLA-DR}^+\text{CD4}^+\text{lin}^-$ cells with a single positive immuno-magnetic selection ($n=2$). $\text{HLA-DR}^+\text{lin}^- \text{IL-3R}\alpha^{\text{hi}}$ cells were also found in adult cervical, axillar, intramammary, mesenteric, and femoral lymph nodes. The frequency ranged from 0.1%-1.8% (average 0.5%, $n=9$). In all cases, cells staining brightly with the anti-IL-3R α were found within the $\text{HLA-DR}^+\text{lin}^-$ population and constituted the majority of these cells (on average $60.2\pm 11.2\%$, $n=9$).

Freshly sorted HLA-DR⁺lin⁻IL-3R α ^{hi} cells showed an immature morphology without cytoplasmic protrusions (Fig. 1D). The majority of the cells died rapidly in culture, but could be partially rescued by the addition of cytokines (GM-CSF and IL-3). Under these conditions, the cells rapidly formed large aggregates of cells as previously reported for tonsillar DC (Fig. 1E). After 3-5 days the cells were more dispersed and showed multiple long processes characteristic of DC (not shown).

IL-3R α ^{hi} cells were found almost exclusively in the T cell-rich extra-follicular regions of the tonsil (Fig. 1F). The localization as well as the cytokine requirements and phenotype of the HLA-DR⁺lin⁻IL-3R α ^{hi} cells (Table I) suggest that they are identical to the "plasmacytoid T cell" DC that were recently characterized by Grouard et al. (33).

IL-3R α ^{hi} DC are immature and appear in lymphoid organs independently of stimuli that cause upregulation of MHC class II and costimulatory molecules. Originally, it was concluded that DC in lymphoid tissues such, as tonsils are activated, mature APC, since isolated cells expressed high levels of MHC class II and costimulatory molecules (31, 32). However, in those studies the cells were isolated after 1-2 days of cell culture. As shown in Fig. 2A and Table I, HLA-DR⁺lin⁻IL-3R α ^{hi} cells in fresh preparations of tonsillar mononuclear cells expressed low levels of CD80 (B7.1), CD86 (B7.2) and HLA-DQ. Overnight culture of unseparated cells in the absence of cytokines was sufficient to induce the mature phenotype (Fig. 2A). Sorted HLA-DR⁺lin⁻IL-3R α ^{hi} cells that were allowed to mature in culture for 36h and kept viable with GM-CSF and IL-3, were potent stimulators of allogeneic CD4⁺ T cells (Fig. 2B). These

results are surprising in view of the hypothesis that DC migrate to lymphoid organs in response to signals that lead to cell activation. The data are, however, in agreement with those of Grouard et al., who also studied freshly isolated cells (33) and with previous reports showing that DC in murine spleens were immature immediately after isolation and differentiated rapidly in vitro (34, 35).

The non-activated phenotype of DC in tonsils may seem like a paradox since tonsils are typically removed after repeated inflammations. One can further not exclude the possibility that the isolated cells had migrated to lymphoid tissue in response to inflammation, but not yet assumed the activated phenotype. To determine whether the presence of HLA-DR⁺lin⁻IL-3R α ^{hi} cells in lymphoid tissue depends on previous exposure to foreign antigens or inflammatory stimuli, we examined whether the cells could be found in fetal lymph nodes. Presumably, these lymph nodes drain sterile, non-inflamed tissues. As shown in Fig. 2C, HLA-DR⁺lin⁻IL-3R α ^{hi} cells were present at high frequencies in the fetal lymph nodes (2.6 ± 1.1 %, n=3). These cells were more frequent than cells expressing high levels of myeloid markers, such as CD13 and CD33 (1.5 ± 0.2 %, n=3). The HLA-DR⁺lin⁻IL-3R α ^{hi} cells in fetal lymph nodes expressed the same combination of markers as those in tonsils (Table I). This cell type is therefore most likely capable of migrating to lymphoid tissue independently of inflammation and foreign antigens and without initiating immune responses.

IL-3R α^{hi} DC are present in peripheral blood.

HLA-DR⁺lin⁻IL-3R α^{hi} cells were readily detectable as a population with low orthogonal light scatter (OLS) in blood from adult donors, and constituted $0.47 \pm 0.14\%$ (n=8) of peripheral blood mononuclear cells (PBMC) (Fig. 3A and 3B, R1). A second population of IL-3R α^{hi} cells was present among PBMC, but these cells were HLA-DR⁻ (Fig. 3B, R2) and were found to be basophilic granulocytes (not shown). The HLA-DR⁺lin⁻IL-3R α^{hi} blood cells expressed the same combination of markers and had similar morphology as shown for the tonsillar counterparts (Table I and not shown). An interesting difference was, however, that whereas tonsillar DC did not express the lymph node homing molecule L-selectin (CD62L), the cells from PBMC were positive for this marker (Table I).

Whereas multiple cell types can stimulate allogeneic T cells, DC are characterized by their higher potency relative to other APC (13, 15, 21, 31, 33, 36-39). The cells also induce proliferation of autologous T cells in vitro (37). The data in Fig. 3C and D demonstrate that HLA-DR⁺lin⁻IL-3R α^{hi} blood cells were up to 100 fold more potent than monocytes in stimulating both allogeneic and autologous T cells. Cells with similar characteristics have previously been identified in blood and were referred to as CD11c⁻ DC or CD33^{dim}CD14⁻CD16⁻ DC (36, 37).

Precursors of IL-3R α^{hi} DC are found among CD34⁺ bone marrow cells and are distinct from the cells that give rise to Langerhans cells in response to GM-CSF and TNF- α .

IL-3R α^{hi} cells were readily identified as a distinct subset of CD34 $^{+}$ fetal bone marrow cells with low orthogonal light scatter (3.1 ± 0.9 %, $n=5$) (Fig. 4A,B). The majority of the CD34 $^{+}$ IL-3R α^{hi} cells were within the CD34 $^{\text{lo}}$ subset, which contains lineage-committed progenitors (Fig. 4B) (23). The CD34 $^{+}$ IL-3R α^{hi} cells had morphology and immunophenotype similar to IL-3R α^{hi} cells in blood and tonsil, but appeared more immature, and mitotic figures were frequently observed (Fig. 4C and Table I). .

Previous studies have demonstrated that DC and Langerhans cells can be derived by culture of CD34 $^{+}$ cells with GM-CSF and TNF- α (13-19). The subset of CD34 $^{+}$ cells that contains these TNF- α -dependent progenitors has not yet been identified. We therefore examined whether they were identical to the CD34 $^{+}$ IL-3R α^{hi} cells. The sorted IL-3R α^{hi} population (Fig. 4A) formed aggregates of cells with DC morphology during culture with either GM-CSF and IL-3 or GM-CSF and TNF- α (not shown), and after 5 days the cells were positive for CD1a (Fig. 4D,E). However, differentiation into DC occurred independently of TNF- α , and the CD1a $^{+}$ cells co-expressed CD45RA, which is absent from Langerhans cells (39) (Fig. 4D,E). Tonsillar HLA-DR $^{+}$ lin $^{-}$ IL-3R α^{hi} cells also obtained a CD1a $^{+}$ CD45RA $^{+}$ phenotype when cultured under the same conditions (not shown). In contrast, sorted CD34 $^{+}$ cells with low levels of the IL-3R α (Fig. 4B) gave rise to few CD1a $^{+}$ cells when cultured with GM-CSF and IL-3, but large numbers of CD45RA $^{-}$ CD1a $^{+}$ cells in the presence of GM-CSF and TNF- α (Fig. 4F,G). Consistent with a phenotype of Langerhans cells, 30-40% of these CD45RA $^{-}$ CD1a $^{+}$ cells contained Birbeck granules (Fig. 4H). The frequency of Birbeck granule-positive cells among CD1a $^{+}$ cultured CD34 $^{+}$ IL-3R α^{hi} cells was less than 4% and may therefore reflect

contamination of sorting gates. Finally, CD34⁺IL-3R α ^{hi} cells cultured with GM-CSF and IL-3 were potent stimulators of allogeneic CD4⁺ T cells, and more potent than CD14⁺ macrophages generated by culture of CD34⁺M-CSFR⁺ cells from the same donor in M-CSF (Fig. 4I).

IL-3R α ^{hi} DC are of myeloid origin.

The distribution of macrophage colony-stimulating factor receptor (M-CSFR) and IL-3R α among CD34⁺ cells suggested that CD34⁺IL-3R α ^{hi} cells may derive from cells in the M-CSFR^{hi} population, which downregulate the M-CSFR as they upregulate the IL-3R α (arrow in Fig. 5A). Since M-CSFR expression on CD34⁺ cells is restricted to granulo-monocytic progenitors, this would indicate that IL-3R α ^{hi} cells belong to the granulo-monocytic lineage (29). To test this possibility, immature progenitors (i.e. CD34^{hi}, see Fig. 4B) with high levels of M-CSFR and low levels of IL-3R α (region in Fig. 5A) were sorted and cultured. After 60h of culture two populations of cells that had downmodulated the M-CSFR were observed, with high and low levels of IL-3R α , respectively (Fig. 5B). These cells were sorted and cultured for five additional days in medium supplemented with GM-CSF and IL-3. At this stage, the cultures from the IL-3R α ^{lo} population contained CD15⁺ granulocytic cells (Fig. 5C) whereas cultures from the IL-3R α ^{hi} population contained CD1a⁺ cells with DC morphology (Fig. 5D and not shown). The latter cells induced strong proliferation of allogeneic T cells compared to equal numbers of CD14⁺ macrophages generated by culture of CD34⁺M-CSFR⁺ cells for 5 days with M-CSF (29) (n=2, not shown). Addition of TNF- α to the medium did not increase the number of CD34⁺IL-3R α ^{hi} cells generated from

M-CSFR^{hi} myeloid progenitors, further suggesting that the cells are distinct from the TNF- α dependent Langerhans cell progenitors (not shown).

Thymus contains small numbers of IL-3R α ^{hi} DC that express low levels of CD34.

Earlier studies have demonstrated that some DC may share the differentiation pathway of the T cell lineage and that DC are generated by progenitors in the thymus (18, 19, 40). We therefore investigated whether IL-3R α ^{hi} DC were present in the thymus. The results showed that less than 0.1 % of the total thymic cell population were IL-3R α ^{hi} DC and that the IL-3R α ^{hi} cells constituted only 15 $\pm$ 2% (n=3) of HLA-DR⁺lin⁻ subset. This may indicate that other DC populations than the IL-3R α ^{hi} cells are predominant in thymus. In addition, the IL-3R α ^{hi} DC in thymus expressed low levels of CD34 compared to bone marrow IL-3R α ^{hi} cells from the same donor (not shown). Similar low levels of CD34 were found on the cells in fetal lymph nodes (Table I). It therefore seems likely that the few IL-3R α ^{hi} DC in the thymus represent cells that have migrated from bone marrow and are distinct from thymic DC progenitors.

IL-3R α ^{hi} DC constitute a separate lineage of DC that follow a differentiation pathway distinct from Langerhans cells.

The data presented here demonstrate that a subset of DC in human lymphoid organs and blood represent a separate lineage of cells. The cells can be readily

identified and isolated on the basis of their high levels of the IL-3R α . Their precursors in the bone marrow appear to follow the myeloid differentiation pathway to the branching point of the granulocytic and monocytic lineages. Progenitors that have committed to this DC lineage are distinct from those that give rise to Langerhans cells when cultured in the presence of GM-CSF and TNF- α . Their progeny furthermore lack several characteristics of Langerhans cells that were previously considered common to DC. Unlike Langerhans cells, the IL-3R α^{hi} DC appear to undergo little differentiation during transit from the bone marrow to lymphoid organs. The IL-3R α^{hi} DC further seem capable of migrating to lymphoid tissue independently of inflammatory stimuli. The results concur with previous reports showing that some DC spend less than 24h in transit from the progenitor pool to lymphoid tissue and that DC turnover occurs constantly during steady state conditions (1, 41-43). Thus, the DC system is constituted by multiple cell types with distinct developmental pathways and functional properties. A large proportion of the cells may enter lymphoid tissue without inducing immune responses.

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Figure 1 A-C. Antibodies to IL-3R α selectively stain DC in extrafollicular regions of human tonsils. (A, B and C) Tonsillar mononuclear cells were stained with anti-HLA-DR PerCP, CD4 PE, anti-IL-3R α biotin+Streptavidin AIPC and a cocktail of FITC-conjugated lineage markers ("lin") for lymphocytes and monocytes (CD3, CD14, CD16, CD19, CD20, CD56 and goat-anti-human IgM). The cells were analyzed by four-color flow cytometry. Dendritic cells were identified as HLA-DR⁺CD4⁺lin⁻, i.e. cells that simultaneously satisfy the criteria of the box regions in (A) and (B), and are represented by large black dots. Dashed lines represent isotype control levels.

Figure 1. (D) Wright-Giemsa staining of a cytocentrifuge slide of freshly FACS-sorted tonsillar HLA-DR⁺lin⁻IL-3R α ^{hi} cells (magnification, 600x) (E) FACS-sorted tonsillar HLA-DR⁺lin⁻IL-3R α ^{hi} cells were cultured for 24h culture with GM-CSF and IL-3 and photographed in situ (magnification, 400x). (F) A frozen section of tonsillar tissue was stained with anti-IL-3R α , biotinylated anti-mouse IgG and streptavidin peroxidase (magnification, 100x). Staining was visualized by diaminobenzidine and hydrogen-peroxide and the section counterstained with methylene blue.

Figure 2. IL-3R α^{hi} DC are immature and appear in lymphoid organs independently of stimuli that cause upregulation of MHC class II and costimulatory molecules.

(A) Mononuclear cells from tonsil were stained with anti-HLA-DR, anti-IL-3R α and "lin" markers, and either CD86, CD80, HLA-DQ or isotype control mAbs before (open bars) and after (black bars) 16h incubation at 37°C in Yssel's medium(30). The HLA-DR⁺lin⁻IL-3R α^{hi} population was analyzed for mean fluorescence intensity (MFI) staining with the markers indicated on the figure, and the bars represent MFI after isotype control levels were subtracted. (B) T cells (10⁵) were co-cultured with indicated numbers of allogeneic IL-3R α^{hi} HLA-DR⁺ cells from tonsil (stimulator cells). T cell proliferation was measured as the total number of CD3⁺ bromodeoxyuridine-positive (BrdU⁺) cells per well at day 6 of co-culture. (C) Fetal lymph node cells were stained with anti-HLA-DR and anti-IL-3R α and lineage markers (not shown) and analyzed by flow cytometry as described in Fig. 1A-C. Data is representative of three experiments.

Figure 3. HLA-DR⁺IL-3Rα^{hi} DC are present in peripheral blood. (A-B) Peripheral blood mononuclear cells were stained with anti-HLA-DR, anti-IL-3Rα and lineage markers (not shown) and analyzed by flow cytometry as described in Fig. 1A-C. R1 and R2 in (B) represent regions used to sort IL-3Rα^{hi} blood cells that were positive or negative for HLA-DR, respectively. (C-D) HLA-DR⁺IL-3Rα^{hi} cells sorted from blood according to R1 in (B) were first cultured separately with IL-3 and GM-CSF for 36h and then incubated with allogeneic (C) or autologous (D) CD4⁺ T cells. T cell proliferation was measured as total number of CD3⁺BrdU⁺ cells per well at day 6 of co-culture with indicated numbers of IL-3Rα^{hi}HLA-DR⁺ cells (■), or CD14^{hi} monocytes (○) from the same donor (stimulator cells). Individual displays show data that are representative of three experiments. OLS; orthogonal light scatter.

Figure 4 A, B, D-G. Proliferating progenitors for IL-3R α^{hi} DC are found as a discrete CD34⁺IL-3R α^{hi} population that is distinct from the cells that give rise to Langerhans cells in response to GM-CSF and TNF- α . (**A, B**) Nucleated fetal bone marrow cells were stained with CD34 and anti-IL-3R α . The cells were analyzed by flow cytometry as described in Fig. 1A-C. The CD34⁺IL-3R α^{hi} population was defined according to the region in (A) (blue dots). CD34⁺IL-3R α^{lo} cells were defined according to the region in (B) (red dots) OLS: orthogonal light scatter. (**D-G**) CD34⁺IL-3R α^{hi} cells (blue) and CD34⁺IL-3R α^{lo} cells (red) were sorted according to the regions in (A) and (B), respectively, and cultured in the presence of indicated cytokines, stained with CD1a and CD45RA after 5 days of culture and analyzed by FACS. L = B-lymphoid cells staining brightly with CD19 (not shown). None of the cultured CD34⁺IL-3R α^{hi} cells (D, E) stained positively with CD19 (not shown). Dashed lines indicate isotype control levels.

Figure 4 C, H. (C) Freshly sorted Wright-Giemsa-stained CD34⁺IL-3R α ^{hi} cells display mitotic figures (magnification, 600x). (H) Transmission electron microscograph (magnification, 5,000x) of a CD1a⁺ cell sorted from CD34⁺IL-3R α ^{lo} cells cultured with GM-CSF and TNF- α , as described in (G). The arrows point to areas containing Birbeck granules, (shown in inserts magnification, 45,000x). Transmission electron microscograph (magnification, 5,000x) of a CD1a⁺ cell sorted from CD34⁺IL-3R α ^{lo} cells cultured with GM-CSF and TNF- α , as described in (G). The arrows point to areas containing Birbeck granules, (shown in inserts magnification, 45,000x).

Figure 4I. (I) CD4⁺ T cells (10⁵) were co-cultured with indicated numbers of allogeneic DC (■) or macrophages (○) from the same donor. The DC were generated by culturing sorted CD34⁺IL-3Rα^{hi} cells for 5 days with GM-CSF and IL-3 (■). The macrophages were generated by culture of CD34⁺M-CSFR⁺ cells for 5 days with M-CSF and purified by FACS sorting of CD14⁺ cells. T cell proliferation was measured as incorporation of ³H-thymidine at day 6 of co-culture. c.p.m; counts per minute. Data is representative of three experiments.

Figure 5. IL-3R α^{hi} DC follow the myeloid differentiation pathway to the branching point of the granulocytic and monocytic lineages.

(A) CD34 $^{+}$ cells were incubated for 12h in serum-free medium to allow upregulation of the M-CSFR, and stained with CD34, anti-M-CSFR and anti-IL-3R α . The arrow shows the suggested differentiation pathway of CD34 $^{+}$ IL-3R α^{hi} cells. The region shows sorting criteria for M-CSFR $^{\text{hi}}$ /IL-3R α^{lo} cells. An additional gate was set to include only CD34 $^{\text{hi}}$ cells, shown in Fig. 4B, to restrict the sort to immature progenitors (29) (B) After a 60h culture of CD34 $^{\text{hi}}$ M-CSFR $^{+}$ cells in serum-free medium (25) containing SCF, G-CSF, GM-CSF, IL-3 and IL-6, the cells were stained with anti-M-CSFR and anti-IL-3R α . The regions indicate criteria for sorting of the two populations that had downmodulated the M-CSFR during the culture period. (C,D) After a 5 day secondary culture of IL-3R α^{lo} M-CSFR $^{\text{lo}}$ cells (green) and IL-3R α^{hi} M-CSFR $^{\text{lo}}$ cells (blue) with IL-3 and GM-CSF, the cells were stained with CD1a and CD15 and analyzed by FACS. The small subset of CD15 $^{\text{lo}}$ cells in (C) represent basophilic granulocytes (27, 29).

	Tonsil	Fetal LN	PBMC	Fetal BM
CD1a	-	-	-	-
CD3	-	-	-	-
CD4	++/+++	++/+++	++	++
CD5	-	-	-	-
CD11b	-	-	-	-
CD11c	-	-	-	-
CD13	-/+	+	-/+	+
CD14	-	-	-	-
CD15	-	n.d.	-	-
CD16	-	-	-	-
CD19	-	-	-	-
CD20	-	-	-	-
CD32	+	+	+	+
CD33	+	+(+)	+(+)	+/++
CD34	-/+	+	-/+	++/+++
CD36	++/+++*	++/+++	++/+++	++/+++
CD40	++	+	++	+
CD45RA	++(+)	++(+)	++(+)	++
CD45RO	-	-	-	-
CD54	++/+++	n.d.	++/+++	+/++
CD56	-	-	-	-
CD58	+	n.d.	+/++	+/++
CD62L	-	+	+/++	+++
CD64	-	-	-	-
CD80	-	-	-	-
CD86	+	n.d.	+	+(+)
HLA-DR	++	++	++	++
HLA-DQ	-	n.d.	-/+	-

Table 1. Expression of surface molecules on HLA-DR⁺lin⁻IL-3R α^{hi} DC from mononuclear tonsillar cells and peripheral blood mononuclear cells (PBMC), fetal lymph node (LN) cells and on CD34⁺IL-3R α^{hi} cells from fetal bone marrow (BM). Cells were stained as described in legends to Figs. 1, 3 and 4. Mean fluorescence intensity levels (MFI) for the IL-3R α^{hi} populations are expressed as "-", indicating MFI in the first decade on a four log scale, which correspond to isotype control levels. The "+", "++", and "+++" indicate MFI in the second, third, and fourth decades respectively. A / sign means that MFI is on the border between two decades. Parenthesis means that the MFI is in the upper end of a decade. * In one out of three experiments tonsillar HLA-DR⁺lin⁻IL-3R α^{hi} DC were negative for CD36. n.d. not determined. The data is a representative of at least three experiments.

CHAPTER II

**The Dendritic Cell System: A
discussion on dendritic cell
development, characterization, and
function**

General Characteristics of Dendritic Cells

Generation of an effective immune response involves two major groups of leukocytes: antigen-presenting cells (APC) and T cells. Professional APC include B cells, macrophages, and dendritic cells, and are specialized to internalize, process, and re-express antigen on their Major Histocompatibility Complex (MHC) molecules for presentation to T cells. Dendritic cells (DC) are distinguishable from other APC due to their ability to stimulate naive T cells circulating through lymphoid tissues and thus initiating primary immune responses (1). Other APC stimulate secondary immune responses since they interact only with primed memory T lymphocytes which have entered the periphery. Another distinguishing feature of DC is their ability to stimulate a more vigorous T cell proliferation than the other APC. These functional characteristics of DC were described shortly after they were identified in lymphoid tissues as a unique population of cells with dendritic-type processes protruding from their cell body and expressing high levels of the MHC type II molecules (2). DC have recently been given the title of "nature's adjuvant" since they have been shown to efficiently induce T cell responses in vivo in the absence of other adjuvants (3).

Activation of resting T cells requires two signals: antigenic and co-stimulatory (4). An antigenic signal is delivered during the engagement of the T cell receptor with the antigen-MHC complex of the APC. This initial interaction must be accompanied by the ligation of receptors on the T cell such as CD28 with co-stimulatory molecules on the surface of mature DC such as CD80 (B7-1) and CD86 (B7-2) (5). The efficient delivery of secondary signals by the co-stimulatory molecules is crucial for the activation of naive T

cells. Therefore, the intimate interaction and the subsequent exchange of signals between DC and T cells is necessary for the induction of a functional T cell response.

When antigen is presented in the absence of co-stimulatory signals, T cells become non-responsive to the APC and are thus considered tolerized. The T cells entering this state are not able to mount an immune response against the antigen presented. Therefore, the absence of the appropriate signals during an APC-T cell interaction results in a tolerogenic rather than an immunogenic response. DC have been implicated in the mediation of tolerogenic responses. Transplantation studies have reported that DC exported by donor allografts to recipients mediate allograft acceptance and acquired transplantation tolerance (6, 7). The DC under these conditions are believed to migrate from the donor organ to the lymphoid organs of the host, where they tolerize the host T cells due to lack of co-stimulation.

Despite the important role of DC in the immune system, they have not been as well characterized as the cells which they activate, the T lymphocytes. Exhaustive efforts in T cell research have resulted in a detailed understanding of their subsets ($CD4^+$, $CD8^+$), their developmental pathways (naive, memory, and cytotoxic T lymphocytes) and their roles in immune responses through using most notably, specific T cell markers as valuable research tools (8-10). Conversely, elucidation of the DC system has suffered from the lack of a DC-specific marker. Present DC classification uses morphologic and phenotypic descriptions which has resulted in the identification of a number of DC and DC precursors with different characteristics; (1) interstitial regions in the heart, lung, kidney, and gut, where they are called interstitial DC; (2)

the skin and mucous membranes where they are called Langerhans Cells; (3) thymic medulla and secondary lymphoid tissue where they are known as interdigitating dendritic cells; (4) blood; and (5) afferent lymph where they are referred to as "veiled cells" (11). However, due to the lack of a DC-specific marker, no connection has been drawn between DC within. Therefore, the relationship between the various DC types remains unclear.

The understanding of DC ontogeny is also unclear and questions regarding their differentiation pathway are unresolved. DC have been described to have a bone marrow origin (12, 13), but their development is poorly understood, and their lineage relationship with other classes of hematopoietic cells is poorly characterized (14) due to the absence of a DC-specific marker. Understanding of the DC differentiation pathway is minimal because no DC-specific marker has been identified which would allow for the distinction of DC from other hematopoietic cells during development. Present descriptions of DC differentiation pathways reflect this problem. It has been proposed that DC are lymphoid-derived and arise from a common progenitor that also gives rise to B cells, T cells, and natural killer cells (15-17). Other evidence indicates that DC have a myeloid origin and share phenotypic characteristics with myeloid precursors (18, 19). Results from a recent study of hematopoietic colony forming units has suggested the existence of a separate DC lineage by identifying a separate colony-forming-unit DC (CFU-DC) (20). However, the progenitor cells giving rise to this colony have not been identified.

The paucity of DC has been another major problem in studying their biology. DC are the least abundant population of leukocytes (3), and major

efforts have been concentrated on generating them in sufficient numbers for study. Such procedures have relied on CD34⁺ bone marrow or cord blood cells as their starting populations which contain progenitors for all hematopoietic cells (21-23). However, the *in vitro* generation of DC from these populations relies on long culture periods requiring exogenous cytokine supplementation which suppresses the outgrowth of other hematopoietic cells. It is not clear to what extent these conditions reflect the normal differentiation of DC in the bone marrow. Enrichment of differentiated DC in blood or lymphoid organs is also used, but has also been difficult due to the lack of a DC-specific marker. Such enrichment relies on extended procedures involving multiple laborious steps where non-DC cell types are removed (24). This approach results in low DC yields and impure populations. In addition, the multiple incubation periods employed by enrichment methods promotes the *in vitro* maturation of the DC and does not permit the examination of freshly isolated cells.

This report describes a DC population which is specifically identified and selected using monoclonal antibodies against the interleukin-3 α chain receptor (IL-3R α). The IL-3R α chain dimerizes with the β chain to form a high affinity receptor for interleukin-3 (IL-3) and is expressed on a variety of cells including those of the myeloid lineage, some B lymphocytes, and endothelial cells (25). IL-3 is a T cell-derived cytokine that has a wide range of differentiation and growth-promoting activities on hematopoietic progenitor cells. It is produced by CD4⁺ activated T cells, activated Natural Killer (NK) cells, and activated mast cells. It is known to act as a critical growth factor for hematopoiesis, acting directly upon stem cells and lineage-

specific precursor cells (25). The IL-3R α^{hi} DC subset is present in lymphoid tissues and blood, and stems from a CD34 $^{+}$ DC-committed progenitor cell population in the bone marrow (Chapter 1).

Dendritic Cells in Lymphoid Tissues

Lymphoid tissues represent the end-organ for DC migration. DC arrive there by draining directly through afferent lymphatics following antigen uptake and processing but have not been observed to re-circulate through the efferent lymphatics (26). Moreover, there is no evidence for direct entry of DC into the blood stream from within the lymph nodes or for transformation into another cell type (27). Early characterization of DC in tonsils showed that they express high levels of MHC class II molecules, co-stimulatory molecules and other molecules such as CD4, CD30, and CD40 (28, 29). Their membrane was described to be irregular, containing dendritic-like processes. The DC were also more effective T cell stimulators than other tonsillar APC as demonstrated in the Mixed Leukocyte Reaction (MLR) (28). Based on these functional and phenotypic characteristics, DC present in lymphoid organs were considered to be "mature".

Lymphoid DC are presumed to be the mature counterparts of peripheral tissue DC, however, direct identification of the same cell type in the two sites has not been made. DC seem to undergo phenotypic and morphological changes during their transition in tissues, therefore, it has been difficult to identify the peripheral sources of lymphoid DC. For example, the skin Langerhans type DC are characterized by having intracytoplasmic

structures termed Birbeck granules (11). However, Birbeck granules have not been observed in DC present in lymphoid organs. Langerhans cells that migrate to lymphoid tissue after antigen uptake apparently lose their Birbeck granules during this period. Therefore, the phenotypic changes associated with maturation and migration of DC has made it difficult to link DC populations from various tissues.

Recent reports have described the presence of un-characteristic DC in lymphoid organs. Studies on mouse spleen leukocytes identified DC expressing low levels of co-stimulatory molecules, in particular CD86 (30, 31). These DC were also less potent T cell stimulators than the mature ones and were described as "immature". However, the immature DC were able to up-regulate their co-stimulatory molecules during a short period (16 hours) in culture and convert into phenotypically mature DC (30). These results suggested that perhaps not all DC populations present in lymphoid tissue are functionally mature, and that DC at different developmental stages may be simultaneously present in lymphoid organs. It is also possible that the mature DC in lymphoid organs described in earlier studies may have been due to enrichment and isolation procedures. Due to the lack of a DC-specific marker, the DC isolation methods involved multiple purification steps requiring culture periods for the depletion of unwanted leukocyte populations. Therefore, immature DC could potentially have been converted to mature DC during the isolation procedures.

The study described in chapter 1 of this report was undertaken to allow for the isolation and characterization of fresh DC. Human tonsillar DC are characterized by the positive expression of HLA-DR and CD4 and lack of

lineage markers for monocytes and lymphocytes (CD3, CD14, CD16, CD19, CD20, and CD56) denoted as "lin⁻" (28, 29). A panel of antibodies to leukocyte differentiation antigens was screened for selective reactivity with this population. A population which represented 85% of the HLA-DR⁺CD4⁺lin⁻ cells was identified that expressed high levels of the IL-3R α chain (Chapter 1 Fig. 1A-C). The reactivity was sufficiently specific to allow for a 200-fold enrichment of the HLA-DR⁺CD4⁺lin⁻ by a single, positive immuno-magnetic selection procedure. Freshly sorted IL-3R α ^{hi} DC were round-shaped cells containing a large nucleus (Chapter 1 Fig. 1D).

The IL-3R α ^{hi} population did not display dendritic processes as do mature DC, and expressed no or low levels of the MHC molecule HLA-DQ and the co-stimulatory molecules CD80 and CD86. This initial morphological and immuno-phenotypical profile suggested that the IL-3R α ^{hi} cells were immature DC. An overnight culture period was, however, sufficient to induce their maturation and up-regulation of these molecules (Chapter 1 Fig. 2A), and their ability to perform as potent APC in MLR's using allogeneic T cells (Chapter 1 Fig. 2B). Extended culture induced the homogeneous expression of CD1a, a marker used for identification of mature DC (Chapter 2 Fig. 1). These results indicate that the IL-3R α ^{hi} population represents a unique DC type which appears in lymphoid organs as immature cells and can be converted to mature DC in culture.

Examination of other lymphoid organs such as the adult cervical, axillar, intramammary, mesenteric, and femoral lymph nodes resulted in the identification of the IL-3R α ^{hi} DC population indicating a wide distribution of this DC population throughout the lymphoid organs (Chapter 2 Fig. 2). In

addition, another DC population in the adult lymph nodes and tonsils was found and is distinguished from the IL-3R α^{hi} DC by the differential expression of the molecule CD11c. The CD11c $^{+}$ DC population also appears as a subset of the HLA-DR $^{+}$ CD4 $^{+}$ lin $^{-}$ cells constituting the remaining 15% of this population. These two DC populations had distinct phenotypic profiles. The IL-3R α^{hi} cells had high expression of the leukocyte antigen isoform, CD45RA, but were negative for CD45RO while the CD11c $^{+}$ DC were CD45RO $^{+}$ CD45RA $^{\text{lo}}$. The CD11c $^{+}$ DC population also expressed higher levels of HLA-DR, CD13 and CD33 (Chapter 2 Table 1). The CD11c $^{+}$ DC were more potent T cell stimulators than the IL-3R α^{hi} DC, yet they also required overnight culture to become functionally mature. The immuno-phenotypic profile of the IL-3R α^{hi} DC suggested that they were identical to the tonsillar CD11c $^{-}$ DC previously described (28, 29). These results demonstrate the presence of two distinct immature DC populations in lymphoid organs; IL-3R α^{hi} DC and CD11c $^{+}$ DC. The two populations have different phenotypic profiles and evidently migrate to separate areas in tonsils. IL-3R α^{hi} DC are found almost exclusively in the T cell-rich extrafollicular regions of the tonsil (Chapter 1 Fig. 1F) whereas the CD11c $^{+}$ DC were recently shown to home to germinal centers (32). The variations among the two DC populations suggests different functional roles.

Dendritic Cell Migration Pattern

DC present in lymphoid organs arrive by migrating from peripheral tissues. This migration is an important aspect of DC biology because it allows the interaction of DC with T cells in the lymphoid organs. The migratory

process has been predominantly studied using the skin Langerhans cells (33). The present DC migration model explains that foreign antigens captured by the Langerhans cells along with inflammatory stimuli within the skin environment stimulate the migratory process (Chapter 2 Fig. 3). Cytokines such as Interleukin -1 (IL-1), Tumor Necrosis Factor-alpha (TNF- α), and Interferon-gamma (IFN- γ) have been shown to be the principle inflammatory mediators (33-36), which also induce phenotypic changes of the Langerhans cells (4). These changes result in the maturation of Langerhans cells from a mainly antigen-processing in the skin to an APC population once they have entered the lymph node micro-environment (37). The mature cells become capable of triggering T cell activation, due to new or increased expression of co-stimulatory molecules (33).

Under steady-state, non-inflammatory, conditions Langerhans cells have been described to reside in the skin for long periods (>9 weeks) where they experience very slow turnover rates (27). However, in response to inflammatory stimuli Langerhans cells leave the skin environment and mature cells can be detected in draining lymph nodes within 24 hours after mobilization (38). The method of skin painting with fluorescent haptens for tracing Langerhans cell migration, has shown that the migratory process is initiated very rapidly after antigen application and also ceases very rapidly (37, 39, 40). These results indicate that Langerhans cells undergo a rapid turnover state when stimulated by inflammatory mediators. Therefore, according to the current model of DC migration, foreign antigen and inflammatory stimuli mediate the rapid mobilization and maturation of Langerhans cells from antigen-processing to APC upon arrival in lymphoid

organs. This DC migration model has been generalized to relate to all DC types.

Whereas Langerhans cells can reside in skin for long periods, DC populations in mucosal organs have been shown to undergo rapid turnover (2-7 days) (41, 42). This difference has been suggested to be due to the frequency of antigen exposure in the various sites. Langerhans cell exposure to "immunizing" dosages of antigen not previously encountered may be low. However, DC populations in mucosal tissues, such as the respiratory and gastrointestinal tracts, are almost in continuous contact with incoming antigen (41). This constant interaction provides a continuous stimulus for the mucosal DC to take up antigen and migrate to regional lymph nodes. The rapid turnover of the mucosal DC populations could be due to the frequent migration of mucosal DC.

Rapid DC turnover also suggests that DC in peripheral reserves must be continuously renewed by bone marrow precursors (42). It has been shown that DC in peripheral blood, which are presumably derived from bone marrow precursors, migrate to lymphoid organs in approximately 24 hours (42). The fast DC turnover has been described by Holt et. al. to occur during steady-state conditions (41), indicating that DC are capable of migration in the absence of inflammatory triggers. Since inflammatory stimuli mediate DC maturation along with migration, then hypothetically, DC that migrate independent of these stimuli may remain immature and do not up-regulate their co-stimulatory molecules. It is hypothesized that in the absence of high expression of co-stimulatory molecules such as CD80 on DC, tolerance of T cells instead of immunity is induced (43). It might therefore be speculated

that DC are able to migrate to lymphoid organs during steady-state conditions, where under these circumstances do not induce immunity due to absence of inflammatory triggers, but rather impart a tolerogenic T cell response.

The discovery of the IL-3R α^{hi} DC with an immature morphology and phenotype in lymphoid tissues suggested that this DC population migrates in the absence of inflammation. Fetal lymph nodes, which presumably drain sterile, non-inflamed tissue, were tested to determine whether the IL-3R α^{hi} DC do, in fact, migrate independent of inflammatory stimuli. The results showed the presence of large frequencies of the IL-3R α^{hi} DC population in the fetal lymph nodes possessing phenotypic characteristics similar to the cells in tonsils and adult lymph nodes (Chapter 2 Fig. 4).

These findings indicate that DC migration may also occur by a pathway other than the currently accepted model. Therefore, an alternative DC migration pattern may exist (Chapter 2 Fig. 5). According to this model, precursor DC present in peripheral tissues migrate to lymphoid organs by "default", where no mediators such as antigen load and inflammatory stimuli are necessary to trigger the mobilization of DC out of their resident tissues. Migration seems to occur habitually during steady-state conditions where the turnover rate is high, enabling a constant flux of DC to lymphoid tissues. The migrating precursor DC do not up-regulate their co-stimulatory molecules in the absence of inflammatory stimuli, and therefore remain immature throughout their migration. When interacting with T cells in lymphoid tissues, the absence of DC co-stimulatory signals thus potentially induces T cell tolerance. This model may provide an explanation for the well established route of tolerance induction through orally administered antigen.

There is a continuous intake of antigen through the respiratory (airborne) and gastrointestinal tracts (food and liquid). The failure of DC that acquire such antigens in inducing T cell responses possibly prevents constant immune reactions due to frequent antigen exposure. DC which migrate through this process are, however, capable of becoming functionally mature and can induce T cell proliferation as is demonstrated by MLR results of the cultured IL-3R α^{hi} DC (Chapter 1 Fig. 2B). This may indicate that they are capable of inducing an immune response if exposed to an inflammatory stimulus coupled with antigen encounter.

Dendritic Cells in Peripheral Blood

It is presumed that DC in peripheral blood (PB) are intermediate stages of bone marrow progenitor cells (44). Early descriptions of DC generation from proliferating progenitors were obtained using PB (45), which is more accessible than bone marrow. The characterization of PBDC has been complicated due to the lack of DC-specific markers. In addition, DC precursors are a rare population in blood (<1%), and isolation of sufficient numbers of these cells has been difficult. As an alternative to studying these more differentiated DC populations, investigators have generated DC from blood CD34 $^{+}$ progenitors by inducing differentiation using cytokines such as Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF) and IL-4 (46, 47). The disadvantage of these procedures is the lack of correlation to populations that have differentiated to DC *in vivo*.

Recently, direct PBDC characterization has been performed where DC subsets were identified by their differential expression of CD33 (bright and

dim), a myeloid marker (48). O'Doherty et. al. also characterized two subsets of blood DC: CD11c⁺CD45RO⁺ which they described as being activated, mature DC, and CD11c⁻CD45RA⁺ DC which were hypothesized to be bone marrow precursors (49). However, the various differentiation markers used to identify these subsets has made it difficult to compare the results.

A cell-surface marker, CD83, was recently described to be specifically expressed on PBDC (50). This marker was also used for DC identification to demonstrate that CD14⁺ monocytes can also be induced by the cytokines GM-CSF, IL-4 (51), and TNF- α (44) to differentiate into mature DC. However, CD83 has only been shown to appear on cells after culture, and therefore is not a valuable marker for identification of fresh DC. This marker is not present on freshly sorted IL-3R α ^{hi} DC and CD11c⁺ DC.

There is a natural inclination to relate PBDC to DC in lymphoid organs, since DC migrating to lymphoid organs are from various sites which have been seeded by bone marrow-derived DC following passage through blood. The migration from the bone marrow to the peripheral tissues is most likely through blood. This has not yet been demonstrated since no marker has been available which could identify DC in various tissues and establish a lineage relationship between them. Following the identification of immature IL-3R α ^{hi} DC in lymphoid organs, it was hypothesized that this DC population may also appear in blood. Immuno-phenotyping experiments of blood from human adult donors identified an HLA-DR⁺lin⁻IL-3R α ^{hi} and an HLA-DR⁺lin⁻CD11c⁺ DC subset which constituted $0.47\% \pm 0.14\%$ and $0.69 \pm 0.06\%$ of peripheral blood mononuclear cells (PBMC), respectively (Chapter 2 Fig 6).

The immuno-phenotypic profile of the IL-3R α^{hi} and the CD11c $^{+}$ DC subsets in PB was for the most part similar to the tonsillar IL-3R α^{hi} and CD11c $^{+}$ DC populations (Chapter 2 Table 1). Interestingly, the IL-3R α^{hi} DC expressed the homing molecule L-selectin (CD62L), whereas the tonsillar subset did not. The presence of this molecule on the PBMC subset possibly indicates their state of active migration whereas the need for this homing molecule disappears once the cells arrive at the lymphoid organs. The PBMC populations of both DC subsets also stained positively for myeloid lineage markers such as CD13, CD33, and GM-CSFR suggesting that they are derived from myeloid progenitors.

It has been hypothesized that mature DC reenter the blood from tissues and function to uptake blood-borne pathogens and present them to splenic T cells (48). Pathogen uptake by blood leukocytes is done by the process of phagocytosis. However, most reports describe the lack of Fc γ receptors (Fc γ R) such as CD64 (Fc γ RI), CD32 (Fc γ RII), and CD16 (Fc γ RIII) on DC, which are important phagocytic receptors on phagocytes (52). The IL-3R α^{hi} and CD11c $^{+}$ DC populations were tested for phagocytic capacities, and the CD11c $^{+}$ demonstrated to be more efficient than the IL-3R α^{hi} DC. The CD11c $^{+}$ PBMC subset expressed higher levels of CD64 (Fc γ RI), CD32 (Fc γ RII), and CD11b (another molecule involved in the phagocytic process) than the IL-3R α^{hi} DC (Chapter 2 Table 1). This perhaps explains the superior phagocytic capacity of the CD11c $^{+}$ DC over the IL-3R α^{hi} DC. However, both DC subsets had much lower phagocytic capacities relative to macrophages which employ this process during their scavenging activities (Chapter 2 Fig. 7). In comparison, the tonsillar CD11c $^{+}$ DC were negative for CD64, CD16, and CD11b. The

phagocytic functions probably diminish during the DC migration to the lymphoid organs where antigen capture and processing capacities decrease and the DC convert primarily into APC.

Development of DC in culture has been described to be dependent on exogenous cytokines such as GM-CSF (45), TNF- α , and IL-4 (46). IL-4 suppresses monocyte growth, thus the cultures are enriched for DC (46). The IL-3R α^{hi} PBDC were sorted and cultured in various cytokine combinations to determine the optimal mix for their development in culture. The cells formed characteristic aggregates when the cultures were supplemented with GM-CSF only (Chapter 2 Fig 8). Addition of TNF- α which has been described to induce the DC maturation (13), did not enhance this effect of the IL-3R α^{hi} DC in culture.

Another population is present in PB expressing high levels of the IL-3R α . Morphological and immuno-phenotypical analysis of this population demonstrated that these cells were basophils, possessing darkly stained intracellular granules, expressing IgE receptors and staining negatively for HLA-DR and CD4. This population can thus be easily separated from the IL-3R α^{hi} DC which expresses high levels of HLA-DR and CD4 (Chapter 2 Fig. 9).

Following culture IL-3R α^{hi} and the CD11c $^{+}$ DC populations were active stimulators of CD4 $^{+}$ T cells in the MLR and more potent than CD14 $^{\text{hi}}$ monocytes from the same donor indicating that they can differentiate into functionally mature DC (Chapter 2 Fig 10). The characterization of the IL-3R α^{hi} DC compares to the CD11c $^{-}$ DC and CD33 $^{\text{dim}}$ CD14 $^{-}$ CD16 $^{-}$ DC in published reports (48, 49). However, while O'Doherty et. al. hypothesized that the "CD11c $^{-}$ " DC could differentiate into the mature CD11c $^{+}$ DC, we did

not find this to be the case. The two populations (IL-3R α^{hi} and CD11c $^{+}$) seem to be distinct since they have not demonstrated any inter-convertibility. Neither population expressed the marker for the other population at any point during their differentiation in culture. Furthermore, the two DC populations exhibited a functional difference in their phagocytic capacities where the CD11c $^{+}$ DC demonstrated more efficient phagocytosis than the IL-3R α^{hi} DC (Chapter 2 Fig. 7). Finally, the two DC populations home to different regions in the lymphoid organs as was demonstrated by Grouard et. al. suggesting different roles for the DC populations as immune regulators. Therefore, the published results suggesting a connection between the two DC populations are questionable.

Altogether, the results from PBMC analysis indicate that the IL-3R α^{hi} and the CD11c $^{+}$ DC populations initially detected in the lymphoid organs also appear in PB. A lineage relationship has been established between the lymphoid and PBDC populations since the corresponding DC populations in the tissues display similar morphological, phenotypical, and functional characteristics. The immuno-phenotypic profile of the PBDC populations also suggests that they are both derived from the myeloid lineage of bone marrow progenitor cells which may have recently left the bone marrow and entered the circulation.

Dendritic Cells in the Bone Marrow

DC have a bone marrow (BM) origin, however, their developmental pathway is still unclear. Presently, no BM precursor cells have been identified which are exclusively committed to DC development. Attempts to define DC

precursors have been through studying their differentiation from bulk CD34⁺ BM progenitor cells. Caux et. al. demonstrated that DC can be generated from CD34⁺ BM cells when induced by GM-CSF and TNF- α (53). The DC generated expressed CD1a, and were described to be of the Langerhans type since they acquired Birbeck granules. However, the DC constituted only a fraction of the cell populations generated. Progenitors for all hematopoietic cells exist among CD34⁺ cells, and other populations such as granulocytes and monocytes expand along with the DC under the influence of the exogenous cytokines (22). Therefore, while DC were shown to be generated from bone marrow cells, their particular subset was not identified.

Since a DC progenitor has not been isolated, the lineage relationship of DC with other hematopoietic cells remains unclear. Some studies describe that DC are lymphoid-derived and share a common progenitor population with T cells, B cells, and natural killer cells (15-17). Conflicting reports indicate that DC stem from a myeloid origin (26). Investigators have demonstrated that DC exhibit typical myeloid-derived characteristics; they lack lymphocyte antigen receptors, and do not rearrange Ig or T cell receptors (54). DC also exhibit functional behaviors of myeloid cells since they express high levels of MHC class I and II molecules, can endocytose protein, and prime T cells (14). DC are also responsive to GM-CSF, which provides further support to their possible derivation from a myeloid lineage (13).

Recently, Young et. al. reported the existence of a separate DC colony-forming unit (CFU-DC) that gives rise to pure DC colonies (20). The investigators observed that GM-CSF alone induced the formation of granulomonocytic colonies from CD34⁺ cells. The addition of TNF- α stimulated the

generation of additional DC colonies without affecting the number of the granulo-monocytic colonies. This study supported the view of the existence of a separate DC lineage. However, the subset of CD34⁺ cells containing these DC-committed progenitors was not identified to further define the putative DC lineage and possible DC differentiation pathway.

Identification of the IL-3R α ^{hi} DC in PB prompted an investigation for their possible bone marrow progenitors. Flow cytometric analysis of fetal BM cells identified a population of CD34⁺IL-3R α ^{hi} cells with similar immunophenotypic characteristics to the IL-3R α ^{hi} DC population present in PB and lymphoid tissues. The BM CD34⁺IL-3R α ^{hi} cells expressed the molecules HLA-DR, CD4, and CD45RA when freshly isolated and maintained their expression following an overnight culture (Chapter 2 Table 1) and (Chapter 2 Fig. 11). Overnight incubation was sufficient for their *in vitro* maturation and up-regulation of co-stimulatory molecules such as CD86. The BM CD34⁺IL-3R α ^{hi} developed into functionally potent DC and induced a stronger T cell proliferation than CD14^{hi} monocytes generated from BM CD34⁺ cells. Functional APC were generated following a 3-day growth of the two populations in medium supplemented with growth factors such as GM-CSF for the IL-3R α ^{hi} DC and M-CSF for supportive growth of monocytes (Chapter 1 Fig. 4I).

To determine if the BM IL-3R α ^{hi} population is identical to the TNF- α -dependent DC population described by Caux et. al. (13, 17, 21, 53, 55), sorted CD34⁺IL-3R α ^{hi} cells were cultured in medium supplemented with GM-CSF and TNF- α or GM-CSF and IL-3. Freshly sorted cells seemed to be actively proliferating, since mitotic cells were frequently observed (Chapter 1

Fig. 4C). After a 5-day culture period, the cells from both medium cultures expressed CD1a, indicative of mature DC, and CD45RA, a molecule not present on Langerhans cells (56). These results demonstrated that the BM CD34⁺IL-3R α ^{hi} cells are progenitors to the IL-3R α ^{hi} DC. In addition, this population did not rely on TNF- α for differentiation into mature DC since mature DC were able to be generated from the cultures supplemented with GM-CSF and IL-3 (Chapter 1 Fig. 4 A,B,D,E). This indicates that the BM CD34⁺IL-3R α ^{hi} population is a separate DC population than the one described by Caux et. al.

A subset of the CD34⁺IL-3R α ^{hi} cells also expressed the Macrophage-CSF receptor (M-CSFR), a molecule which appears on progenitor cells that differentiate along the granulo-monocytic pathway (57) (Chapter 1 Fig. 5A). Olweus et. al. described that the differentiation of monocytic and granulocytic cells along the hematopoietic granulo-monocytic lineage passes through a stage where the precursor cells are CD34^{hi}M-CSFR^{hi}. They noted that during differentiation a subset of cells which down-modulated their M-CSFR developed into granulocytic cells while the cells which maintained high M-CSFR expression developed into monocytic cells (57). The CD34⁺IL-3R α ^{hi} cells expressed heterogeneous levels of the M-CSFR (Chapter 1 Fig. 5A). It was therefore hypothesized that the IL-3R α ^{hi} DC possibly follow the same differentiation pathway as the monocytic and granulocytic cells. To determine this, CD34^{hi}M-CSFR^{hi} cells were sorted and cultured in medium supplemented with a cocktail of cytokines including stem cell factor (SCF), Granulocyte-CSF (G-CSF), GM-CSF, IL-3, and IL-6 to allow differentiation of all subsets within this population. After a 60 hour culture period, the subset

of cells which had down-modulated their M-CSFR were analyzed for IL-3R α expression and further divided into cells which were IL-3R α^{hi} or IL-3R α^{lo} (Chapter 1 Fig. B). These two subsets of IL-3R α cells were sorted and cultured for five days in medium supplemented with GM-CSF and IL-3. The IL-3R α^{hi} cells developed into CD1a $^{+}$ CD15 $^{-}$ exhibiting dendritic morphology whereas the IL-3R α^{lo} cells developed into CD15 $^{+}$ CD1a $^{-}$, indicative of granulocytic cells (Chapter 1 Fig. C-D).

The identification of the differentiation of the IL-3R α^{hi} DC prompted an investigation search for the TNF- α dependent Langerhans cell precursors. IL-3R α^{hi} M-CSFR $^{\text{lo}}$ and IL-3R α^{lo} M-CSFR $^{\text{hi}}$ cells were sorted following the initial sort of the M-CSFR $^{\text{hi}}$ cells (Chapter 1 Fig. A-B) and cultured in medium supplemented with GM-CSF and TNF- α . The IL-3R α^{hi} M-CSFR $^{\text{lo}}$ developed into the IL-3R α^{hi} -derived DC as expected but interestingly the IL-3R α^{lo} M-CSFR $^{\text{hi}}$ developed into the Langerhans-type DC. These two DC populations were distinguishable by Transmission electron microscope analysis revealed the presence of Birbeck granules within the cytoplasm of the DC derived from the IL-3R α^{lo} M-CSFR $^{\text{hi}}$ precursors whereas the IL-3R α^{hi} -derived DC lacked those granules (Chapter 1 Fig. 4 H)

These results demonstrate the existence of a DC-specific lineage whose precursor population is a distinct subset of CD34 $^{+}$ bone marrow cells (CD34 $^{+}$ M-CSFR $^{\text{lo}}$ IL-3R α^{hi}). This subset is of myeloid origin and differentiates along the granulo-monocytic pathway and delineates sequentially from the monocytic and granulocytic cells. The progenitor cells within this subset are terminally differentiated to DC which were identified in peripheral blood and lymphoid organs. Differentiation of the Langerhans

cells seems to follow the monocytic pathway and is distinct from the CD34⁺IL-3R α ^{hi} cells.

Dendritic Cells in Thymus

Thymic DC have may be involved in the deletion of self-reactive T lymphocytes (15). A population of thymic cells being CD34⁺CD45RA⁺lin⁻ and which expressed high levels of HLA-DR were described to give rise to T, B, natural killer (NK) cells, and DC (16, 17). Due to the presence of DC in the thymus, thymic cells were examined for the presence of IL-3R α ^{hi} DC. Immuno-phenotyping of thymic mononuclear cells identified the IL-3R α ^{hi}HLA-DR⁺lin⁻ cells. However, this subset represented less than 0.1% of the total cell population, and unlike what was observed in the lymphoid organs, constituted only 15% of the HLA-DR⁺lin⁻ cells in the thymus (Chapter 2 Fig. 12 A). This indicates that other DC populations are predominant in the thymus and possibly have a more pronounced role than the IL-3R α ^{hi} DC.

Although all DC are ultimately of bone marrow origin, the previously described thymic DC (16, 17) and the IL-3R α ^{hi} DC seem to follow separate differentiation pathways. Since multipotent precursors to T, B, NK cells and DC were identified in the thymus, it seems likely that this progenitor population is generated in the bone marrow but migrates to the thymus while still multipotent. In contrast, CD34⁺IL-3R α ^{hi} progenitors become committed to the DC lineage in the BM before they enter the circulation.

Analysis of the expression of CD34 on IL-3R α ^{hi} DC present in the thymus supported the idea that these cells migrated from the BM rather than developed from more immature progenitors in the thymus. The CD34 molecule is expressed on progenitor cells and is down-regulated during cellular differentiation. The thymic IL-3R α ^{hi} DC had lower levels of CD34 than their bone marrow counterparts indicating that they were further along in their differentiation (Chapter 2 Fig. 12 B,C).

The IL-3R α ^{hi} DC and the lymphoid-derived DC may also have distinctive roles in self-reactive T cell deletion. The DC which are generated from the lymphoid progenitor populations which also give rise to T, B, and NK cells may function to recognize T cells that react with "self" surface molecules. Therefore, the T cell clones which react with MHC molecules expressed on the lymphoid-derived DC may be deleted. Whereas the myeloid-derived IL-3R α ^{hi} DC may function to present "self" antigens to T cells in order to identify the T cell clones which react with antigen/MHC complexes on the IL-3R α ^{hi} DC. Further characterization of the thymic DC populations is necessary to define their possible roles in T cell selection and other thymic functions.

Future Projects

DC are the professional antigen-presenting cells that are most efficient in activating T cells and thus play a crucial role in the immune system. DC are the least abundant population of leukocytes in blood, and the lack of a DC-specific marker has presented difficulties in their isolation in sufficient

numbers for experimentation. The identification of a subset of DC that expresses high levels of the IL-3R α has provided a tool for the quantitation as well as the isolation of pure DC to enable further characterization and understanding of their role in the immune system. Projects to further characterize DC would be:

1) *The origin of the CD11c⁺ DC*

This study did not characterize the CD11c⁺ DC in bone marrow. Further studies should be aimed at characterizing their differentiation pathway, their lineage origin (myeloid or lymphoid) and their relationship with the IL-3R α ^{hi} DC, Langerhans DC, and monocytes.

2) *DC quantitation of patients with immuno-compromising diseases*

The HIV-1 virus infects the immune system and causes critical loss of CD4⁺ T cells. DC have also been shown to be infected with HIV-1 and can be involved both in the transmission of the infection to T cells and in T cell priming to HIV antigens (58). The IL-3R α and CD11c markers can be used as effective tools for DC quantitation. Presently, AIDS patients are monitored only for their T cells counts. The availability of DC markers will allow the simultaneous quantitation of T cells and DC. The addition of DC counts as a parameter for monitoring the progression of AIDS may offer valuable information on the effects of the HIV virus on DC and vice versa.

3) *Investigation into DC activation of T cell subsets.*

Stimulation of CD4⁺ T helper cells (T_H) induces two types of responses; T_H1 or T_H2. CD4⁺T_H cells which exhibit T_H1 responses secrete IL-2, IFN- γ , and

TNF- α , whereas the Th2 subsets secrete IL-4, IL-5, IL-6, IL-10, and IL-13 upon stimulation (10). In addition, it has been shown that DC can initiate CD8⁺ T cytotoxic cell responses (59, 60). It is, however, not known what role DC play in inducing Th1 versus Th2 responses. Moreover, since the DC system is constituted of multiple cell types (IL-3R α ^{hi} DC, CD11c⁺ DC, Langerhans DC) it is not known which DC populations initiate different T cell responses. Therefore, to further understand the dynamics of the DC system, it is relevant to investigate their roles in initiating immune responses and more specifically their interaction with T cell subsets.

4) *Identification of the IL-3R α ^{hi} DC in mouse models*

The IL-3R α ^{hi} DC have not been identified in any mouse models. Animal models permit more detailed *in vivo* studies which cannot be performed in humans. Therefore, the possible presence IL-3R α ^{hi} DC subset in mice would expand the experimental capacities for understanding IL-3R α ^{hi} DC.

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Figure 1. Human tonsillar mononuclear cells were immuno-magnetically enriched for HLA-DR⁺CD4⁺lin⁻ cells and FACS-sorted for the IL-3R α ^{hi} DC. Sorted cells were cultured in culture medium supplemented with GM-CSF and IL-3. Seven-day cultured cells were stained with CD1a, a marker used to assess for mature cell development including DC and macrophages. CD14 appears on mature macrophages but not mature DC. Stained samples were analyzed by four-color flow cytometry using CELLQuest[®] software for data acquisition and PAINT-A-GATE[®] software for data analysis. The above panels show approximately 8,000-10,000 events. Panel (A) displays the forward scatter (FSC) and orthogonal light scatter (OLS) properties of live-gated cells. The blue events are indicative of the CD1a⁺CD14⁻ DC as shown in panel (B) and constitute 98% of live cells. Panel (C) shows the positive HLA-DR expression of both CD1a⁺ DC and CD14⁺ macrophages. Dashed lines indicate isotype control levels.

Figure 2. Flow cytometric analysis of mononuclear cells in adult lymph nodes. The IL-3Rα^{hi}HLA-DR⁺ population (R1) have similar immunophenotypic characteristics to the human tonsillar IL-3Rα^{hi}HLA-DR⁺ DC. This demonstrates a widespread distribution of the IL-3Rα^{hi} DC among lymphoid tissues. This population constitutes 0.3-0.5% of the lymph node mononuclear cells.

Figure 3. DC differentiation and migration model based on the Dendritic Langerhans cells. DC derive from bone marrow progenitor populations which enter the circulation as precursor cells and seed peripheral tissues such as skin, gut, lungs, kidneys, and the heart as immature DC (round cells). According to this model, DC remain in the tissues until they are stimulated by foreign antigen, such as infectious microorganisms, and also inflammatory stimuli such as TNF- α and IFN- γ released by neighboring cells in response to infections. These events activate the immature DC precursors to begin a maturation process. DC activation involves the development of dendritic protrusions, effective uptake and process of foreign antigen, and migration to neighboring lymph nodes. During migration, DC convert from antigen-processing cells to antigen-presenting cells. The activated DC drain through the afferent lymphatics and migrate to T cell-rich areas in the lymph nodes where they present the foreign antigen to naive T cells to induce an immune response. It is presumed that DC do not re-circulate since they have not been observed in the efferent lymphatics. Therefore, the lymph nodes represent the end-organ for DC migration.

Figure 4. Flow cytometric analysis of IL-3R α^{hi} DC in fetal lymph nodes. Fetal lymph node mononuclear cells were stained with antibodies against IL-3R α , HLA-DR, and a cocktail of lineage markers including CD3, CD14, CD16, CD19, CD20, CD56, and goat-anti-human IgM ("lin"). The cells were analyzed by flow cytometry as described in Fig. 1. **(A)** Forward Light Scatter (FSC) and Orthogonal Light Scatter (OLS) of live cells. **(B)** The IL-3R α^{hi} DC present in fetal lymph nodes are indicated by the green population. The DC express HLA-DR also shown in **(C)** but are negative for the lineage markers. Approximately 30,000 events were acquired. Dashed lines are indicative of isotype control levels.

Figure 5. An alternative model for DC migration. This model suggests that DC migration to lymph nodes occurs in the presence or absence of inflammatory mediators. DC in peripheral tissues which are triggered by inflammatory mediators such as $\text{TNF-}\alpha$ and $\text{INF-}\gamma$ and foreign antigen migrate to lymphoid organs as activated mature DC. The DC cluster naive T cells and induce an effective antigen-specific immune response. However, DC migration also occurs during steady-state conditions where no inflammatory mediators are present. DC precursors in peripheral tissues take up foreign or self antigen and begin their migration. The DC precursors remain immature and do not up-regulate co-stimulatory molecules such as CD80 and CD86. Therefore, when interacting with T cells, the necessary co-stimulatory signals are not available to induce an immune response and the T cells thus become tolerized to the antigen presented.

Figure 6. Flow cytometric analysis of IL-3Rα^{hi} and CD11c⁺ DC populations in peripheral blood. Mononuclear cells from peripheral blood were stained with antibodies against IL-3Rα, CD11c, HLA-DR PerCP, and a cocktail of lineage markers (CD3, CD14, CD19, CD20, CD56, and human IgM) termed "lin" and analyzed by flow cytometry as described in Fig. 1. Both DC populations are distinguishable by surface phenotyping. (A) The IL-3Rα^{hi} DC are negative for the CD11c antigen and the CD11c⁺ DC express low levels of the IL-3Rα antigen. (B) Both DC populations are within a subset of mononuclear cells which express HLA-DR and are negative for lineage markers. The CD11c⁺ DC express higher levels of HLA-DR and (C) have slightly higher orthogonal (OLS) scatter but similar forward scatter (FSC) properties than the IL-3Rα^{hi} DC. The panels are gated on live cells and include approximately 15,000 events. Dashed lines are indicative of isotype control levels.

Figure 7. Measurement of phagocytosis by macrophages, and CD11c⁺ and IL-3Rα^{hi} DC. Peripheral blood mononuclear cells were depleted of CD14⁺ macrophages via immuno-magnetic separation and stained with anti-CD11c, anti-IL-3Rα, anti-HLA-DR (not shown), and anti-"lin" (CD3, CD14, CD19, CD20, CD56, and goat-anti-human IgM cocktail). The CD11c⁺ and IL-3Rα^{hi} DC were FACS-sorted and along with the initially depleted CD14⁺ macrophages were cultured separately for 5 days in medium supplemented with GM-CSF (for DC) or M-CSF (for macrophages). Cultured cells were harvested and incubated with fluorescein isothiocyanate (FITC)-labeled β-glucan particles for four hours to assay for the phagocytic capacity of the cells. Cells were washed and analyzed by flow cytometry for ingestion of particles. All panels display the type of cells and the percentages of those cells which have ingested at least one fluorescinated particle. **(A)** the majority (88%) of the macrophages ingested particles. The blue, red, and green events indicate cells which ingested one, two, or three particles respectively. **(B)** In comparison, less than one half (44%) of the CD11c⁺ DC ingested particles and **(C)** only 2% of the IL-3Rα^{hi} DC ingested particles.

Figure 8. *In vitro* culture appearance of peripheral blood IL-3R α^{hi} DC. Peripheral blood mononuclear cells were stained with anti-IL-3R α , anti-CD4, and anti-lineage markers and FACS-sorted for IL-3R α^{hi} CD4 $^{+}$ lin $^{-}$ cells. Sorted cells were cultured in medium supplemented with GM-CSF. The cells were photographed following a 24-hour culture period. The DC begin aggregation immediately and form clusters in culture as indicated in the photograph (magnification, 400X).

Figure 9. Flow cytometric analysis of peripheral blood mononuclear cells. The IL-3R α molecule is a valuable marker for identifying a subset of DC (IL-3R α ^{hi} DC). The expression of this marker aids in the identification and enrichment of this DC subset from mononuclear cells. Basophilic granulocytes also express high levels of the IL-3R α molecule and have similar orthogonal light scatter (OLS) and forward light scatter (FSC) to the IL-3R α ^{hi} DC as shown in (A). The two cell types can, however, be immunophenotypically distinguished by the use of other markers such as CD4, expressed highly on the IL-3R α ^{hi} DC but not on basophilic granulocytes (not shown). (B) The IL-3R α ^{hi} DC also express HLA-DR (red events), however, basophilic granulocytes are negative for this molecule (blue events). The use of such markers in addition to the IL-3R α molecule is useful for identification and isolation of the IL-3R α ^{hi} DC. The events are gated on live cells where approximately 30,000 cells were acquired. The dashed lines indicate isotype control levels.

Figure 10. T cell stimulatory capacity of the IL-3Rα^{hi} DC, CD11c⁺ DC, and CD14^{hi} monocytes isolated from the same donor and tested in the mixed leukocyte reaction (MLR) to assay the stimulatory capacity of T cells by these "stimulator cells". CD3⁺CD4⁺ T cells (10⁵) were co-cultured with varying numbers of allogeneic stimulator cells. To measure T cell proliferation, bromodeoxyuridine (BrdU), which incorporates into DNA specifically during the S-phase of the cell cycle, was added to the cultures. T cell proliferation was measured as the number of CD3⁺BrdU⁺ cells. The degree of T cell proliferation is correlated to the potency of the stimulator cells. The CD11c⁺ DC (■) were more potent than the IL-3Rα^{hi} DC (●) at lower numbers but equal at higher numbers. However, both DC population were more potent than monocytes (◇).

Figure 11. Flow cytometric analysis of fetal bone marrow CD34⁺ cells. Mononuclear cells were incubated overnight in serum-free medium to allow up-regulation of surface markers. **(A)** Incubated cells were stained and gated on CD34⁺ cells with low orthogonal light scatter (OLS) as indicated by the dark events. Panels **(B-I)** show the staining of various markers against IL-3Rα on OLS^{lo}CD34⁺ gated cells. The cluster of cells which is identified in the red boxes are indicative of the IL-3Rα^{hi} DC. Dashed lines indicate isotype control levels. The IL-3Rα^{hi} populations which appear above the isotype control levels on the panel are considered to be also positive for the particular markers in each panel. This panel demonstrates that a majority of the IL-3Rα^{hi} DC express HLA-DR, CD4, CD13, CD45RA, and CD86, are negative for CD64, and CD11c and form a continuum of M-CSFR⁺ and M-CSFR⁻ cells.

Figure 12. Flow cytometric analysis of IL-3Rα^{hi} DC in fetal thymus. Fetal thymic mononuclear cells were stained with anti-IL-3Rα, anti-HLA-DR, and anti-lineage markers (not shown) and FACS-analyzed as described in Fig. 1. **(A)** A population of HLA-DR⁺lin⁻IL-3Rα^{hi} cells was identified (R1), indicating the presence of the IL-3Rα^{hi} DC in the fetal thymus. However, this population represented a minority (15%) of the HLA-DR⁺lin⁻ population. The thymic IL-3Rα^{hi} DC may originate in the bone marrow and migrate to the thymus as more differentiated cells than the IL-3Rα^{hi} DC in the bone marrow. This is suggested by the relatively higher density of CD34 on fetal bone marrow IL-3Rα^{hi} DC **(B)** as compared with the fetal thymus IL-3Rα^{hi} DC **(C)** from the same donor.

	Tonsil		Fetal Lymph node		PBMC		Fetal BM
	IL-3R α^{hi}	CD11c $^+$	IL-3R α^{hi}	CD11c $^+$	IL-3R α^{hi}	CD11c $^+$	IL-3R α^{hi}
CD1a	-	-	-	-	-	-	-
CD3	-	-	-	-	-	-	-
CD4	++/+++	++/+++	++/+++	+++	++	++	++
CD5	-	+ / ++	-	+	-	+(+)	-
CD11b	-	-	-	++	-	+ / ++	-
CD11c	-	+++	-	+++	-	+++	-
CD13	- / +	++/+++	+	++	- / +	+++	+
CD14	-	-	-	-	-	-	-
CD15	-	-	n.d.	n.d.	-	-	-
CD16	-	-	-	-	-	-	-
CD19	-	-	-	n.d.	-	-	-
CD20	-	-	-	-	-	-	-
CD32	+	n.d.	+	+	+	++	+
CD33	+	++	+(+)	++	+(+)	+++	+ / ++
CD34	- / +	- / +	+	+	- / +	- / +	++/+++
CD36	++/+++*	+ / ++	++/+++	++/+++	++/+++	++	++/+++
CD40	++	+++	+	+ / ++	++	n.d.	+
CD45RA	++(+)	-	++(+)	+	++(+)	+	++
CD45RO	-	+ / ++	-	+++	-	n.d.	-
CD54	++/+++	+++	n.d.	(+) +	++/+++	n.d.	+ / ++
CD56	-	n.d.	-	n.d.	-	n.d.	-
CD58	+	n.d.	n.d.	n.d.	+ / ++	n.d.	+ / ++
CD62L	-	-	++	+	+ / ++	n.d.	+++
CD64	-	-	-	+	-	++	-
CD80	-	+	-	-	-	-	-
CD83	-	-	n.d.	n.d.	-	-	n.d.
CD86	+	n.d.	n.d.	n.d.	+	n.d.	+(+)
IL-3R α	+++	+	++++	+	+++	+	+++
HLA-DR	++	++/+++	++	++/+++	++	n.d.	++
HLA-DQ	-	n.d.	n.d.	n.d.	- / +	n.d.	-
GM-CSFR	++	++/+++	n.d.	n.d.	++	++	n.d.

Table 1. Expression of surface molecules on HLA-DR $^+$ lin $^-$ IL-3R α^{hi} DC and HLA-DR $^+$ lin $^-$ CD11c $^+$ DC on freshly isolated human mononuclear cells from tonsils, fetal lymph node cells, peripheral blood mononuclear cells (PBMC), and fetal bone marrow (BM). Mean fluorescence intensity (MFI) levels for the DC populations is expressed as "-" indicating MFI in the first decade (of four), which corresponds to isotype control levels. The "+", "++", and "+++" indicate MFI's in the second, third, and fourth decades, respectively. /= MFI is on the border between two decades. ()= MFI is in the upper end of the lower decade. * In one out of three experiments, tonsillar HLA-DR $^+$ lin $^-$ IL-3R α^{hi} DC were negative for CD36. n.d. indicates not determined.

IMAGE EVALUATION TEST TARGET (QA-3)

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