Development and validation of an *in vitro* model of dendritic cell identification and activation

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Declaration

I, the undersigned hereby declare that the work contained in this dissertation is my own original work and that I have not previously, in its entirety or in part, submitted it at any university for a degree.

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Summary

The aim of this study was to investigate the effect of MBV and Coley's Toxin on dendritic cells *in vitro.* The dendritic cell system of antigen presenting cells is the initiator and modulator of the immune response. The principle function of the dendritic cells is to present antigens to resting naïve T lymphocytes: these cells are the only APCs that prime naïve T cells and only mature DCs can carry out this function.Previous studies done on dendritic cells showed that bacterial peptides can induce the maturation of dendritic cells. With the results of these studies in mind we hypothesized that these two vaccines will also induce the maturation of dendritic cells.

Chapter 1 is a literature review on the immune system explaining the organs and cells of the immune system. Chapter 2 includes a full description of DCs, the MBV and Coley's toxin. Also included in this chapter is a short explanation of the principle of the technique being used for the identification and maturation of both mDCs and pDCs, namely the technique of flow cytometry.

Chapter 3 describes the method for the phenotypic identification of DCs: the subsets are distinguished by their absence of expression of several lineage markers for lymphocytes, monocytes and NK cells and the expression of CD11c (in the case of myeloid DCs) and CD123 (in the case of plasmacytoid DCs). The inclusion of HLA-DR in addition to the previous described markers allows the discrimination of $CD123^{+}$ DCs from basophils. The assay requires three tubes per sample which enables quick analysis of these rare subsets with a small sample volume. This assay was applied to peripheral blood samples obtained from healthy individuals and individuals with cancer, HIV and HIV and TB co-infected

patients. Our results showed that the maturation status of DCs in HIV and lymphoma were low but those measured in the case of HIV + TB patients were even higher than in the control group.

Chapter 4 and 5 describe the *in vitro* activation and maturation status of DCs following their incubation with bacterial-derived products. Interactions between DCs and microbial pathogens are fundamental to the generation of innate and adaptive immune responses and upon contact with bacteria or bacterial components such as lipopolysaccharide (LPS), immature DCs undergo a maturation process that involves expression of costimulatory molecules, HLA molecules, and cytokines and chemokines, thus providing critical signals for lymphocyte development and differentiation. In this study, we investigated the response of human DCs to MBV and Coley's Toxin. Previous studies showed DCs can be activated with killed *Streptococcus pyogenes.* With this study in mind it was hypothesized that the MBV and Coley's Toxin used in this study might modulate DC maturation. The results of this study showed that the MBV and Coley's toxin did induce the maturation of both pDCs and mDCs as measured by increased surface expression of costimulatory molecules such as CD80 and CD83.

Chapter 6 presents the measurement of cytokines released after the PMBCs had been were incubated with Coley's Toxin and Mixed Killed bacteria. The BD™ Cytometric Bead Array (CBA) flex set was used for the simultaneous detection of multiple soluble analytes. The results indicated that both Coley's Toxin and the MBV activated the DCs and subsequently induced TH1 as well as a TH2 responses in the T cells present in the cell cultures.

Finally, a general conclusion discussing the significance and implications of our results as well as possible future research required is discussed in Chapter 7. DCs are potent antigen presenting cells (APCs) which play a critical role in the regulation of the immune response. There is great interest in exploiting DCs to develop immunotherapies for cancer, chronic infections, immunodeficiency diseases and autoimmune diseases.

Opsomming

Die doel van die studie was om die effek van ‘n gemengde bakteriële vaksiene en Coley se toksiene op dendritiese selle te toets *in vitro.* Die dendritiese sel sisteem speel ‘n belangrike rol in die modulering en reaksie van die immuun sisteem.Die hoof funksie van dendritiese selle is om antigene bloot te stel aan naïewe ongeaktiveerde T selle. Slegs volwasse dendritiese selle kan die T selle aktiveer. Vorige studies het bewys dat bakteriële peptiedes die veroudering van die dendritiese selle kan induseer. Met die resultate in gedagte het ons gehipotiseer dat die twee vaksienes ook die maturasie van dendritiese selle kan induseer.

Hoofstuk 1 is ‘n literatuur studie wat handel oor die organe en selle van die immuun sisteem. Hoofstuk 2 gee n volle beskrywing van dendritiese selle, die gemengde bakteriële vaksiene en Coley se toksiene. Ingesluit in die hoofstuk is die beskrywing van die prinsiep van die tegniek, vloei sitometrie, wat gebruik word vir die identifikasie en veroudering status van die dendritiese selle.

Hoofstuk 3 beskryf ‘n vloei sitometrie metode vir die fenotipiese identifikasie van dendritiese selle. Dendritiese sel tipes kan onderskei word deur die afwesigheid van sekere merkers vir limfosiete, monosiete en NK selle. Plasmasitoïede dendritiese selle druk CD123 uit en miloïede dendritiese selle druk CD11c uit. HLA DR is ook ingesluit saam met die bogenoemde merkers om die dendritiese selle te onderskei van basofiele.

Vir elke toets word slegs drie buise geprosesseer en dus kan die subklasse vinning geanaliseer word. ’n Klein volume bloed word benodig vir die toests. Perifêre bloed is gebruik vir die toets op bloed monsters van 10 gesonde individue en individue met kanker,

HIV en HIV en TB. Die resultate van die studie het getoon dat die maturasie status van die dendritiese selle in HIV en limfoom was, maar in die geval van HIV en TB pasïente was die maturasie status selfs hoër as die van die kontrole groep.

Hoofstuk 4+5 beskryf die aktivering en maturasie status van die dendritiese selle na inkubasie met die bakteriële produkte. Interaksie tussen dendritiese selle en patogene speel ‘n belangrike rol in die aktivering van die immuunstelsel. Wanneer dendritiese selle in aanraking kom met bakterieë of bakteriële komponente, matureer die dendritiese sel wat lei tot the uitdrukking van stimulerings molekules, HLA molekules end die uitskeiding van sitokiene. Die uitdrukking van die molekules lei tot limfosiet ontwikkeling en differensiasie. In die studie het ons gekyk na die reaksie van menslike dendritiese selle in die teenwoordigheid van die gemende bakteriële vaksiene en Coley se toksiene. Vorige studies het bewys dendritiese selle word geaktiveer deur *Streptococcus pyogenes.* Met die resultate in gedagte het ons gehipotetiseer dat die gemengde bakteriële vaksiene en Coley se toksiene ook die maturasie van dendritiese selle kan induseer. Die resultate van die studie het bewys dat die gemengde bakteriële vaksiene en Coley se toksiene die veroudering van beide pDCs en mDCs induseer. Die uitdrukking van verouderings merkers CD80 en CD83 is gemeet.

Hoofstuk 6 beskryf ‘n vloei sitometrie metode om die sitokiene te meet wat afgeskei word nadat selle geinkubeer het in die teenwoordigheid van Coley se toksiene en die gemengde bakteriële vaksiene.Die BD^{TM} CBA Flex set metode het dit moontlik gemaak om meer as een sitokiene te meet in net een buis Die resultate het getoon dat albei die vaksienes ‘n TH1 en TH2 reaksie veroorsaak.

Laastens volg‘n algemene afleiding waar ons kyk na die toepassing en implikasies van die resultate asook toekomstige navorsings moontlikhede,word bespreek in Hoofstuk 7 Dendritiese selle speel ‘n kritiese rol in die regulering van die immuun reaksie. Verdere studies kan nou gedoen word om dendritiese selle terapeuties toe te pas vir die behandeling van kanker, autoimmuniteit, immuun onderdrukkende siektes en kroniese siektes.

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Abbreviations

Ags	Antigens
AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen presenting cell
APC	Allophycocyanin
BCG	Bacille Calmette-Guérin
Ca	Calcium
CCR5	Chemokine (C-C motif) receptor 5
CD	Cluster of differentiation
CLMF	Cytotoxic lymphocyte maturation factor
CLR	C-type lectin receptor
CMI	Cell mediated immunity
CTL	Cytotoxic T cell
CTLA 4	Cytotoxic T-lymphocyte-associated protein 4
CXCR4	Chemokine (C-X-C motif) receptor 4
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-grabbing nonintegrin
DEC 205	Dendritic and epithelial cells, 205 kDa
ELISA	Enzyme linked immunosorbent assay
FDC	Follicular Dendritic cells
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GM –CSF	Granulocyte- macrophage colony stimulating factor

$gp120^{SU}$	Surface glycoprotein gp120
$gp41^{TM}$	Transmembrane glycoprotein 41
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HLA-DR	Human leukocyte antigens Class II,DR
ICAM	Intercellular Adhesion Molecule – 1(CD54)
ICOS	Inducible T-cell costimulator
IELs	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IgE	Immunoglobulin E
IL- 1	Interleukin 1
IL -2	Interleukin 2
IL- 4	Interleukin 4
IL- 5	Interleukin 5
IL 6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-15	Interleukin 15
IL-16	Interleukin 16
IL-18	Interleukin 18
ILT	Immunoglobulin-like transcript receptor
LC	Langerhans cell
LFA	Lymphocyte function-associated antigen-1
lin	Lineage

LPS	Lipopolysaccharide
LN	Lymph node
MBV	Mixed killed bacterial vaccine
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MIIC	MHC class II rich compartment
MR	Mannose receptor
MMR	Macrophage Mannose Receptor
mRNA	Messenger ribonucleic acid
MRV	Mixed Respiratory Bacterial Vaccine
NK	Natural killer
NKSF	Natural killer cell stimulatory factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritc cell
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PLC	Phospholipase C
PKC	Protein kinase C
PMA	Phorbal myristate acetate
PMNs	Polymorphonuclear cells
RPMI 1640	Roswell Park Memorial Institute Medium 1640
RNA	Ribonucleic acid
SMAC	Supramolecular activation clusters
SSC	Side scatter

TAAg	Tumor-associated antigens
TAP	Transporters for Antigen Presentation
TDSFs	Tumor-derived soluble factors
TCR	T cell antigen Receptor
TH	T helper
TiDCs	Tumor-associated immature DCs
TiDCs-Cp	TiDCs-captured apoptotic cells
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

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Previous Publications:

A part of this thesis has been presented at the Federation of Infectious Diseases Societies of Southern Africa (FIDSSA) Congress 2007, held 28-31 October 2007 at Spier.

The Abstract for this presentation was published in the following journal:

Clark A, Bouic P (2007): The effects of different bacterial vaccine preparations on *in vitro* dendritic cell activation and maturation. SA J. Epidem. Infec. 22 (2,3):56

Chapter 1

General introduction to the immune system

1.1 Introduction

The human body has natural barriers to prevent entry by microbes, but when these barriers are broken, pathogens can enter the body. The human body provides an ideal environment for many microbes and therefore they try to pass the skin barrier and enter. The immune system is a network of cells, tissues, and organs that have evolved to defend you against such "foreign" invasions. The innate immune system kicks in first and phagocytic white blood cells begin to attack the invading microbes within minutes. The microbes are killed by phagocytosis and other protein components including the complement components which facilitate the process of phagocytosis. Natural killer cells can detect certain virally infected cells and lyse them. The innate immune system is often sufficient to kill and destroy invading microbes. If this system fails to clear the infection then the adaptive or acquired immune response takes over (Janeway CA *et al.* 1996). The connection between the two systems is mediated by cytokines.

At the heart of the immune response is the ability to distinguish between "self" and "non-self". Every cell in the body carries the same set of distinctive surface proteins that distinguish you as "self". Normally the immune cells do not attack the body's own tissues, which all carry the same pattern of self-markers. This set of unique markers on human cells is displayed on the major histocompatibility complex (MHC). There are two classes: MHC Class I proteins, which are present on all cells, and MHC Class II proteins, which are present on certain specialized cells. Any non-self substance capable of triggering an immune response is known as an antigen. An antigen can be a whole non-self cell, a

bacterium, a virus, an MHC marker protein or even a portion of a protein from a foreign organism. The distinctive markers on antigens that trigger an immune response are called epitopes. When tissues or cells from another individual enter your body carrying such antigenic non-self epitopes, your immune cells react. The immune cells recognize epitopes presented on the MHC when they distinguish between self and non-self. An MHC protein serves as a recognizable scaffold that presents pieces of a foreign protein (peptides) to immune cells.

1.2 The arms of the immune system

1.2.1. Innate (or natural) immunity

Kabelitz D *et al.* (2007) explains that this part of the immune system is made up of several components:

- Physical barriers are the first line of defense against infection. The mucous membranes and the skin provide a continuous surface
- Physical or physico - chemical factors such as temperature, pH and oxygen tension limit microbial growth. In the stomach the acid environment combined with microbial normal flora inhibits gut infection
- Invasion is also blocked by protein secretions, like lysozyme. Other factors like complement, interferons and molecules like C – reactive protein are important in protection against infection
- Phagocytic cells are critical in the defense against bacterial and simple eukaryotic pathogens. Macrophages and polymorphonuclear leucocytes recognize bacterial and yeast cell walls through broadly specific receptors and this recognition is greatly enhanced by activated complement (opsonin) (Anderson KV,2000).

Complement

The complement system has two pathways: the classical and the alternative pathway. Both pathways have a similar terminal sequence which creates the membrane attack complex (MAC).This enzyme complex punches a hole in various cell surfaces. Both pathways have by- products namely anaphylatoxins which contribute to an inflammatory response. The complement system consists of a series of about 25 proteins which are normally present in plasma in inactive form and become activated by classical or alternative pathways. The alternative pathway is triggered by a variety of substances, including bacterial polysaccharides.There is no formation of antigen-antibody complexes or the participation of C1, C4 or C2. In the absence of these complexes, there is a spontaneous conversion of C3 to C3b. In normal conditions the C3b binds to inhibitory proteins and sialic acid present on the surface of the body's own cells and the C3b is inactivated. However, bacteria and other foreign materials that may get into the body lack these proteins and have little or no sialic acid. The C3b binds a protein called Factor B forming a complex of C3b•Bb, which is a C3 convertase. The C3 convertase activates more C3. The C3b•Bb•C3b, which is a C5 convertase, start the assembly of the membrane attack complex.

The Classical pathway of the complement system helps to clear the body of antigen-antibody complexes. Complement proteins (only activated C3a, C5a and C4a) cause blood vessels to become dilated and leaky, causing redness and swelling during an inflammatory response. Complement proteins circulate in the blood in an inactive form. The so-called "complement cascade" is set off when the first complement molecule, C1, binds to the antigen-antibody complex. The C4 and C2 are cleaved into C3 convertase. C3 convertase splits C3 into C3a and C3b. C3a is released and increases vascular permeability.C3b forms

a complex which splits C5 into C5a and C5b. C5a increases vascular permeability and is highly chemotactic to polymorphonuclear and mononuclear leucocytes. The end product is a cylindrical complex that punctures the cell membrane and by allowing fluids and molecules to flow in and out, dooms the target cell (Janeway CA *et al*. 1996).

1.2.2. Adaptive immunity:

The first encounter with an antigen is known as the primary response. Re- encounter with the same antigen causes a secondary response that is more rapid and powerful. The difference between the innate and the acquired immune system lies in the antigen specificity of lymphocytes. Lymphocytes express cell surface receptors that recognize discrete parts of the antigen known as antigenic epitopes (Barton GM *et al*. 2002). Adaptive immunity can be divided into two branches, the cellular or cell–mediated immune response and the humoral immune response. These two interconnected immune functions work together through finely tuned checks and balances to mount an appropriate defense.

In response to bacterial invasion, B–cells of the humoral arm proliferate and produce large amounts of appropriate antibodies that flag invaders for elimination from the body. The cellular immune response employs specialized T–cells to recognize and destroy host cells showing signs of cancer or infection by viruses or parasites. The relative mobilization of each branch of the immune system depends on the specific disease or condition, and the nature of the response can be influenced by the pathogen itself and where it enters the body (Abbas *et al*. 2003).

1.3 Organs of the immune system:

1.3.1 Primary lymphoid organs:

Bone Marrow and Thymus:

- Stem cells in bone marrow give rise to cells of all lineages produced by the bone marrow.
- Cell lineages produced by the bone marrow:
    - Cells of innate immunity for example monocytes, macrophages, DCs and granulocytes
    - Antigen specific cells or acquired immunity for example T cells, B cells and NK cells
- The thymus is a greyish organ located in the thoracic cavity just below the neck. The main function of the thymus is to develop immature T-cells into immunocompetent T-cells. Pre-T cells are produced in the bone marrow and transported to the thymus via the blood. The pre-T cells are then taken into the cortex of the thymus. Here, a series of molecular events take place allowing the cells to recognize certain antigens. Some of the cells recognize self-components, and these are eliminated by a process of negative selection. Those that fail the selection undergo apoptosis and those that live proceed to the medulla and eventually into the blood stream where they act upon foreign agents in the body.

1.3.2 Secondary immune organs:

Spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT):

- The spleen is a flattened organ at the upper left of the abdomen. The white pulp in the spleen, provides lymphocytes and hence antibodies for the cellular and humoral specific immune defenses.
- Small, bean-shaped lymph nodes sit along the lymphatic vessels, with clusters in the neck, armpits, abdomen and groin. Each lymph node contains specialized compartments where immune cells come together and encounter antigens. Immune cells and foreign particles enter the lymph nodes via incoming lymphatic vessels or tiny blood vessels. All lymphocytes exit lymph nodes through outgoing lymphatic vessels. Once in the bloodstream, they are transported to tissues throughout the body. Immune cells patrol everywhere for foreign antigens, then gradually drift back into the lymphatic system to begin the cycle all over again.
- MALT includes nodules of immune- system tissue embedded in the mucosa of the digestive tract and the airways and lungs. These tissues include the tonsils, adenoids, appendix and Peyer’s patches of the intestine. MALT is specialized for production of IgA antibody which is secreted across mucosal surfaces.

The organs of your immune system are connected with one another and with other organs of the body by a network of lymphatic vessels. Lymphocytes can travel throughout the body using the blood vessels. The cells can also travel through a system of lymphatic vessels that closely parallels the body's veins and arteries. Cells and fluids are exchanged between blood and lymphatic vessels, enabling the lymphatic system to monitor the body for invading microbes. The lymphatic vessels carry lymph, a clear fluid that flows through the body's tissues.

1.4 Cells of the immune system

Cells destined to become immune cells arise in the bone marrow from stem cells. Some develop into myeloid progenitor cells while others become lymphoid progenitor cells. The myeloid progenitors develop into the cells that respond early and non-specifically to infection. Neutrophils engulf bacteria upon contact and send out warning signals. Monocytes turn into macrophages in body tissues and demolish foreign invaders. Granule-containing cells such as eosinophils attack parasites, while basophils release granules containing histamine and other allergy-related molecules. Lymphoid precursors develop into the small white blood cells called lymphocytes. Lymphocytes respond later in infection. They mount a more specialized attack after antigen-presenting cells such as DCs (or macrophages) display their catch in the form of antigen fragments (epitopes). The B cell turns into a plasma cell that produces and releases specific antibodies into the bloodstream. The T cells coordinate the entire immune response and eliminate the viruses hiding in the infected cells.

1.4.1 B cells:

B cells play a major role in the immune response to the presence of a foreign antigen. B cells fights foreign antigens by the production of antibodies and differentiate in the bone marrow from lymphoid stem cells. When an antigen-specific antibody on a B cell matches up with an antigen, a remarkable transformation occurs. The antigen binds to the antibody receptor, the B cell engulfs it, and allows phagocytes to digest and destroy the antigen completely. After a special helper T cell joins the action, the B cell becomes a large plasma cell factory that produces identical copies of specific antibody molecules. The plasma cells will disappear with time from the blood plasma, but a certain population of cells derived

from the original B cellswill be retained in a dormant state and allow a more rapid and effective immune response to occur if the same antigen appears again. These cells are called memory cells and are induced during immunization. They will be reactivated should the B cell come into contact with the identical antigen.

Each antibody is made up of two identical heavy chains and two identical light chains, shaped to form a Y. The sections that make up the tips of the Y's arms vary greatly from one antibody to another; this is called the variable region. The variable region attaches to the specific antigen. The stem of the Y links the antibody to other participants in the immune defenses. This area is identical in all antibodies of the same class--for instance, all IgEs--and is called the constant region. This portion fixes complement and play a role in the binding of the antibody to receptors found on macrophages and various other cells. This leads to the start of a cascade that leads to more antibody production. Antibodies can cause toxic cells to clump together and cause agglutination so that they may be more effectively removed by the innate immune response. The clump of antibodies and toxic cells may become so large that it becomes insoluble, which also facilitates its removal (precipitation). The antibodies may actually neutralize and cover up the toxic portion of the foreign cell and are occasionally even able to directly attack and kill the toxic cell through a process called lysis. The actions listed above are all direct effects possible from antibody-antigen binding. However, most often the constant portion of the immunoglobulin initiates a signal cascade that results in the release of substances other than the initial antibody that cause the aggregation, neutralization, or lysis of the toxic cell.

Immunoglobulins:

Immunoglobulins G, D, and E are similar in appearance. IgG, the major immunoglobulin in the blood, is also able to enter tissue spaces; it works efficiently to coat microorganisms, speeding their destruction by other cells in the immune system. IgD is inserted into the membrane of B cells, where it somehow regulates the cell's activation. IgE is normally present in only trace amounts, but it is responsible for the symptoms of allergy. IgA guards the entrance to the body. It concentrates in body fluids such as tears, saliva, and secretions of the respiratory and gastrointestinal tracts. IgM usually combines in star-shaped clusters. It tends to remain in the bloodstream, where it is very effective in killing bacteria.

1.4.2 T cells:

T cells belong to a group of white blood cells known as lymphocytes and play a central role in cell-mediated and humoral immunity. Dhodapkar MV *et al.* (1999a) explains T cells can be distinguished from other lymphocyte types, such as B cells and NK cells by the presence of a special receptor on their cell surface that is called the T cell receptor (TCR). These cells develop in the thymus and contribute to immune defenses in two major ways. Some help regulate the complex workings of the overall immune response, while others are cytotoxic and directly come in contact with infected cells and destroy these infected cells. There are two subpopulations of T cells ($CD8^+$ or $CD4^+$) that develop and their development in the thymus can be traced by surface markers. The cells with a CD4 marker are called helper T cells (TH cells) and the CD8 positive cells are called suppressor cells. The $CD8^+$ cells develop into cytotoxic T cells. These cells have a T cell receptor, but they perform very different functions in the immune system (Iwakasi A *et al*. 2004).

T cell receptors recognize antigen, but not in the same way that antibodies do. Antibodies will recognize antigen in its native form, but antigen recognition by the T cell receptor requires the antigen to be digested, degraded and presented on the surface of another cell (an antigen presenting cell or APC) in the context of Major Histocompatibilty Complex (MHC). A piece of the antigen is found on the surface groove of the MHC molecule and is expressed on the surface of the APC (Kadowaki *et al*. 2001; Netea *et al*. 2004).

TH cell activation takes place through the T cell receptor complex. The primary signal is the antigen-MHC II and T cell receptor-CD3 interaction while the co-stimulatory signal occurs with cytokines,CD40L and CD28 (Kadowaki N *et al*. 2001; Netea MG *et al*. 2004). This interaction initiates a cascade of biochemical events in the T cell that eventually results in growth and proliferation of the T cell. This occurs primarily through an increase in Interleukin-2 (IL-2) secretion by the T cell and an increase in IL-2 receptors on the T cell surface. IL-2 is a potent T cell growth cytokine which, in T cell activation, acts in an autocrine fashion to promote the growth, proliferation and differentiation of the T cell recently stimulated by antigen. The T cell receptor is an antigen recognition molecule and therefore the T cell that best responds to the antigen presented is the one that gets turned on. Activated TH cells then continue to become effector cells whose role includes B cell help and cytokine production. The generation of an immune response, both humoral by B cells and cell-mediated by cytotoxic T cells ($CD8^+$), depends on the activation of TH cells. The importance of these $CD4^+$ cells has become obvious as these are the cells affected in AIDS.

Mature B cells that have already seen antigen require contact with a T cell in order to become plasma cells or memory cells. T cells provide signals to the B cell through contact

of the T cell receptor -complex and MHC-antigen. In addition, the activated T cell produces cytokines such as and IL- 4, 5, 6 and 10 which stimulate B cell proliferation and differentiation into antibody secreting B cells. The type of cytokines produced by the T cells helps the plasma cells to produce different classes of antibodies. In response to bacterial invasion, B–cells of the humoral arm proliferate and produce large amounts of appropriate antibodies. The cellular immune response employs specialized T–cells to recognize and destroy host cells showing signs of cancer or infection by viruses or parasites. The relative mobilization of each branch of the immune system depends on the specific disease or condition, and the nature of the response can be influenced by the pathogen itself and where it enters the body.

The balance between the cellular and humoral arms of the immune system is modulated by a highly integrated network of molecular and cellular interactions driven by cytokines, small proteins that act as intercellular chemical messengers (Koch F *et al.*1996). They are the chief communication signals of the T cells. Lymphocytes, including both T cells and B cells, secrete cytokines called lymphokines, while the cytokines of monocytes and macrophages are called monokines. Many of these cytokines are also known as interleukins because they serve as a messenger between white cells, or leukocytes. When cytokines attract specific cell types to an area, they are called chemokines. These are released at the site of injury or infection and call other immune cells to the region to help repair the damage and defend against infection. Cytokines encourage cell growth, promote cell activation, direct cellular traffic, and destroy target cells--including cancer cells (Syme R *et al.* 2001).

T cell subsets:

T lymphocytes differ from B lymphocytes, because T cells do not secrete antibodies. The T cell itself becomes capable of recognizing a specific foreign agent because it expresses cell surface markers known as T cell antigen receptors (TCRs).These antigen-specific TCRs are associated with three more general surface molecules

- **CD3** molecules are found on all mature T cells. The transmembrane area of the CD3 peptide transmits a signal to the T cell's cytoplasm, notifying the cell that an antigen has been bound. This signal causes the secretion of the lymphokines responsible for the further recruitment and differentiation of other responsive cells.
- **CD4** molecules are found on T-helper cells. These molecules recognize and bind class II MHC proteins (found on DCs, monocytes, macrophages, and B cells) and transmit the signal for the T-helper cell to secrete even more lymphokines, including the very important interleukin-2. Helper cells secrete IL-4, IL-5 and IL- 6 (B cell growth factors) which promote the proliferation and maturation of B cells. Helper cells recognize antigen presented on the surface by macrophages in the form of antigenic peptide complexes with class II MHC molecules. Once activated, they divide rapidly and secrete small proteins called cytokines that regulate the immune response. These cells are a target of HIV infection; the virus infects the cell by using the CD4 protein to gain entry. The loss of TH cells as a result of HIV infection leads to AIDS.
- **CD8** molecules are found on T-killer cells. These molecules recognize and bind class I MHC molecules and transmit the signal for the T-killer cell to secrete proteins such as perforin that punch holes in the membrane of the foreign cell and directly cause its lysis and death. Suppressor cells recognize antigen presented on the surface as antigenic peptide complexes with class I MHC molecules. Class I molecules are found

on all cells and present antigens that are synthesized within host cells, such as viral or tumour antigens.

- **Memory T cells** are a subset of antigen -specific T cells that occurs in the body after an infection has been cleared. They quickly expand to large numbers of effector T cells upon re-exposure to the known antigen, thus providing the immune system with "memory" against past infections. Memory T cells comprise two subtypes: central memory T cells and effector memory T cells. Memory cells may be either $CD4^+$ or $CD8^+$.
- **Regulatory T cells** (T_{reg} cells), formerly known as suppressor T cells, are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell mediated immunity towards the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus (Fehervari Z *et al:*2004). Two major classes of regulatory T cells have been described, including the naturally occurring T_{reg} cells and the adaptive T_{reg} cells. Naturally occurring T_{reg} cells (also known as $CD4^+CD25^+FoxP3^+$ T_{reg} cells) arise in the thymus, whereas the adaptive T_{reg} cells (also known as Tr1 cells or TH3 cells) may originate during a normal immune response. Naturally occurring T_{reg} cells can be distinguished from other T cells by the presence of an intracellular molecule called FoxP3.
- **γδ T cells** (Girardi M, 2006) represent a small subset of T cells that possess a distinct TCR on their surface. The majority of T cells have a TCR composed of two glycoprotein chains called α- and β- TCR chains. However, in γδ T cells, the TCR is made up of one γ-chain and one δ-chain. This group of T cells is much less common (5% of total T cells) than the αβ T cells, but are found at their highest abundance in the gut mucosa within a population of lymphocytes known as intraepithelial lymphocytes (IELs). However, γδ T cells are not MHC restricted and seem to be able to recognise

whole proteins rather than requiring peptides to be presented by MHC molecules on antigen presenting cells (Holtmeier W *et al*. 2005).

T cell development in the thymus:

All T cells originate from hematopoietic stem cells in the bone marrow. These cells populate the thymus and expand by cell division to generate a large population of immature thymocytes. The earliest thymocytes express neither CD4 nor CD8, and are therefore classed as double-negative ($CD4^-CD8^-$) cells. As they progress through their development they become double-positive thymocytes ($CD4^+CD8^+$), and finally mature to single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) thymocytes that are then released from the thymus to peripheral tissues. About 98% of thymocytes die during the development processes in the thymus by failing either positive selection or negative selection, while the other 2% survive and leave the thymus to become mature immunocompetent T cells.

Positive selection:

Double-positive thymocytes move deep into the thymic cortex where they are presented with self-antigens complexed with MHC molecules on the surface of cortical epithelial cells. Only those thymocytes which bind the MHC/antigen complex with adequate affinity will receive a vital "survival signal." The other thymocytes die by apoptosis and their remains are engulfed by macrophages. This process is called positive selection. Whether a thymocyte becomes a $CD4^+$ TH cell or a $CD8^+$ T cell is also determined during positive selection. Double-positive cells that are positively selected on MHC class II molecules will become $CD4^+$ cells, and cells positively selected on MHC class I molecules will become $CD8^+$ cells.

Negative selection

Thymocytes that survive positive selection migrate towards the boundary of the thymic cortex and thymic medulla. While in the medulla, they are again presented with self-antigen in complex with MHC molecules on antigen-presenting cells (APCs) such as DCs and macrophages. Thymocytes that interact too strongly with the antigen receive an apoptosis signal that causes their death; the vast majority of all thymocytes initially produced end up dying during thymic selection. A small minority of the surviving cells are selected to become regulatory T cells. The remaining cells will then exit the thymus as mature naive T cells. This process is called negative selection, an important mechanism of immunological tolerance that prevents the formation of self-reactive T cells capable of generating autoimmune disease in the host.

T cell activation:

T cells differentiate in the thymus and when mature, leave the thymus and circulate in the blood and lymph. Following mitogenic or antigenic stimulation, resting T cells are transformed into blast cells capable of division. T cells are activated by specific binding of the T cell receptor to the immunogenic complex of antigenic peptide class I or II MHC molecules. Stimulated by IL- 2, activated T cells proliferate and differentiate into functional classes (Akira S *et al*. 2001). During the activation of $CD4^+$ T cells both the T cell receptor and CD28 are engaged on the T cell by the Major histocompatibility complex peptide and B7 family members on the APC respectively. Both are required for production of an effective immune response. (Lambrecht BN, 2001)

The first signal is provided by binding of the T cell receptor to a short peptide presented by the major histocompatibility complex (MHC) on another cell. (See Figure 1.1) This

ensures that only a T cell with a TCR specific to that peptide is activated. The partner cell is usually a professional antigen presenting cell (APC), usually a DC in the case of naïve responses, although B cells and macrophages can also be important APCs. The peptides presented to $CD8^+$ T cells by MHC class I molecules are 8-9 amino acids in length; the peptides presented to $CD4^+$ cells by MHC class II molecules are longer, as the ends of the binding cleft of the MHC class II molecule are open.

The second signal comes from co-stimulation, in which surface receptors on the APC are induced by a relatively small number of stimuli, usually products of pathogens, but sometimes breakdown products of cells, such as necrotic-bodies or heat-shock proteins. The only co-stimulatory receptor expressed constitutively by naïve T cells is CD28, so co-stimulation for these cells comes from the CD80 and CD86 proteins on the APC (See Figure 1.1).Other receptors are expressed upon activation of the T cell, such as OX40 and ICOS, but these largely depend upon CD28 for their expression. The second signal activates the T cell to respond to an antigen. Without it, the T cell becomes anergic (decrease in response to an antigen) and it becomes more difficult for it to activate in future. This mechanism prevents inappropriate responses to self, as self-peptides will not usually be presented with suitable co-stimulation (Akira S *et al.* 2001).

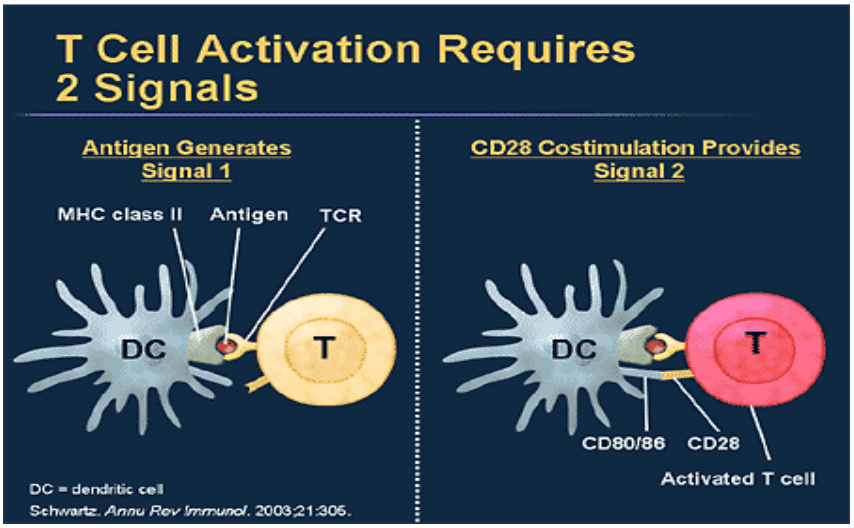

Figure 1.1 T cell activation by an antigen -presenting DC (Website: www.csa.com)

MHC Class I Presentation:

- Ackermann AL *et al.* (2003) states MHC I glycoproteins are present on all cells in the body, acting to present endogenous antigens that originate from the cytoplasm.
- Proteosome further degrade the antigens in the cytosol and enter the Endoplasmic Reticulum, where they can bind to MHC I proteins, before being transported via the Golgi apparatus to the cell surface.
- Once at the cell surface, the membrane-bound MHC I protein displays the antigen for recognition by special immune cells known as cytotoxic T cell lymphocytes

MHC Class II presentation to stimulate $CD4^+$ T Helper cells:

- Antigen is taken up by phagocytosis or receptor mediated endocytosis into APCs only to endosomes where proteolysis occurs.
- The peptides enter a vesicle containing MHC Class II where they bind and are transported to the cell surface.

TH17 Cells:

Experiments done by Afzali B *et al.* (2007) have demonstrated that naive $CD4^+$helper T cells can develop into at least four types of helper T cells, namely TH1, TH2, TH17 and regulatory T cells (T_{regs}) These experiments described that a discrete population of $CD4^+$ helper T cells are a source of IL-17. These cells have been named Th17 cells. IL-17 has a proinflammatory role and has been implicated in many inflammatory conditions in humans and mice, while T_{regs} have an anti-inflammatory role and maintain tolerance to selfcomponents.

1.4.3 Natural killer cells and cytotoxic T cells:

At least two types of lymphocytes are killer cells - cytotoxic T cells and natural killer cells. Both types contain granules filled with potent chemicals and both types kill on contact. They bind their targets, aim their weapons, and deliver bursts of lethal chemicals. Natural killer cells are lymphoid cells found in the blood and peripheral lymphoid organs. Natural killer cells are capable of killing virus-infected cells or tumour cells in the absence of prior immunization and without MHC restriction. Natural killer cells and cytotoxic T cells produce pore forming molecules called cytolysin or perforin which has structural and functional similarity to components of the complement system. The cytolysin or perforin binds to the cell surface membranes and forms transmembrane channels, leading to the osmotic death of the target cells (Wentworth PA *et al.* 1997).

Natural killer cells have many more granules in their cytoplasm when compared to other killer lymphocytes. These granules are thought to be involved in the direct lysis of foreign substances induced by these cells. Like the other lymphocytes, NK cells are very

responsive to the lymphokine IL-2 and will not proliferate without it. To attack, cytotoxic T cells need to recognize a specific antigen bound to self-MHC markers, whereas natural killer (NK) cells will recognize and attack cells lacking these. This gives NK cells the potential to attack many types of foreign cells.

1.4.4 Phagocytes:

Some immune cells have more than one name. For example, the name "phagocytes" is given to the large immune cells that can engulf and digest foreign invaders, and the name "granulocytes" refers to immune cells that carry granules loaded with killer chemicals. Phagocytes include monocytes, which circulate in the blood; macrophages, which are found in tissues throughout the body; DCs, which are more stationary, monitoring their environment from one spot such as the skin; and neutrophils, cells that circulate in the blood but move into tissues when they are needed (Figdor CG *et al.* 2004; Filgueria L. 1996).

Macrophages are versatile cells; besides acting as phagocytic cells, they secrete a wide variety of signaling cytokines (called monokines). Neutrophils are both phagocytes and granulocytes: they contain granules filled with potent chemicals. These chemicals, in addition to destroying microorganisms, play a key role in acute inflammatory reactions. Other types of granulocytes are eosinophils and basophils, which degranulate by spraying their chemicals onto harmful cells or microbes. The mast cell is a twin of the basophil, except it is not a blood cell. Rather, it is responsible for allergy symptoms in the lungs, skin, and linings of the nose and intestinal tract. A related structure, the blood platelet, is a cell fragment. Platelets, too, contain granules. They promote blood clotting and wound repair, and activate some immune defenses.

Monocytes have important properties:

- Monocytes express a myeloid receptor (CD14) which serves as recognition molecule for a wide variety of bacterial envelope molecules, such as LPS from Gram positive organisms and components of Mycobacterial and Gram positive cell walls. Ligation of this receptor leads to macrophage activation.
- Monocytes can act as antigen presenting cells for T cells.
- Monocytes are activated by T cell derived cytokines leading to increased phagocytosis and microbicidal activity (increased activity of degradative enzymes, nitrogen and oxygen free radical production and prostaglandins.)
- Monocytes express receptors for antibody and complement which means that they bind immune complexes

1.4.5 Granulocytes: (Wardlaw *et al.* 1995*)*

There are three types of granulocytes

- Neutrophils, also known as polymorphonuclear leukocytes, express receptors for immunoglobulin and complement and are involved in the acute inflammatory response.
- Eosinophils carry receptors for IgE and are involved in the destruction of IgE coated parasites. They contribute to the reponse to allergens.
- Basophils express high affinity receptors for IgE and are stimulated to secrete the chemicals responsible for immediate hypersensitivity following antigen induced aggregation of these receptors.

1.4.6 Dendritic cells

These cells will be discussed in detail in Chapter 2 since they are the main focus of this dissertation.

1.5 Cytokines

Cytokines are small soluble factors released by cells that influence the functions and communicate with other cells. Cytokines, which are regulated by hormones generated by the endocrine system, can be classified as either TH1 or TH2 depending on their role (Arai K *et al.* 1990; Liles WC *et al.* 1995). TH1 and TH2 cells are thought to derive from a non-polarised, naive TH0 precursor that makes a wide range of cytokines: this TH0 cell can differentiate after activation in the presence of IL-12 and IL-18 (from DCs) into TH1 cells that secrete IL-2, IFN-γ and TNF however in the presence of IL-4 (derived from B cells or lymphoid DCs) the TH0 cells differentiate into TH2 cells that secrete IL-4, IL-5, IL- 6 and IL-10 (Karnitz LM *et al.*1995; Joyce DA *et al.*1996; O'Garra A *et al.* 1998). Generally, in healthy individuals the immune system has a balanced expression of TH1 and TH2 cytokines. If a foreign invader triggers an adaptive cellular or TH1–-type response, the feedback mechanism within the immune system greatly reduces the humoral or TH2–type response. Once the invader is controlled or eliminated, a combination of hormones and cytokines act quickly to return the system back towards homeostasis through the same feedback mechanism.

TH1 cells drive the cellular immunity to fight viruses and other intracellular pathogens, eliminate cancerous cells, and stimulate delayed-type hypersensitivity skin reactions. TH2 cells drive humoral immunity and up-regulate antibody production to fight

extracellular organisms. The cytokines produced by the two subsets also have a cross-regulatory role (Bueno C *et al.* 2001). (See Figure 1.2) In other words, an activated TH2 cell will down regulate the TH1 cells. Likewise, TH1 cytokines down regulate the TH2 responses (Lucey DR *et al.* 1996; Moser M *et al.* 2000).

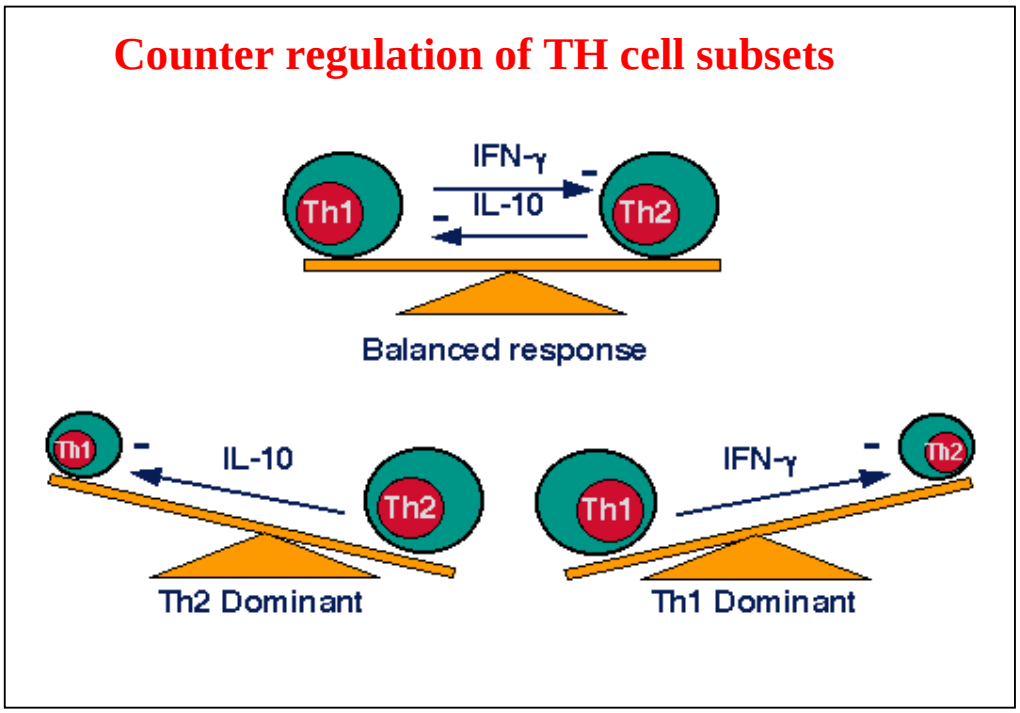

Figure 1.2: TH1/TH2 balance **(**Website: http://inet.uni2.dk/~iirrh/IIR/03Th/Th.htm)

Only the most important cytokines in relation to this thesis will be discussed.

1.5.1 TH1 cytokines:

Interferons (IFN) as stated by Baron S *et al.* (1991) modulate the activity of virtually every component of the immune system.Type I interferons include more than 20 types of interferon-alpha, interferon-beta, interferon omega, and interferon tau (Liu *et al.* 2005b). There is only one type II interferon, interferon-gamma (Colonna M *et al.* 2002a).Type I interferons, which can be produced by virtually any virus-infected cell is better able to induce viral resistance in cells, whereas type II interferon is produced by activated

T-lymphocytes as part of an immune response and functions mainly to promote the activity of the components of the cell-mediated immune system such as CTLs, macrophages, and NK cells.

Interferons are among the best studied cytokines. IFN is produced by immune-activated cells or virus-infected cells in response to the double-stranded RNA (dsRNA) that many viruses produce as a part of their life cycle. Interferons exert their antiviral activity by binding to uninfected neighboring cells and induce them to produce enzymes that degrade mRNA. This not only prevents translation of viral mRNA into viral protein, but it also eventually kills the host cell that produces the viruses. Interferons also promote the body's defenses by enhancing the activities of CTLs, macrophages, NK cells, and antibody-producing cells (Colonna M *et al.* 2002a).

Interferon-gamma (IFN-γ) is secreted by T cells (cytotoxic and TH1) and Natural Killer cells, activate macrophages and increase the expression of class II MHC on APC. IFN-γ stimulated macrophages are more phagocytic, they are more capable of killing intracellular pathogens and they have increased ability to present antigen. IFN-γ secreted by TH1 cells has a cross regulatory role in controlling TH2 function, and will induce a antibody class switch to IgG. It actually can inhibit the activities of the TH2 pathway by inducing IL-12 production by macrophages. This cytokine has a role in many different types of immune reponses such as inflammation, antibody production and viral infection. In summary IFN-γ induces MHC-I and MHC-II production. It activates and increases the antimicrobial and tumourcidal activity of monocytes, neutrophils, and NK cells and stimulates the synthesis of adhesion factors on endothelial cells and leukocytes for diapedesis. (Farrar MA *et al.* 1993)

Interferon-alpha (IFN-α) is expressed by T-lymphocytes, B-lymphocytes, NK cells and monocytes/macrophages.

Interferon-beta (IFN-β) is expressed by virus-infected cells, fibroblasts, macrophages, epithelial cells, and endothelial cells (Eloranta ML *et al.* 1997). This cytokine influences antiviral and antiparasitic activity and induces MHC-I antigen expression. (Siegal FP *et al.* 1999)

Interleukin-1 (IL-1) has many functions on many different cells and is secreted by a number of cells including macrophages, monocytes and DCs. An important stimulus for IL-1 production by the macrophage is the presence of microbial products. IL-1 (originally described as T cell activation factor) helps to activate T helper cells by acting as a co-stimulator with the antigen presenting cell receptors. It also helps promote the maturation and clonal expansion of B cells. IL-1 is an important part of the inflammatory response; it promotes both the inflammation and catabolic processes. One way it mediates this is by increasing the expression of cell adhesion molecules on endothelial cells of the vasculature which allows translocation of immune cells from the blood vessels into the tissue. One very interesting action of IL-1 is its action on the hypothalamus. Here IL-1, and some other cytokines (including IL-6, the IFN and TNF), bind to receptors on the endothelial cells within the hypothalamus and appear to 'reset' the thermoregulatory centre to increase the core body temperature thereby causing fever. IL- 1 activates B-lymphocytes, NK cells, polymorphonuclear leukocytes, endothelial cells, smooth muscle cells, and fibroblasts. IL-1 induces fever, sleep and neutrophilia and stimulates synthesis of proinflammatory cytokines and acute-phase proteins. It also induces coagulation and stimulates the synthesis of collagen and collagenase for scar tissue formation.

Tumour necrosis factors: Bazzoni F (1995) explains TNF plays a key role in bridging the innate and the adaptive immune systems. The release of TNF causes a range of activities that are important in immune responses to viruses and bacteria.

These activities include:

- Activation of neutrophils and macrophages to destroy microbes
- The enhancement of cytokine release by mononuclear phagocyte system cells
- The stimulation of the recruitment of neutrophils and monocytes to sites of infection
- The amplification of the expression of MHC Class I molecules to enhance the presentation of viral peptides in intracellular infections.
- With the presence of IFN-γ, it induces the expression of MHC Class II molecules

Tumour necrosis factor-alpha (TNF-α) is secreted by activated mononuclear phagocytes, natural killer cells, mast cells and antigen- stimulated T cells. TNF-α is a potent mediator of inflammatory and immune functions caused by bacteria and other infectious microorganisms. It is cytotoxic for some tumour cells, induces fever and sleep. It stimulates the synthesis of collagen and collagenase for scar tissue formation and adhesion factors on endothelial cells and leukocytes for diapedesis. TNF-α activates macrophages and promotes inflammation and catabolic processes. TNF-α is a chemoattractant for phagocytes and promotes neutrophil degranulation and is responsible for endotoxin-induced septic shock. It triggers apoptosis (Kikuchi K *et al.* 2003). The most potent inducer of TNF-α by macrophages is the lipopolysaccharide (LPS) of bacterial cell walls. Therefore infections by Gram negative bacteria produce large amounts of this monokine.

Tumour necrosis factor-beta (TNF-β; lymphotoxin) carries out many of the same activities as TNF-α. It is primarily produced by TH1 cells and B-lymphocytes (Kikuchi K *et al.* 2003).

Interleukin-12 (IL-12) described by Dalod M *et al.* (2002) activates cytotoxic T cells. IL -12 also stimulates natural killer cells and TH1 cells to proliferate. It activates TH1 induction and maturation and induces interferon-gamma production. (Biron *et al.*1995). IL-12, also known as natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF) is produced by macrophages and B lymphocytes and has been shown to have multiple effects on T cells and natural killer (NK) cells (Hendzrak JA *et al.*1995; Kato T *et al.* 1997). These include inducing production of IFN-γ and TNF by resting and activated T and NK cells. Evidence indicates that IL-12, produced by macrophages in response to infectious agents, is a central mediator of the cell-mediated immune response by its actions on the development, proliferation, and activities of TH1 cells (Scott P: 1993). These activities of IL-12 are antagonized by IL-4 and IL-10, factors associated with the development of T helper cells into TH2 cells and mediation of the humoral immune response (Stern AS *et al.*1996; Trinchieri G *et a*l. 1995).

Interleukin-8 (IL-8) is a pro-inflammatory cytokine (chemokine) derived from endothelial cells, fibroblasts, macrophages, and monocytes. It causes chemotaxis of neutrophils and T-cell lymphocytes.

1.5.2 TH2 Cytokines

Interleukin-4 (IL-4) is a cytokine secreted by cells. It has many biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of $CD4^+$ T-cells into TH2 cells. It is a key regulator of humoral pathway/arm of adaptive immunity. IL-4 induces immunoglobulin class switching to IgE, and up-regulates MHC class II production. It is a growth and differentiation factor for activated B-lymphocytes and activated T-lymphocytes. (Hochrein H *et al*. 2000; Lee JD *et al.*1995).

Interleukin-5 (IL-5) is primarily produced by TH2 cells and stimulates the proliferation of activated B-lymphocytes and their differentiation into plasma cells. It stimulates antibody secretion and immunoglobulin class shift. It also induces growth and differentiation of eosinophils.

Interleukin-6 (IL-6) functions in both the innate and adaptive immunity. It is secreted by vascular endothelial cells, fibroblasts, T and B cells and mononuclear phagocyte system cells. IL-6 is released in response to infection, burns, trauma, and neoplasia, and its functions range from key roles in acute-phase protein induction to B- and T- cell growth and differentiation (Chomarat P *et al.* 2000). IL-6 can have direct effects on cells, can mediate the effects of other cytokines, can be agonistic or antagonistic in conjunction with other cytokines, and interact with glucocorticoids. IL-6 is induced by bacteria, viruses, bacterial products and chemicals that induce inflammatory reactions. The IL-6 receptor is found on many cell surfaces, including resting normal T-cells, activated normal B-cells, myeloid cell lines, hepatoma cell lines and myeloma cell lines. IL-6 stimulates the acute-phase reaction, which enhances the innate immune system and protects against tissue damage (Barton B, 1996; Syme R *et al.*2001). The inflammatory response aims to dilute,

neutralize or remove the threatening agent and initiate the process of repair or recovery. Inflammatory agents stimulate monocytes to secrete IL-6 which in turn induce hepatocytes to synthesize acute phase proteins, like CPR, clotting and complement factors and therefore IL-6 plays a key role in innate immunity. It also plays an important role in adaptive immunity. Interleukin-6 is especially important in the early stages of T-cell differentiation. In this phase, it reinforces the effect of IL-2 and promotes the differentiation of CD4 cells into TH2 cells. It controls the growth and proliferation of early progenitor cells in the thymus and bone marrow and is later important in both T-cell and Natural Killer (NK) cell activation. IL-6 also functions as the required second signal in both antigen- or mitogen-activated T-cells. This protein holds a very important role in the life of NK cells. It is first an activator and later stimulates them to perform a more effective lysis of a pathogen. IL-6 provides support for continued development throughout the life of a natural killer cell. Interleukin-6 is very important in the stimulation of differentiation and proliferation of B-cells. It plays a big role in the induction of permanent differentiation of B-cells into plasma cells. IL-6 enhances the release of antibodies by acting as a growth factor for already differentiated plasma cells. It stimulates mostly the release of IgG and IgA antibodies from these cells (Chomarat P *et al.* 2000; Syme R *et al.*2001).

Interleukin-10 (IL-10) is a small protein that plays a big role in the regulation of the immune system. The two major activities of IL-10 are the inhibition of cytokine production by macrophages and inhibition of the accessory functions of macrophages during T cell activation. The effects of these actions cause IL-10 to play mainly an anti-inflammatory role in the immune system. It is a multifunctional cytokine that modulates the function of many cells, including T-lymphocytes, B-lymphocytes, NK cells, monocytes/macrophages, and neutrophils. IL-10 is mainly produced by the TH2 subset of $CD4^+$ helper cells. Some

activated B cells, TH1 cells, activated macrophages, and some nonhematopoietic sources (e.g. keratinocytes, colon carcinoma, melanoma cells) also produce IL-10 (Duramad O *et al.* 2003).

1.6 General

Immunology interfaces with medicine at many points, the most prominent currently being infectious diseases, cancer, transplantation, allergy, and autoimmunity. In each of these instances, there is a need for treatments that increase (immunize) or decrease (tolerize) the immune response to the disease causing antigens. The DCs comprise several subsets that induce and regulate the immune responses against foreign and self-antigens, and can therefore function as initiators of protective immunity and inducers of central or peripheral tolerance. The different subpopulations of DCs interact with and also influence other cell populations of the immune system, such as T and B lymphocytes and natural killer cells. The factors that determine the given DCfunctions depend on the state of maturation and the local microenvironment. The interactions between DCs and microorganisms are rather complex, but progress in the past few years has shed light on several aspects of these interactions. The ultimate goal of this research is to control DC biology to gain improved antigen specific control of the immune system.

Chapter 2

The Immunobiology of Dendritic cells

Abstract

The most efficient antigen-presenting cells (APCs) are mature, immunologically competent DCs. DCs are capable of evolving from immature, antigen-capturing cells to mature, antigen-presenting, T cell-priming cells; converting antigens into immunogens and expressing molecules such as cytokines, chemokines, costimulatory molecules and proteases to initiate an immune response. The ability of DCs to regulate immunity is dependent on DC maturation. A variety of factors can induce maturation following antigen uptake and processing within DCs, including: whole bacteria or bacterial-derived antigens. The following chapter will provide an overview of the different types of the DCs and the process of DC maturation (*e.g.* cell surface receptors, costimulatory molecules, intracellular proteins, cytokines, chemokines and their corresponding receptors, and proteases).

2.1 Introduction

Immunology has long been focused on antigens and lymphocytes, but the presence of these two parties does not always lead to immunity. The DC system of antigen presenting cells (APCs) is the initiator and modulator of the immune response (Bonosia R *et al.*2006; Hart DNJ *et al*. 1997). First visualized as Langerhans cells (LCs) in the skin in 1868, the characterization of DCs began only 30 years ago. Ralph Steinman first described DCs and shortly thereafter it became clear that DCs existed in all lymphoid and non- lymphoid tissues. It was not until the 1980's that it became widely accepted that DCs were "professional" antigen presenting cells. In the 1990's researchers showed how to generate large numbers of DCs from $CD34^+$ bone marrow precursors or from $CD14^+$ monocytes *in*

vitro (Grabbe S *et al*. 2000; Libscomb MF *et al*. 2002).There is a great interest in exploiting DCs to develop immunotherapies for cancer, chronic infections and autoimmune disease (Ludewig B *et al*. 1998a; O'Neil D *et al*. 2004).

2.2 Features indicating the important roles for DCs in the control of immunity:

(Nelson CA *et al.* 1994; Morgan DJ *et al*. 1999; Banchereau J *et al.* 2000a)

Potency: Studies done on DCs showed small numbers of DCs and relatively small amounts of antigen were able to stimulate strong T cell responses in culture.

Position: DCs situated *in vivo* in the tissues and airways are positioned to capture antigens. DCs can migrate to lymphoid tissues, via lymphatics, to enter the T cell areas. There, antigen bearing DCs are located to select antigen – reactive T cells from the circulating pool of lymphocytes.

Priming: Mature DCs lack many antigen – eliminating functions, such as the capacity to kill microorganisms or make antibodies. DCs can be pulsed *ex vivo* with antigens and be reinfused to autologous recipients. T cells recognize antigen in the context of the MHC haplotype of the priming DCs. The activated T cells leave the lymph node via efferent lymphatics; enter the blood and finally, the inflammatory site where antigen was initially deposited.

2.3 What is a dendritic cell?

To stimulate a cellular immune response against for example a tumour, one needs more than just antigen and lymphocytes. Another cell is required that can bind and present tumour antigen(s) to T lymphocytes in a way that is immunogenic. If successful, this means that the immune system reacts against, rather than ignores, all other cells bearing the same antigen. In the early 1970's Steinman first purified a novel leukocyte from mouse lymphoid tissue. These cells were called a "DC," on the basis of its unusual morphology of veiled dendrites (Faratian D *et al*: 2000). DCs were also distinguished from other antigen-presenting cells, such as macrophages and B cells, on the basis of their capacity to stimulate T-cell responses, even when the other antigen-presenting cells had been removed (Austyn JM:2000b).

DCs have been referred to as "professional" antigen presenting cells, since the principle function of the DCs is to present antigens to resting naïve T lymphocytes. DCs are capable of capturing antigens, processing them and presenting them on the cell surface along with appropriate costimulation molecules. Unlike B lymphocytes that can respond to soluble or native antigen by transforming into antibody-secreting plasma cells, T lymphocytes can only respond to peptide fragments of protein antigens bound to surface MHC molecules. MHC molecules are of two types, class I and class II (Banchereau J *et al.* 2001b). Class I MHC molecules bind intracellular or endogenous antigens that have been cut into smaller peptides in the cytosol. Class II MHC molecules bind extracellular or exogenous antigens endocytosed by the antigen-presenting cell. MacDonald KP *et al. (*2002) states there are 3 generally accepted stages of differentiation for all DC subtypes: DC precursors, immature DCs and mature DCs. These cells are traditionally divided into 2 populations by staining with anitibodies to CD11c and CD123 (O'Doherty U *et al.* 1994; Romani N *et*

*al.*1994a; Thomas R *et al.* 1994c; Howard CJ *et al*. 1997; Pulendran B *et al*. 1999; Huang FP *et al*. 2000; Shortman K *et al*. 2002b). These studies showed that DCs play an important role in the induction and maintenance of the adaptive immune response, but now it is evident that DCs also have important roles in innate immunity. These features make DCs very good candidates for therapy against various pathological conditions including malignancies.

2.4 Types of dendritic cells:

Myeloid DCs: (mDCs)

mDCs have a monocytoid appearance and are $CD11c^{+}$ $CD123^{-}$ (Olweus J *et al*. 1997). These cells are major stimulators of T cells and are found in many tissues, where they may be classified in two principal subtypes: Langerhans cells and interstitial, dermal or submucosal DCs (Thomas R *et al*. 1993a; Thomas R *et al*. 1994b). Myeloid precursor DCs leave the blood and home to various tissues in the response to chemoattractant gradients. Immature mDCs are located at sites where most pathogens enter the body. Upon exposure to a virus or bacterial antigen, immature mDCs bind and internalize the virus or pathogen and release inflammatory cytokines and chemokines. This initiates the recruitment of more immature mDCs and other leucocytes to the site of infection. mDCs at first release IL-12 and stimulate a TH1 response (Cella M *et al*. 2000b). Within 24 hours, the production of the IL-12 decreases and IL-4 secreting T cells are generated to decrease the TH1 response. mDCs in lymph nodes produce chemokines that recruit T cells, B cells, more mDCs and pDCs.

Plasmacytoid DCs: (pDCs)

pDCs as described by McKenna K: (2005) have certain characteristics similar to mDCs, but have morphological features like plasma cells and are found in blood and lymphoid organs (Colonna M *et al.*2004b). These cells accumulate in inflammatory sites. The pDCs lack most myeloid markers. These cells are CD $11c^{-}$ $CD123^{+}$and depend on IL- 3, not GM-CSF, for maturation (Facetti F *et al.*1998; Galibert L *et al.* 2001; Diebold SS *et al.* 2003).

pDCs leave the blood through high epithelium venules (HEV) (Langenkamp A *et al.*2000). The expression of CD62L on plasmacytoid DCs allows them to migrate via the HEV's. In the lymph nodes the pDCs are stimulated to mature through the expression of CD40L on recently activated T cells (Brei're F *et al.* 2002). The pDCs secrete IL-12 and express high levels of INF-α and are important in innate antiviral immunity as explained by Cao W *et al.* (2007) and Asselin –Paturel C *et al.* (2005). These DCs can activate antitumour and antiviral antigen responses, but their potential as immunotherapeutic adjuvants is largerly unexplored, because they are difficult to obtain in large quantities. Fiebiger E *et al.* (2001) demonstrated that pDCs only minimally express cathepsins S and D, which are lysosomal proteases involved in antigen processing and thus leading to T cell priming.

pDCs precursors are the major producers of type I interferon and have the unique ability to link innate and adaptive immunity. After producing large amounts of type I IFN in response to microbial stimulation, they can differentiate into DC capable of stimulating naive T cells and modulate the adaptive immune response (Kawamura K *et al*. 2006; Comeau MR *et al.* 2002).

Originally, scientist thought that the different subsets of DCs had unique functions: mDCs induced immunity and pDCs mediated tolerance. However, there is increasing evidence that it is not the DC subset but, more likely, the DC maturation state, activation state and the microenvironment that determine the outcome of the immune response

2.5 DCs differentiation and trafficking pathways:

Robinson SP *et al.* (1999) explains that DCs perform specific functions and travel from one site to the next: bone marrow derived DCs circulate as precursors in blood before they enter the tissues where they become immature DCs and monitor their environment (See Table 1.1). Interstitial DCs and Langerhans cells are found at sites that come in contact with the external environment, for example the mucosal surfaces and the skin. Immature DCs in the peripheral tissues are able to migrate towards inflammatory sites where they take up and break down available antigens. They then emigrate through the lymphatics to draining lymph nodes and there they home to T cell rich areas and interact with T cells and initiate an immune response (Sallusto F *et al.* 2000d).

Table 2.1 Different locations of DCs

Location	**Type of DC**
Epidermis	Langerhans: Express the C-Type lectin Langerin, have unique intracellular organelles called Birbeck granules and are found in the epidermis and oral, respiratory and genital mucosa
Dermis, Interstitium	Interstitial
Circulatory system	Blood
Afferent Lymph	Veiled
Lymph nodes	Lymphoid or Interdigitating

Compiled by A. Clark: May 2007

2.6 Function of DCs:

McLellan AD (2000) explains that the most important role of DCs is the priming of adaptive immunity and organizing immune responses against all classes of pathogens and also against tumours. The function of DCs falls broadly into three categories, each of which involves antigen presentation (Belz GT *et al*. 2002).

2.6.1 T cell activation:

The most prominent role of DCs is to process and present antigens to activate both $CD4^+$ and $CD8^+$ cells, since DCs are the only cells capable of activating naïve T cells (Maldonado-Lopez R *et al*. 2001; Sallusto F:2002e; Sallusto F *et al.* 2002f). Immature DCs originate in the bone marrow and migrate throughout the body. These cells lay dormant, waiting to interact with invading pathogens or other foreign bodies. So, the function of an

immature DC is to find and capture foreign bodies and antigens (Jarrossay CA *et al.* 2001). Once captured, the antigen is processed either by an exogenous or endosomal pathways or by endogenous or proteosomal pathways (Mempel TR *et al*. 2004).

2.6.2 Immune tolerance:

Tolerance is the inability of the immune system to respond to specific antigens. Central tolerance occurs in the thymus for T cells and in the bone marrow for B cells. The primary central tolerance in T cells is the induction of T cell death. DCs are found in the thymus, where newly produced T cells are educated to become $CD4^+$ T cells and $CD8^+$ cells and undergo selection to eliminate immunity against self components. Low affinity reactive T cells are positively selected and allowed to survive and reach the periphery. T cells that respond to DCs carrying self-peptides are destroyed in the thymus by negative selection. This process involves T cells which recognize MHC or peptides with high avidity. DCs contribute to this negative selection process along with thymic epithelial cells. The mechanisms of peripheral tolerance include T cell death, anergy and active suppression by T regulatory cells: DCs induce apoptosis in T cells or by producing IL-10, which induces T regulatory cells. DCs might also contribute to tolerance by inducing anergy in responder T cells (Steinman RM *et al.* 2002b).

2.6.3 B cell stimulation or function:

DCs produce a number of cytokines and factors which are critical to the activation and differentiation of B cells. The follicular DCs (FDCs) which are found in the germinal centers of lymph nodes are important in the maintenance of B cell memory. FDCs present native antigens to potential memory cells, of which only B cells with high affinity B cell receptors can bind. These cells are present in follicles of any secondary lymphoid organ.

The B cells can in turn take up the antigen from FDCs and present it to T cells. The reservoir of antigen and antibody complexes on FDCs is believed to be able to last a very long time (McHeyzer- Williams M *et al.* 2003).

DCs appear in the periphery as immature DCs where they capture and process antigens. Upon capturing an antigen they undergo maturation and present antigen peptides along with MHC on their cell surfaces. Mature DCs then migrate to the lymphoid organs, namely the spleen and lymph nodes, where they express lymphocyte co-stimulatory molecules and secrete various cytokines. This results in selection of rare T lymphocytes with the correct antigen specific T-cell receptors (TCR) which proliferate and mature into effector cells. These newly formed effector cells then circulate through the body, carrying out immune responses.

DCs must therefore:

- be able to internalise antigen, for example by phagocytosis, to allow processing to occur
- be capable of processing antigen by proteolysis, the primary mode of antigen degradation
- be capable of transcribing the products of MHC class II (and/or Class I) genes and expressing these at the cell surface in sufficient quantity
- be capable of associating the processed peptide fragments, several amino acids long, with the MHC molecules and expressing these on the cell surface
- be capable of providing the necessary regulatory signals in the form of cytokines to the responding cells.

2.7 Migration of DCs *in vivo*

DCs travel to the lymphoid tissues such as the spleen and lymph nodes after activation (Macagno A *et al.* 2007). The movement of DCs from the blood to peripheral tissue and the movement from peripheral tissue into lymphoid tissue requires chemoattractants called chemokines. Macagno *A et al.* (2007) states chemokines are differentially produced at peripheral tissue sites by endothelial cells, epithelial cells and leukocytes in response to a inflammatory stimuli. Chemokines are also produced by endothelial cells and leucocytes within secondary lymphoid organs to regulate encounters between DCs, T cells and B cells (Banchereau J *et al*. 2001b). DCs are always present in afferent lymph nodes that access the T cell areas. The two subsets namely myeloid and plasmacytoid can enter lymphoid organs with possible different final destinations such as the B cell and T cell areas. Sallusto F *et al*. (2000d) showed in his study that immature DCs migrate toward increasing concentrations of inflammatory chemokines. The DCs are also exposed to increasing concentrations of proinflammatory cytokines like TNF-α, IL -1 and the pathogen products that caused the inflammatory response. In response to these DCs mature and switch the usage and expression of chemokine receptors from inflammatory to lymphoid homing receptors (MacPherson GG, 1993).

In most tissues, DCs are present in a so-called ‘immature’ state and are unable to stimulate T cells (Lutz MB *et al*. 2002). Although these DCs lack the requisite accessory signals for T cell activation, such as CD40, CD54, CD80 and CD86, they are extremely well equipped to capture antigens in peripheral sites. Once they have acquired and processed the foreign antigens, they migrate to the T cell areas of lymph nodes and the spleen, undergo maturation and stimulate an immune response (De Vries IJ *et al*. 2003).

2.8 Life cycle of DCs:

Kohrgruber N *et al.*(1999) explains that like other cell types within the immune system, immature DCs are continuously produced from hematopoietic stem cells within the bone marrow as two subsets or families of $CD34^+$ progenitor DCs: mDCs and pDCs (Banchereau J *et al.* 2001b). The pDCs migrates to the thymus to continue differentiation.

Mostly newly generated immature DCs migrate along the following route: bone marrow, blood, nonlymphoid tissues, lymphatics, and then draining lymph nodes (See Figure 2.1). These DCs appear to have a short life span of 3–4 days. DCs mediate the transport of antigens from sites of peripheral inflammation to lymphoid organs and then present the antigens to naïve T cells. Immature DCs are endocytic and thus can capture antigens. They become mature DCs after stimulation with lipopolysaccharide (LPS), other pathogen-derived substances, and inflammatory cytokines, resulting in the upregulation of CD40 and cell surface receptors that act as co-receptors in T cell activation, such as CD80 and CD86 (Bender A *et al.* 1996).

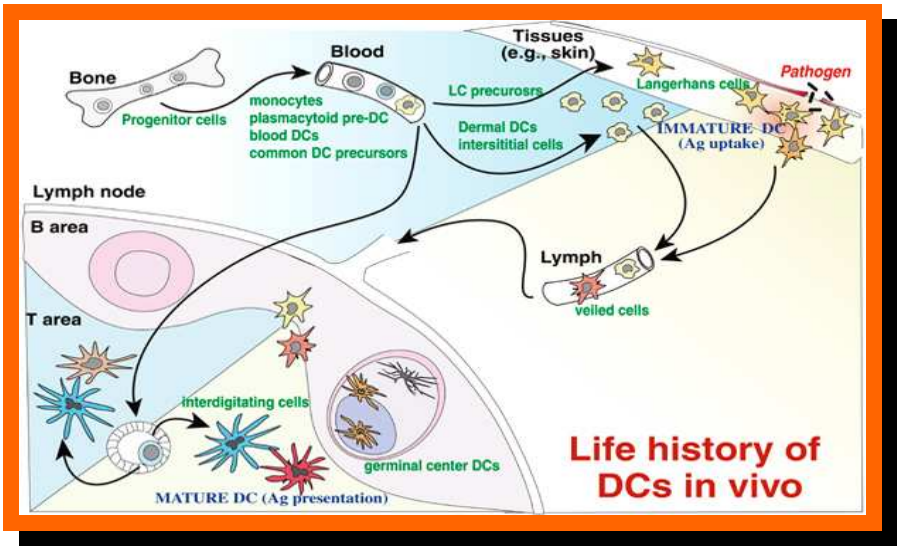

Figure 2.1: The life cycle of DCs *in vivo* (Website: www.zoo.zool.kyoto-u.ac.jp/imm/lab/lab-e.html)

2.9 Maturation of DCs:

The initiation of a productive immune response by T cells requires co-stimulation. Mature DCs arise from immature precursors which are the cells that exhibit endocytic activity (Trombetta ES *et al*. 2003). Maturation is a terminal differentiation process that transforms DCs from cells specialized for antigen capture into cells specialized for T-cell stimulation, (Chapuis F *et al*. 1997; Rescigno M *et al.* 1998) accompanied by changes in the expression of numerous cell-surface antigens that reflect the changing functional role of the cells (Reis e Sousa C *et al.* 2004b; Sozanni S *et al.* 1998).

Maturation is characterized by reduced phagocytic uptake, the development of cytoplasmic extensions or “veils”, migration to lymphoid tissues, and enhanced T-cell activation potential (Romani N *et al*. 1996b; Rovere P *et al*. 1998; Kalinski P *et al.* 1999a). Maturation regulates antigen processing by lowering the pH of endocytic vacuoles, activating proteolysis and transporting the peptide – MHC complexes to the cell surface. Immature DCs, such as Langerhans cells in the skin, have high cell-surface expression of receptors that efficiently capture antigen for uptake and processing. Hermann TL *et al*. (2005) showed in their study that the maturation of DCs is regulated by the enzyme, Calmodulin kinase II.

Mature DCs in secondary lymphoid tissues, such as lymph nodes and spleen, have a lower capacity to capture antigens, but are extremely efficient in antigen presentation and stimulating naïve T cells (Aiba S, 1998). During maturation, DCs switch their pattern of chemokine receptor and adhesion molecule expression, allowing them to migrate to the secondary lymphoid tissues. In addition, the maturing DCs increase cell surface expression of peptide-loaded MHC class II as well as adhesion and co-stimulatory molecules, such as

CD80 and CD86, to allow for a productive response of the T cell. In turn, T cells activate the DCs through the CD40-CD40L interaction to produce cytokines (Caux C *et al*. 1994b). Once primed, the DCs migrate to secondary lymphoid compartments to present Ag-peptide complexes to naïve $CD4^+$ T cells and $CD8^+$ cytotoxic T cells (Allan RS *et al.* 2006). Following education by Ag-loaded DCs in lymph nodes, naïve $CD4^+$ T cells differentiate into memory helper T cells, which support the differentiation and expansion of $CD8^+$ CTLs and B cells (Veeraswamy RK *et al.* 2003). Helper T cells exert anti-tumour activity indirectly through the activation of important effector cells such as macrophages and CTL's, which are capable of eradicating tumour cells or virus-infected cells directly.

Steinman RM *et al*. (2007) explains during the transition from an immature DC to plasmacytoid-derived DC, a radical morphological transformation occurs. The round plasmacytoid cells acquire a typical dendritic morphology that is tightly associated with the specialized effector functions of DC in T cell stimulation. The dendrites enable a DC to efficiently interact with several T cells. In addition, as the number of dendrites increases, so does the surface area of the DC–T cell contact, thereby strengthening the immunological response. During DC differentiation, the cells acquire an antigen-presentation capacity; express a broad range of co stimulatory molecules that are important in the DC–T cell cross-talk, and lose their type I IFN production potential.

Maturation of DCs can be mediated by inflammatory cytokines (IL- 1 and TNF and IFN-γ), T cell products (CD40 Ligand), microbial constituents (liposaccharide and CpG oligonucleatides) and trauma (necrosis, transplantation) (Pan J *et al.* 2004). Maturation leads to the development of many features critical for priming, for example maturing DCs produce IL-12 and express different chemokine receptors to regulate the movements and

interactions with lymphocytes *in vivo*. It is clear that the maturation of DCs is crucial for the initiation of immunity (Ricciardi-Castagnoli P, 1997). Helper T lymphocytes trigger complete DC maturation through CD40-CD40L dependent and independent interactions (Caux C *et al*. 1994b). This maturation process is characterized by reduced Ag-capture capacity and increased surface expression of MHC and co-stimulatory molecules. However, the maturation of DCs is completed only upon interaction with T cells. It is characterized by loss of phagocytic capacity and expression of many other accessory molecules that interact with receptors on T cells to enhance adhesion and signalling (co-stimulation); for example CD58, CD54, CD80, CD86 and CD83. Expression of one or both of the costimulatory molecules CD80 and CD86 on the DCs are essential for the effective activation of T lymphocytes, and, for IL-2 production (Hellman P *et al.* 2007). These co-stimulatory molecules bind the CD28 molecules on T lymphocytes. If this fails to occur at the time of Ag recognition by the TCR, an alternative T lymphocyte function may result, namely induction of anergy.

CD40L is one of the most potent inducers of DC maturation and is mainly expressed on activated CD 4^+ T lymphocytes. CD40 is a costimulatory protein found on antigen presenting cells. CD40 binds to CD154 (CD40L) on T cells to activate the antigen presenting cell and produce a variety of downstream effects (O' Sullivan B: 2003). During effective DC- T cell interactions, T cells become activated and they upregulate CD40L. CD40L can then interact with CD40 on mature DCs to trigger IL-12 release, required for TH1 polarization. In the macrophage, the primary signal for activation is IFN-γ from TH1 type CD4 T cells. The secondary signal is CD40L on the T cell which binds CD40 on the macrophage cell surface. As a result, the macrophage expresses more CD40 and TNF receptors on its surface which helps increase the level of activation. The activated

macrophage can then destroy phagocytosed bacteria and produce more cytokines (Durie FH *et al.* 1994; Schoenberger SP *et al.* 1998).

The B cell can present antigens to helper T cells. If the T cell recognizes the peptide presented by the B cell, the T cell synthesizes CD40L. The CD40L binds to the B cell's CD40 receptor which causes resting B cell activation. The T cell also produces IL-4 which directly binds to B cell receptors. As a result of this interaction, the B cell can undergo division, antibody isotype switching, and differentiation to plasma cells. The end result is a B cell which is able to mass produce specific antibodies against an antigenic target (Alderson MR *et al*. 1993).

Viney JL (1999) states human monocyte -derived DCs were found to induce TH1 differentiation, whereas DCs derived from plasmacytoid cells induced TH2 differentiation. DCs are a major source of many cytokines, (Penna G *et al*. 2002) namely, IFN-α, IL-1, IL-6, IL-7, IL-12 and IL-15 and also produce macrophage inflammatory protein all of which are important in the elicitation of a primary immune response. Also, there is evidence that the cytokine secretion pattern of the plastic-adherent monocyte-derived DCs can be induced along the TH1 (IL-12) or TH 2 (IL-10) cytokine secretory pathway. DC research has become increasingly popular as investigators explore their potential use in the development of better vaccines (to viruses, tumours, etc.), as well as treatment of autoimmune and allergic disorders.

2.10 Antigen uptake, processing and presentation

Albert ML *et al.* (1998b) states immature DCs can be activated by pathogens, stress and inflammation which induce mobilization of cells to draining lymph nodes where they act as highly potent professional APCs (Schuurhuis DH *et al.* 2006). DCs have many uptake receptors that deliver antigen to processing compartments. Many of the receptors on the DCs are lectins with carbohydrate recognition capacity for example DEC205, mannose receptors (MMR) and CD 209/DC –SIGN. CD 209 is known to bind a number of microbes including HIV and mycobacteria. CD 207/ Langerin degrade HIV in DCs and therefore play a role in reducing the transmission of HIV to T cells.

These cells present their intracellular content to $CD4^+$ helper and $CD8^+$cytotoxic T cells (Guermonprez P *et al*.2002; Gunzer M *et al*. 2000). DCs capture bacteria, viruses, dead or dying cells, proteins, and immune complexes through phagocytosis, endocytosis, and pinocytosis in clathrin- coated pits. Austyn *et al.* (1996a); Mellman I *et al.* (2001); Palucka K. *et al.* (2002) explain that most immature DCs show three types of endocytosis: macropinocytosis, phagocytosis and chlartin-mediated endocytosis.

Immature DCs have several features that allow them to capture antigen. (See Table2.2) Firstly, they can take up particles and microbes by phagocytosis. Phagocytosis is triggered by the attachment of extracellular particles to surface receptors, which in turn signal particle uptake. Secondly, they can form large pinocytic vesicles in which extracellular fluid and solutes are sampled; a process called macropinocytosis (Sallusto F *et al.* 1995a). In contrast to what has been found with other cell types, macropinocytosis in DCs is constitutive and allows continuous internalization of large volumes of fluid (Levine TP *et al.* 1992).

Steinman RM *et al.* (2007) explains that pDCs express activating FcR, TLR7 and TLR 9 for maturation. mDCs in the epidermis express DEC205 and Langerin and induce strong killer T-cell responses. Some mDCs in the dermis express DC- SIGN and mannose receptor which can activate antibody forming B cells. Endocytosis occurs through several pathways which include Fc receptors and lectins like the macrophage mannose receptor (MMR) and DEC 205 (Levine TP *et al.* 1992). Steinman RM (2001a) states in particular that the receptors are found in different intracellular sites. As expected, the MMR is abundant within peripheral early endosomes, through which most adsorptive endocytosis receptors recycle. In contrast, DEC-205 is abundant in late endosomes or lysosomes, which in DCs are rich in antigen presenting MHC class II products. [Figure2.2 (a) + (b)]

The second pathway where DCs process exogenous antigens, is called crosspresentation (Heath WR *et al.*2001). This pathway permits DCs to elicit CD8 as well as CD4 T-cell responses to exogenous antigens such as apoptotic or necrotic tumour cells, virus-infected cells, and immune complexes (Albert ML *et al.* 1998a). Cross-presentation is linked to specific DC antigen uptake receptors, which may be targeted in strategies to load exogenous antigens onto both MHC I and II.

Lipid and glycolipid antigens expressed on pathogens or self tissues are presented by DCs to T cells on CD1 molecules, which are structurally similar to MHC I but specialized to bind lipids instead of peptides. Once DCs have captured Ags, which also provide the signal to mature, their ability to capture more Ag rapidly declines. The captured Ags enter the endocytic pathway of the cell. In macrophages, most of the protein substrates are directed to the lysosomes, organelles with only a few MHC class II molecules, where the Ags are completely digested into 8-18 amino acids in length. DCs are able to produce large

amounts of MHC class II-peptide complexes. This is due to the specialized, MHC class II rich compartments (MIICs) that are present in immature DCs. During maturation of DCs, MIIC's convert to non-lysosomal vesicles and discharge their MHC-peptide complexes on to the cell surface (Turley SJ, 2000a).

Steinman RM (2001a) explained that antigen processing by DCs occurs primarily through an exogenous or endosomal pathway and an endogenous or proteosomal pathway. Harshyne LA *et al*: (2001) showed in his study that DCs degrade antigens within a MHC class II rich endosomal compartment. For MHC Class I peptide complexes to form, the peptides are derived by cathepsin S from newly synthesized proteins in the cytoplasm (Shen L *et al.* 2004). These peptides are processed via the proteoasome and transported into the endoplasmic reticulum via TAP (Transporters for antigen processing). Antigens acquired endogenously (ie, synthesized within the DC cytosol) are typically processed and loaded onto MHC I, whereas antigens acquired exogenously (from the extracellular environment) are processed onto MHC II (Brossart P *et al*. 1997; Mitchell DA *et al.* 1998; Bajenoff M *et al*. 2003).

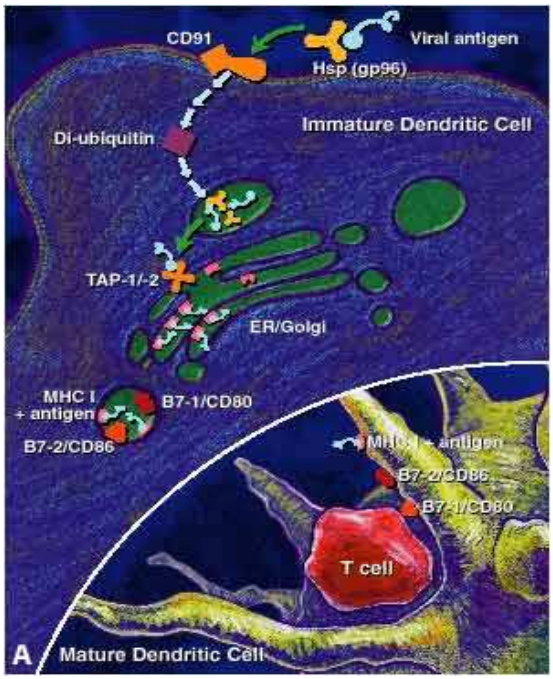

Figure 2.2 (a) The endogenous pathway. Pathogen-derived or selfproteins within the cytosol of antigen-presenting cells (APCs) are enzymatically digested into peptides, mainly by cytosolic proteases called proteasomes, and are then transported by TAP molecules into the endoplasmic reticulum (ER). In the ER lumen, peptides bind to MHC class I molecules, which are subsequently transported via the Golgi to the plasma membrane. (Larsson M *et al*:2001a)

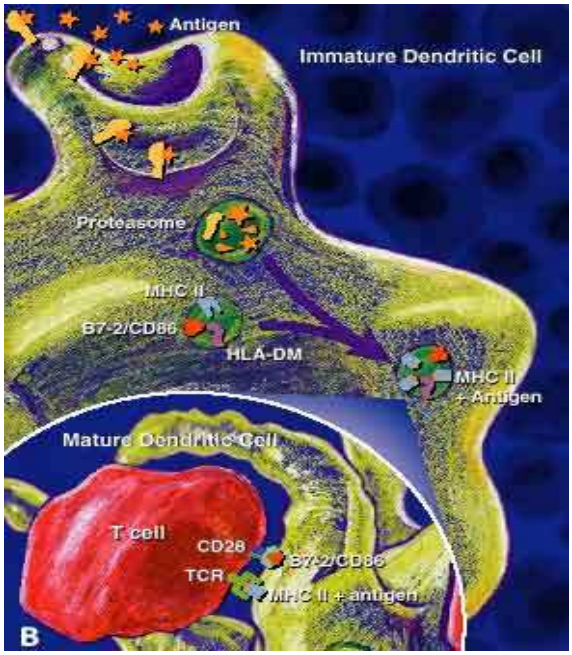

Figure 2.2 (b) The exogenous pathway. Exogenous antigens are derived from proteins that are endocytosed and processed by proteases. Peptides bind to newly synthesized MHC class II molecules in specialized antigen-processing vesicles (MHC class II enriched compartment; MIIC), and the complexes are externalized to the plasma membrane(Larsson M *et al*:.2001a)

DCs are also capable of antigen presentation on CD1 molecules. CD1 molecules are present on mDCs and are a family of nonpolymorphic histocompatibility antigens associated with β2M. CD1 molecules present lipid and glycolipid antigens of both, endogenous and exogenous origin. Antigen processing and presentaion by CD1 is different from that described above for MHC Class 1 and II. These molecules are synthesized in the ER and are expressed on the plasma membrane following traffic to the surface via vesicular transport. (See Figure 2.3)

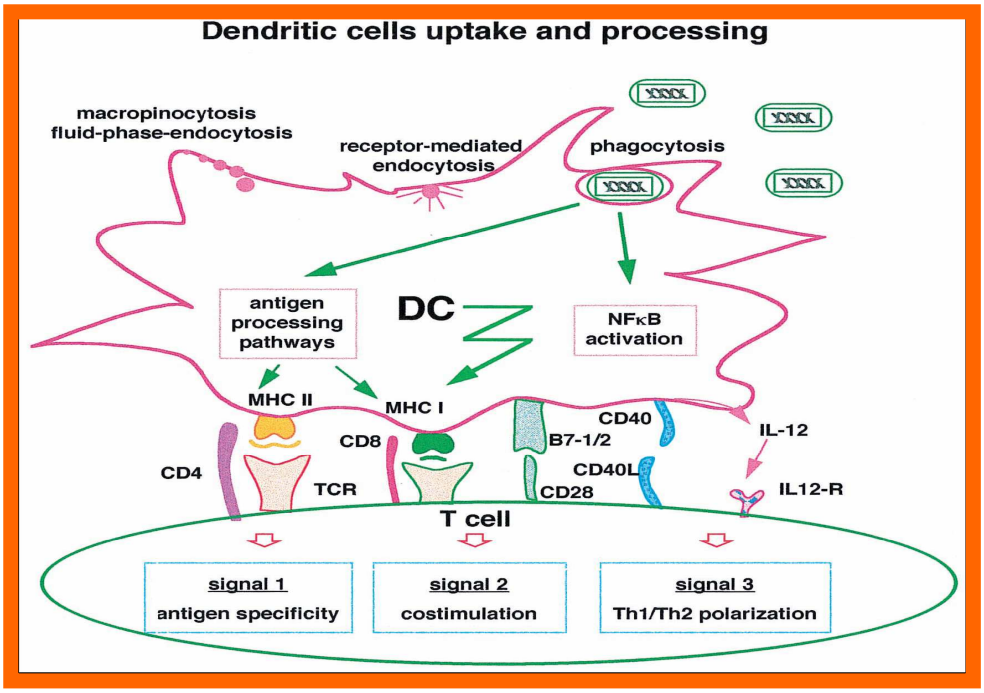

Figure 2.3: Different pathways of antigen uptake, processing and presentation (Rescigno *et al: 2000)*

2.11 Characteristics of immature and mature DCs:

Immature DCs

- High intracellular MHC II in the form of MIICs
- Expression of CD1a
- Active endocytosis for certain particulates and proteins; presence of FcgR and active phagocytosis
- Deficient T cell sensitization *in vitro*
- Low/absent adhesive and costimulatory molecules (CD40/54/58/80/86)
- Low/absent CD25, CD83, p55
- Responsive to GM-CSF
- Maturation inhibited by IL-10

Mature DCs

- Cell shape: Numerous processes (veils, dendrites)
- Motility: Active process formation and movement
- Antigen capture: Macrophage mannose receptor
- Antigen presentation: High MHC class I and II expression
- Abundance of molecules for T cell binding and costimulation, (e.g. CD40, CD54, CD58, CD80 and CD86)
- Cytokines: Abundant IL-12 production; resistance to IL-10 DC-restricted molecules: p55, CD83
- Absence of macrophage-restricted molecules and function: CD14, bulk endocytic activity (pinocytosis, phagocytosis)
- Stability: No reversion/conversion to macrophages/lymphocytes

2.12 DC interactions with lymphocytes

Brocker T *et al.* (2004) explains that during the development of an adaptive immune response, the phenotype and function of DCs play an important role in initiating tolerance, memory and polarized TH1 and TH2 differentiation. Other factors that drive primary immunity are the concentration of antigen, the concentration of cytokines and the genetics of the host that may limit how the interacting cells may respond. She also states that DCs are the only APCs that prime naïve T cells. The study of Patterson S (2000b) showed that the relevant signals by DCs at the DC- T cell interface is likely the most efficient and physiologically relevant mechanism for initiating an immune response.

Only mature DCs prime naïve T cells. (Figure 2.4 and Figure 2.5) Under normal conditions the peripheral DCs reach the lymph nodes and help to maintain peripheral tolerance. DCs initiate or "prime" T-cell responses in secondary lymphoid organs such as lymph nodes, spleen, or mucosal lymphoid tissues. In the lymph nodes the number of DCs that display an antigen peptide is very low. T lymphocytes "scan" the surface of the DCs to find one that expresses their specific MHC peptide ligand. Naïve T cell activation is triggered upon TCR engagement with APCs expressing the right costimulatory molecules. The strength of the T-cell response is dependent on many factors including the state of DC maturation, and the type of maturation stimulus (Shortman K *et al.* 1997a).

Al- Alwan MM *et al.* (2001) mentioned in his study that the contact zone between APCs and the T lymphocytes is referred to as the "immunological synapse." Adhesion and signaling molecules distribute in concentric rings defined as the central and peripheral supramolecular activation clusters (c-SMAC, p-SMAC) at the interface between T cells and APCs (Grakoui A *et* al. 1999). Recent observations indicate that $CD4^+$ T-cell help at

the time of priming is required to generate $CD8^+$ T-cell memory. This effect is thought to be mediated by CD40- CD40L interactions between $CD4^+$ T cells and DCs (Morse M *et al.* 2002). DCs also interact directly with B cells and lymphocytes of the innate immune system. Activated mDCs can directly induce B-cell proliferation, immunoglobulin isotype switching, and plasma cell differentiation through the production of the B-cell activation. Activated pDCs can induce the differentiation of CD40- activated B cells into plasma cells through the secretion of IFN-α and IL-6. Activated NK cells can kill immature, but not mature, DCs and can stimulate DCs to induce protective $CD8^+$ T-cell responses.

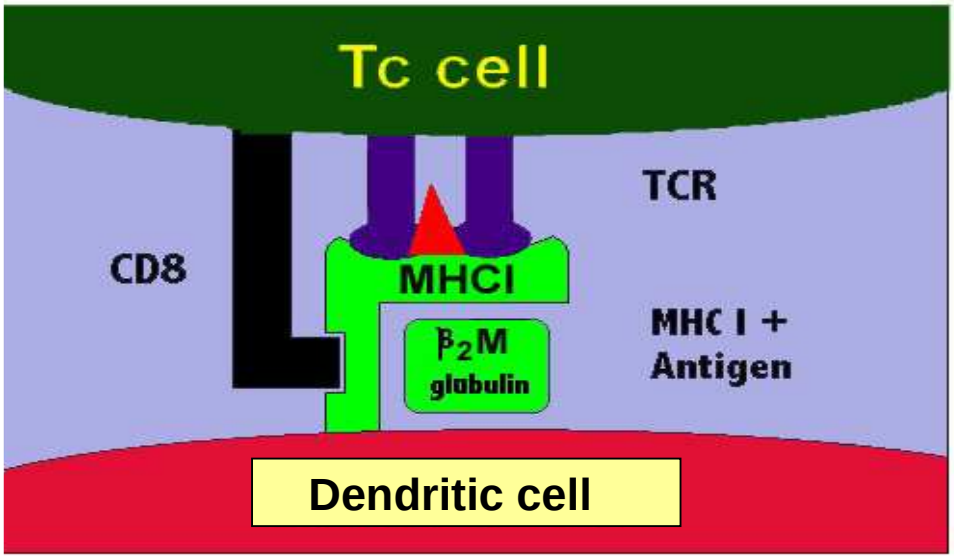

Figure 2.4: Interaction between infected cell and Cytotoxic T cell

(Website: home.comcast.net/.../C/ClassIIpath.gif)

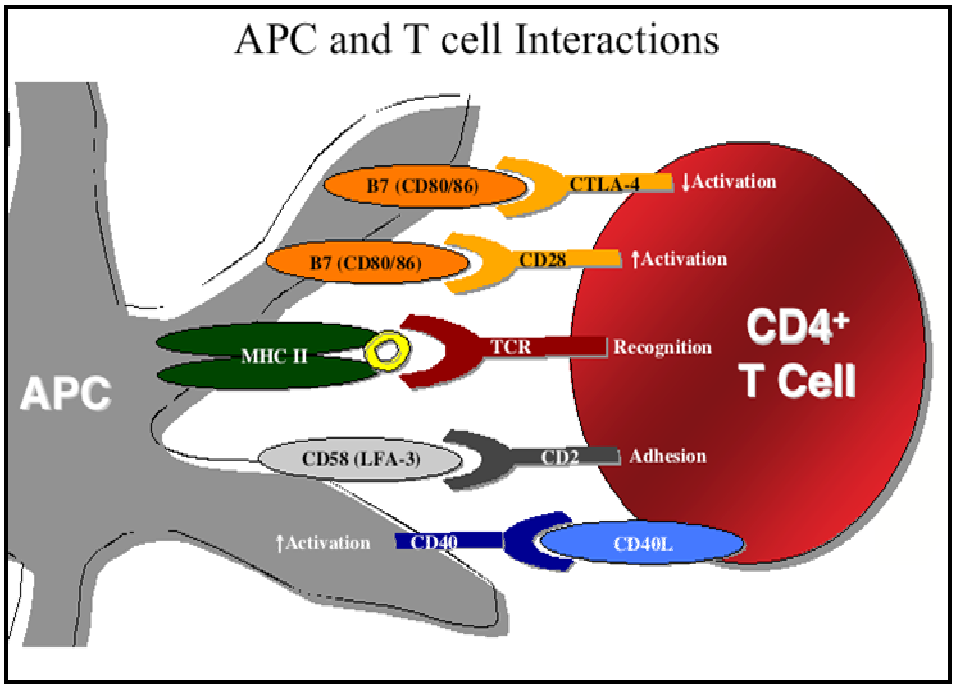

Figure 2.5 Interaction between dendritic and CD 4^{+} cell

(Website: home.comcast.net/.../C/ClassIIpath.gif)

During the development of an adaptive immune response, the phenotype and function of DCs play an important role in initiating tolerance, memory, and polarized TH1 and TH2 differentiation. As discussed, DC subsets have been proposed as playing differing roles in defining the outcome of an immune response. Important factors other than signals delivered by DCs that drive primary immune responses are concentration of antigen in the microenvironment, concentration of cytokines and other soluble factors present in the fluid phase in the vicinity of the APC-T cell interface. Delivery of the relevant signals by DCs at the DC-T cell interface is likely the most efficient and physiologically relevant mechanism for initiating an immune response. CD4 and CD8 T cells respond to peptide antigen displayed on MHC class II and MHC class I molecules, respectively. In the absence of sufficient costimulation, T cells exhibit no response to an antigen or antibody (anergy) or undergo apoptosis.

Cell surface receptors not only facilitate antigen uptake, but also mediate physical contact between DCs and T cells (Miggin SM *et al.* 2006). The soluble cytokine profile secreted by DCs varies with the different stages of DC development and maturation thus influencing the different effector functions characteristic of immature versus mature DCs. A wide variety of cytokines may be expressed (not necessarily simultaneously) by mature DCs including IL-12, IL-15, IL-18, IFN-α , IFN-γ, IL-4, IL-10, IL-6, IL-17, IL-16, and TNF-α. The exact cytokine range expressed will depend on the nature of the stimulus, maturation stage of the DC and the existing cytokine microenvironment. The distinct cytokine patterns released by mature DCs ultimately determine their TH1/TH2 polarizing capacities. Antigens that prime DCs to secrete IL-12 will typically induce TH1 differentiation, whereas antigens that inhibit IL-12 production (e.g. IL-10) will promote TH2 differentiation. Experiments have shown that within 48 hours of antigen stimulation,

all T cells specific for that antigen are retained in the draining lymph node. By day five following antigen stimulation, effector T cells leave the lymph nodes in large numbers and travel to the infection site.

2.13 DCs and B lymphocytes

DCs are known to have major effects on B-cell growth and immunoglobulin secretion. B cells and DCs are both APCs and both are essential for antibody responses but for entirely different reasons; DCs activate and expand T-helper cells, which in turn induce B-cell growth and antibody production. DCs stimulate the production of antibodies directly and the proliferation of B cells that have been stimulated by CD40L on activated T cells.

Differences between DCs and B cells as APCs

- DCs have higher levels of MHC II and accessory molecules
- DCs can make large amounts of IL-12
- DCs can internalize antigens by means of Fc and multilectin receptors; B cells have antigen-specific immunoglobulin receptors and inhibitory FcgR

2.14 DCs in Cancer

The cytotoxic T-lymphocytes are one of the critical effector cells that are able to lyse and destroy tumour cells. Receptors on the surface of T-cells recognise proteins called tumour associated antigens on the surface of tumours. However, the process is complex and in order for the T-cell to become activated, it must recognise the tumour associated antigen and it must have a co-stimulatory signal in order to kill off a cancer cell, as in the absence of this the T-cells become tolerant to the antigen and the tumour continues to grow.

Animal studies done by Esche C *et al.* (1999) showed evidence that DCs can induce immunity to tumours. In tumour cells, the tumour-derived soluble factors (TDSFs) such as vascular endothelial growth factor (VEGF) act as a strong stimulator of immature DCs. At the primary tumour site the induced mDCs and pDCs are recruited through chemokines and their receptors where they are functionally changed into tumour-associated immature DCs (TiDCs) that contribute to tumour immunity (Schuler *et al*:1997). The TiDCs are able to capture apoptotic tumour cells by phagocytosis through a complex composed of the receptor CD36. The TiDCs-captured apoptotic cells (TiDCs-Cp) become more stable and resistant to apoptosis than immature DCs, in which they contribute to the stimulation of immune tolerance. Importantly, despite the fact that the TiDCs-Cp can be matured by inflammatory signals, the MHC-tumour antigen cannot be presented efficiently to naive T cells, resulting in the continued induction of immune tolerance.

The potential to use the patient's own immune system to kill tumour cells shows a growing interest in immunotherapy. One such approach uses bone marrow-derived DCs (DCs), phenotypically distinct and very potent antigen-presenting cells, to present tumour-associated antigens and, thereby, generate tumour-specific immunity (Reis e Sousa *et al.*2003a). Recent studies aim to take cells, such as DCs, out of a patient (or out of a healthy donor) and effectively activate them *in vitro* with bacterial antigens and then (re)infuse them into the patient. This enables the DCs to mature and eventually present the same bacterial antigens as appear in the patient body to the naïve T cells. When these special mature DCs are injected back to the patient's system, they present the bacterial antigens to the patient's immune system, and those T cells that can respond mount an attack on the patient's infected or cancer cells. The idea being that the (re)infused DCs can then

activate the immune system (T cells) to destroy the infected or cancer cells in the patient - now that they know what they are looking for (Berard F *et al*. 2000).

The CTLs arrest in close contact to tumour cells expressing their associated antigen. In regions where most tumour cells are dead, CTLs resume migration, sometimes following collagen fibers or blood vessels. CTLs also infiltrate tumours in depth, but only when the tumour cells express the cognate CTL antigen. In tumours that do not express the associated antigen, CTL infiltration is restricted to peripheral regions, and lymphocytes neither stop moving nor kill tumour cells (Fong L *et al*. 2000; Gabrilovich DI *et al*. 1996). Antigen expression by tumour cells therefore determines both CTL motility within the tumour and deep tumour infiltration.

A number of studies done demonstrated the safety and feasibility of DC immunotherapies. By modifying DCs, researchers are able to trigger a special kind of autoimmune response that includes a T cell attack of the cancer cells (Colaco CA, 1999; Nouri – Shirazi M *et al*. 2000) (Figure 2.6). Other studies showed that a cancer antigen alone is not enough to activate the DCs, and therefore scientists first fuse a cytokine to a tumour antigen with the hope that this will send a strong antigenic and activation signal (Geiger J *et al*. 2000). Next, they culture a patient's DCs in the incubator and let them take up this fused cytokine-tumour antigen. This enables the DCs to mature and eventually display the same tumour antigens as appear on the patient's cancer cells (Hermans IF *et al*. 1998a). When these *in vitro* matured DCs are given back to the patient, the cells present the acquired tumour antigens to the pasient’s T cells that can mount an attack on the patients cancer cells (Jenne L *et al*. 2000; Dhodapkar MV *et al*. 2000b).(Please refer to Figures 2.6 and 2.7).

Steinman RM *et al.* (2007c) states that DCs can be used for the prevention and treatment of cancer for the following reasons:

- The attack on cancer by the immune system is broad enough to include multiple targets, including mutant proteins expressed by the cancer
- DCs have the potential to activate and increase the different arms of the cell-mediated resistance such as NK cells and γδ T cells, each of which recognizes different changes in cancer cells
- DCs generated *ex vivo* and DCs in systemic lymphoid organs retain their immunizing capacities in cancer patients. These features of DCs can be used to generate therapeutic immunity or to prevent cancer during the early stages of disease.

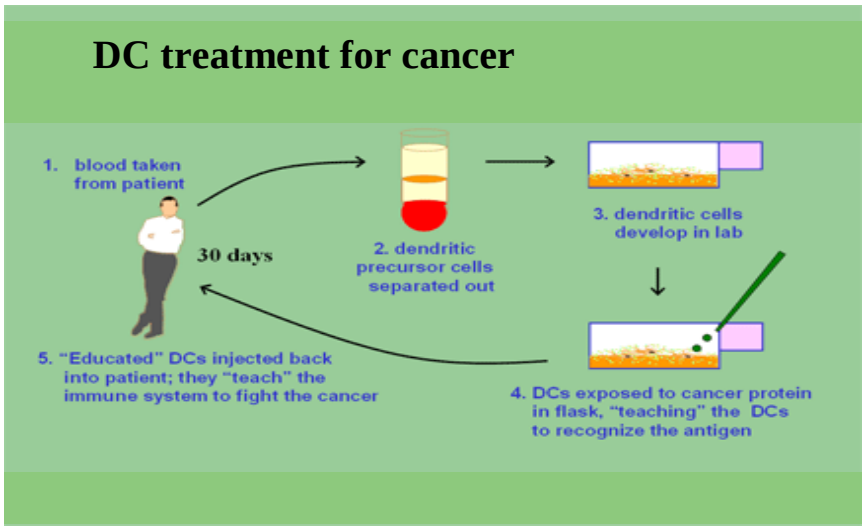

Figure 2.6: Teaching the patient's own DCs (Website:www.nwbio.com)

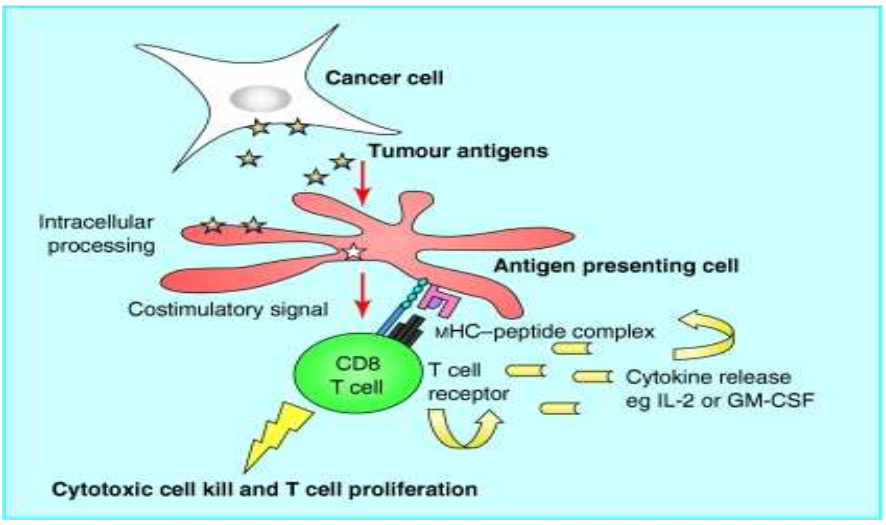

Figure 2.7: The DC processes the antigen and presents it to cytotoxic T cells. The activated T cells proliferate and secrete cytokines. This results in the production of a cascade of immune effector cells which will now recognize the cancer cell and destroy it. (Website: www.nwbio.com)

2.15 DCs and their role in HIV pathogenesis:

DCs are professional antigen presenting cells that sample the environment at sites of pathogen entry. Sentinel immature DC develop into mature effector DC upon activation by microorganisms, and migrate to the draining lymph nodes where they stimulate naïve TH cells. HIV-1 has been proposed to make use of this migratory process, being captured by the DC and delivered to the lymph node where the virus is transmitted to $CD4^+$ T cells (Donaghy H *et al*. 2006c). The lymph node then becomes the principal site of virus production (Lore K *et al*. 2005). DC can capture HIV through several receptors, of which DC-SIGN is the best studied example. After capture, the virus dissociates from DC-SIGN and resides in an unidentified non-lysosomal compartment (Sol- Foulon N *et al*. 2002). After T cell encounter, HIV is recruited to the site of T cell interaction. This so-called "infectious synapse" depends on strong cell-cell adhesion (Garcia E *et al.* 2005; Lore K *et al*. 2005; Piqueras B *et al.* 2006).

Groot F *et al.* (2006b) explained that the mDCs including dermal DCs, Langerhans cells and interstitial DCs are associated with HIV-1 capturing during sexual transmission, whereas pDCs play an important role in the innate immune response to viruses, including HIV-1. They also discovered that differently matured mDCs have different HIV-1 transmission efficiencies (Smed- Sorensen A *et al.* 2005). The study also revealed that regardless of their state of maturation, pDCs inhibit HIV-1 replication in T cells through the secretion of IFN-γ. This study showed that the two main DC subsets have opposing roles in HIV-1 infection of T cells. Several other studies (Barron MA *et al*. 2003; Donaghy H *et al*. 2001a; Pacanowski J *et al*. 2001) showed that the numbers of mDCs and pDCs are reduced in HIV infected patients.

2.16 DCs and autoimmunity:

Bayry J *et al.* (2003) explain that autoimmune disorders develop when the immune response targets the body's own cells and tissues. In autoimmunity the breakdown of tolerance occurs. DCs acquire autoantigens, mature and defectively stimulate normally inactive autoreactive T cells that express a TCR either specific for or able to crossreact with the presented self-peptide. In an autoimmune response, these "self-proteins" are being presented to T cells which cause the T cells to become "self-reactive". Researchers believe that targeting DCs may stop the faulty immune response at a higher "upstream" level since T-cells frequently receive their information from DCs. Studies done by Bayry J *et al.* (2003) explained DCs can be pulsed with the endogenous antigen to produce the relevant "self-peptide". The relevant self-peptide is different for each individual because MHC products are highly polymorphic and each individual MHC molecules might bind different peptide fragments. The "self-peptide" may then be used to design competing peptides or to induce tolerance to the self protein in the individual in need of treatment.

Autoimmune disease occurs when autoreactive T cells cause tissue damage which can lead to infection. Antigen presenting cells migrate to the sites of infection and express co-stimulatory molecules and cytokines that allow naïve T cells to react to self peptides expressed by the tissue cells. In the case of autoimmunity, the most well known cell types that are being dysregulated are the T cells and B cells. B cells produce large quantities of antibodies, and various mechanisms can lead to tissue injury, for example, by the formation of large deposits of immune complexes. Studies done lately showed that DC play a much larger role in autoimmune responses than originally thought (Turley SJ: 2000). DCs cannot differentiate between the antigens they capture at the site of inflammation and thus will accidently present self-antigens along with pathogen-specific antigens. DCs also play a

central role in peripheral T cell tolerance, by inducing T cell anergy or unresponsiveness to self-antigens. The activation of naive T cells by DCs requires 2 signals, antigen presentation and costimulation. Disruption of the co-stimulatory pathways has been shown to be effective in blocking the pathogenic process in several models of autoimmune diseases. Due to its immune inhibitory function, DCs have great potential for treatment of autoimmune diseases, for organ transplantations, and for immunotherapy. The CD40/ CD40L plays an important role in the activation of T cells and therefore it will provide an ideal target for therapeutic intervention studies.

Recent studies by Belz GT *et al.* (2002) showed that DCs can induce tolerance in the steady state. This evidence is critical to the prevention of the autoimmune response. Since most autoimmune diseases are associated with an ongoing immune response, it is important to make use of the ability of specific DCs populations and convert the immune response from a pathogenic response to a protective one.

2.17 Identification of DCs by flow cytometry:

What is Flow cytometry?

A flow cytometer, called a Fluorescence Activated Cell Sorter (FACS), has several key components (Givan AL, 2001; O'Gorman MRG *et al*. 2001)

- A light or excitation source, typically a laser that emits light at a particular wavelength;
- A liquid flow that moves the suspended cells through the instrument and past the laser;
- A detector, in this case a photomultiplier tube, which is able to measure the brief flashes emitted as cells flow past the laser beam. (Figure 2.8)

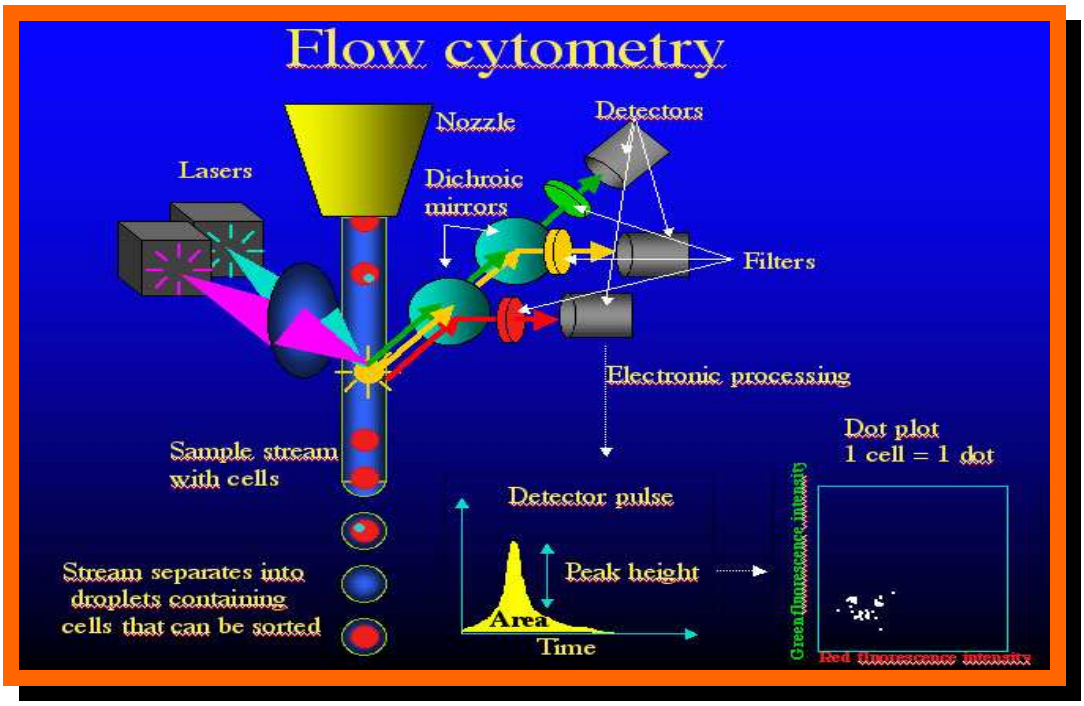

Figure 2.8 Basic picture to show the components of a typical flow cytometer (Website: www.scq.ubc.ca)

In a flow cytometer, single cells move past the excitation source and the light hitting the cells is either scattered or absorbed and then re-emitted (fluorescence). This scattered or re-emitted light is collected by the detector.

Bakke AC (2001) explains that various fluorescent dyes are commercially available and the most common fluorescent dyes are:

- fluorescein isothiocyanate (FITC)
- phycoerythrin (PE)
- peridinin chlorophyll protein (PerCP)
- allophycocyanin (APC)

The amount a cell scatters or fluoresces light is measured by the detector and subsequently needs to be displayed for interpretation. These profiles of cells are normally displayed as dot plots or histograms (Bleesing JJ *et al.* 2001).

2.18 Identifying DCs

The primary subsets of DC precursors that have been described in human blood are distinguished by the absence of expression of several lineage markers for monocytes, lymphocytes and NK cells, and the differential expression of CD11c and CD123 (Van Voorhuis WC *et al.* 1982).

- Plasmacytoid DCs lin 1^- $CD11c^-$ $CD123^+$
- Myeloid DCs lin 1^- $CD11c^+$ $CD123^-$

These include the presence of large amounts of class II MHC antigens and the absence of various lineage markers such as CD3, CD14, CD19, and CD56 (Shortman, K *et al*. 2002b). The inclusion of HLA-DR in addition to CD11c, CD123 and lineage cocktail allows the discrimination of $CD123^+$ DCs from basophils (Macey MG *et al.*1998). DCs also express costimulatory molecules including CD80 and CD86, which are upregulated during DC activation. CD86 is a marker of early DC maturation, while CD80 only appears in mature DCs. Two other markers for mature DCs are CD83 and CMRF-44. CD83 also stains activated B cells and CMRF-44 will also stain macrophages and monocytes (Zhou LJ *et al.* 1995). The identification of DCs by surface phenotyping may be accomplished by demonstrating a high level of MHC class II or a costimulatory molecule such as CD80 and the absence of the lineage markers.

We included he lineage cocktail 1 (lin 1) to distinguish between DCs and other lymphocytes. This cocktail contains antibody clones which stain neutrophils, monocytes, lymphocytes and eosinophils. It includes CD3, CD14, CD16, CD19, CD20 and CD56. Basophils and peripheral blood dendritic cells can be distinguished from other leucocytes by their lack of staining with lin 1.This cocktail can be used for studies of dendritic cell subsets and basophils in peripheral blood.

2.19 Cytokine production by DCs:

Another important measure of the functional potential of DCs is their ability to produce certain cytokines upon stimulation. Nakahara S *et al.* (2003) showed DCs stimulated with LPS, TNF-α and killed *Streptococcus pyogenes* produced a significant amount of IL-12 and IFN-γ. Other studies (Prussin C. *et al.*1995; Macatonia SE *et al*. 1995b; Tang HL *et al*. 1999) demonstrated that mature DCs express IFN-α, IL-12 and IL-6. Through their secretion of particular cytokines (and chemokines) at particular times, DCs contribute to regulating their own migration, the recruitment of other immune cells, and the polarization of the T-cell response.The major cytokines that play a role in DCs are IL-12, IL-4, IL-10, IL-6, TNF-α and IFN-γ. Langenkamp A *et al*. 2000; Fiebieger E *et al*. 2001 state in their studies that some stimuli trigger IL-12 production that leads to a TH1 response. Other stimuli fail to do so and favor the TH2 response. His study showed that soon after stimulation DCs primed TH1 responses and at later time points the same cells primed a TH2 response.

Flow cytometric analysis of cytokine production by DCs:

The method used is known as multiplexing, which means the simultaneous assay of many analytes in a single sample (Oliver KG *et al.* 1998; Savary CA *et al.* 1998). The BD™ Cytometric Bead Array (CBA) is used to detect a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes (Camilla C *et al.* 1998; Chen B *et al.* 1999b; Cook E *et al.* 2001). This method is combined with flow cytometry to create a powerful multiplexed assay (Bueno C *et al.* 2001; Fulton R *et al.* 1997; McHugh TM *et al.*1994; Chen R *et al.* 1999). For this study we will determine the levels of cytokines secreted by DCs during their maturation process. These cytokines include IFN-γ, IL- 12, IL -10, IL- 6, TNF-α, IL- 4, IL-2, IL-8 and IL-1β. See full methodology in Chapter 6.

2.20 Mixed Killed Bacteria: Candidates for activation and maturation of DCs

Mixed bacterial vaccines are produced from disease causing organisms. Selected species of the disease causing bacteria are grown in culture, washed with 0.9% saline, centrifuged, resuspended in salt solution and killed with phenol. The vaccine used in this study is Mixed killed Bacterial Vaccine (MBV) obtained from Dr. Brigitte Riedelsheimer (Düsselsdorf, Germany.) It contains whole bacteria, but no phenol. The concentration of the bacteria in the vaccine is 10^9/ml CFU/ml

Staphylococcus aureus, Streptococcus pneumoniae, Moraxella (Neisseria) catarrhalis, Klebsiella pneumoniae, Heamophilus influenza, Corynebacterium diphtheria, Streptococcus pyogenes, Pseudomonas aeruginosa, Proteus vulagaris, Proteus mirabilis, Candida krusei and *Candida albicans* and include the following bacterial species

Nakahara S *et al.* (2003) and Veckman V *et al.* (2004) showed in their studies that DCs can be stimulated with killed *Streptococcus pyogenes. Streptococcus pyogenes* causes pharyngitis, scarlet fever and impetigo or cellulites. The results of this study showed that DCs incubated with the *S. pyogenes* peptide did produce TH1 type cytokines and can efficiently induce potent CTL responses, specific to the epitope peptide of tumour – associated antigen presented on the DCs (Celluzzi CM *et al*. 1996; Fujimoto T *et al*. 1997). With this study in mind it was hypothesized that the MBV used in this study might modulate DC maturation. Hopefully the matured DCs will present all these epitopes to the T cells and activate the immune system.

In the study of Nakahara S *et al*. (2003), they state that a potent immune response could be efficiently induced only with mature DCs. Maturation of DCs can be influenced by many cytokines like TNF-α and GM-CSF. Other studies done by Sallusto F *et al.* (1999c) showed that these cytokines alone cannot induce full maturation of DCs. In addition to these cytokines, studies by Cella M *et al*. (1997) and Rissoan MC *et al*. (1999) described that bacteria can stimulate DC maturation. A study done by Romagnoli G *et al*. (2003) showed that *Candida albicans* triggered DC maturation and activation. The study also showed that DC maturation induced by Candida triggered a TH1 response. The cells secreted IFN-γ and IL-2, but no IL-4 and IL-10. It has been hypothesized that the MBV and Coley's Toxin might modulate DC maturation and hopefully induces potent Cytotoxic T cells, specific to the peptide presented by the DCs.

2.21 Coley's Toxins

From 1891 until his death in 1936, Coley studied the effects of inoculation with live bacterial cultures on human malignancy. Coley developed a vaccine consisting of extracts of killed Gram-positive *Streptococcus pyogenes* and Gram-negative *Serratia marcescens*, which became known as `Coley's toxins'. These toxins produce many of the symptoms of bacterial infections, such as fever and chills, without the need to worry about producing an actual infection (Bickels J *et al.*2002). A key aspect that Coley found to be necessary for tumour regression was the induction of a mild to moderate fever. Basu S (2003b) states that even brief exposure to elevated temperatures has a powerful effect on the immuno-stimulatory capacity of DC. Elevation of body temperature can cause maturation of DC in skin and other tissues. The mature DCs are better presenters of antigen to the naive T cells in the lymph nodes. The elevation of cytokine levels such as TNF- α, frequently related with fever, also contributes to DC maturation.

Coley's Toxins were used successfully against different types of cancer. After an injection, high fever usually develops, but this is intended. The cancer tissue may become necrotic, which then must be removed by drains. One of the biologically active ingredients in Coley toxins is a lipopolysaccharide (LPS) which causes hyperthermia. This purified LPS induces a fever for three to four hours that enhances lymphocyte activity and boosts tumour necrosis factor (TNF). Coley and other researchers have emphasized that some tumour types appear to be more susceptible to the toxins than others, in particular, sarcomas or other tumours of mesenchymal origin such as renal and ovarian carcinomas and possibly some lymphomas.

Recent microscopic studies of human cancers have found that there are generally high concentrations of immune cells within the tumour. Unfortunately, these immune cells are not receiving the proper signals to fight the cancer. Thus, the cancer continues to grow and spread despite the presence of many immune cells. Coley found that when his vaccine caused a fever, the cancer would start to shrink and the number of immune cells in the blood would increase dramatically. Fevers are routinely suppressed with drugs such as acetaminophen, aspirin or ibuprofen. The immune system over time becomes tolerant to a particular dose and therefore Coley would slowly increase the dose until a fever was once again produced. Doses were increased to compensate for the reticuloendothelial system's increasing capacity to clear the contents of Coley's Toxins. He also found that even though the tumour would start to shrink when the vaccine caused a fever, if he stopped injecting the vaccine too early, the cancer would start to grow again. Therefore, the immune cells could be stimulated to fight the cancer, but only for a limited time. Coley's Toxins were used against different types of cancer from the year 1893 to 1963. In 1963 Coley's Toxins were assigned "new drug" status by the Food and Drug Administration (FDA), making it illegal in the U.S. to prescribe this kind of therapy outside of clinical trials. However, as Coley's Toxins are known and thus not patentable, no clinical trials have been financed since then. The illegalization of Coley's Toxins in the United States has led to a loss of unwritten knowledge of how to exactly create and apply Coley's Toxins.

Why was his treatment not successfully assimilated into the practice of medicine? The literature lists the following reasons:

- There were several preparations in use, and they were of very uneven quality. Many physicians relied on inferior and variable commercial preparations. The toxins were never standardized.

- The administration of the toxins was an imprecise art that took many weeks or months. Many surgeons had neither the patience to learn it nor the required hospital beds for the extended time that was often needed (Bickles J *et al.2002)*

The Coley's Toxin used in this study, obtained from Dr Brigitte Riedelsheimer, (Düsselsdorf, Germany) consists of *Streptococcus pyogenes* and *Serratia liquefaciens* peptides at a concentration of 10^9 CFU/ml (Heat killed). *Streptococcus pyogenes* infections are sometimes accompanied by the release of toxins from the bacteria.

2.22 The effects of fever on immune cell functions:

Effects of fever on components of the immune response have been reported including effects on innate and acquired immunity and on cytokine expression and function.
Van Oss CJ *et al*. (1980) showed in their study that human polymorphonuclear cell (PMN) motility and phagocytosis are potentiated in the case of fever, but PMN chemotaxis is not enhanced, and bactericidal capacity is only weakly and inconsistently increased by exposure to these temperatures. They also confirmed in their study that macrophage functions are enhanced during episodes of fever, including expression of Fc receptors, phagocytosis, pinocytosis and killing of intracellular bacteria. However, like PMNs, macrophages have markedly reduced function at temperatures > 41 °C. By contrast, the cytotoxic activity of human natural killer (NK) cells has been shown to be reduced in the case of fever, demonstrating that the effects of fever on immune function are cell specific.
Basu S *et al*. (2003) proved in his study that elevation in body temperature causes maturation of DCs in the skin and other tissues. With these facts in mind we hypothesized that the Coley's toxin would enhance the maturation and activity of the DCs.

2.23 Aim of this study

The DC system of APCs is the initiator and modulator of the immune response. DCs have been referred to as “professional” APCs, since the principle function of the DCs is to present antigens to resting naïve T lymphocytes. DCs (only mature DCs) are the only APCs that prime naïve T cells and only mature DCs can prime naïve T cells. The aim of the study was the following:

1. To develop a method of detecting circulatory DCs by phenotypic analysis using flow cytometry
2. To use the MBV and Coley’s Toxin to determine whether these bacterial products could induce the activation and maturation of the DCs
3. To analyse the cytokine profile of DCs in order to determine whether the activation /maturation process gave rise to a beneficial biological outcome *in vitro*

DC based therapies have potential application across a wide variety of diseases, ranging from infectious diseases such as HIV to cancer, inflammatory disease and autoimmune diseases. The market potential for a successful treatment is large (Cerundolo V *et al.* 2004). Mohamdzadeh M *et al.* (2004) states that market opportunities include the manipulation of DC activity for the development of effective therapeutic vaccines, the modulation of DC’s activity to manage autoimmune diseases and targeting the underserved markets through the development of novel therapies that make use of the immune system to prevent and treat disease. These cells have the potential to be used as enhancers of responses to infectious diseases and due to their capacity to induce a primary immune response DCs can be used as vectors (vaccine carriers) for immunotherapy (Paczesny S *et al.* 2003).

Chapter 3

Identification of dendritic cells in health and disease by making use of Flow Cytometry

Abstract:

This chapter describes a flow cytometric method for the phenotypic identification of DCs in normal individuals and in different diseases. In this chapter we also investigated the maturation status of DCs in different diseases. DC subsets are distinguished by different markers. These cells do not express several lineage markers for lymphocytes, monocytes and NK cells. The DC expresses CD11c (in the case of mDCs) and CD123 (in the case of pDCs). The inclusion of HLA-DR in addition to the previous described markers allows the discrimination of CD123 $^{+}$ DCs from basophils. Whole blood samples (heparinized) are needed for the assay. The assay requires two tubes per sample which enables quick analysis of these rare subsets with a small sample volume. This assay was applied to peripheral blood samples obtained from healthy individuals and individuals with cancer, HIV and HIV and TB infected patients. There is a great interest in exploiting DCs to develop immunotherapies for cancer, chronic infections and autoimmune disease,

3.1 Introduction:

Identified by Ralph Steinman more than 30 years ago (Steinman *et al.*1973) based on its curious morphology with long dendritic processes, this cell was difficult to study and define until the advent of modern culture techniques. In this chapter we will describe all the flow cytometry markers that can help in identifying DCs. The assay is designed to detect two different subsets of peripheral blood DCs, $CD123^{+}$ and $CD11c^{+}$. The inclusion

of HLA-DR in addition to CD11c and CD123 allows the discrimination of $CD123^+$ DCs from basophils. Basophils express similar levels of CD123 as the $CD123^+$ DC subset, but can be discriminated by their lack of HLA-DR expression (Ida JA *et al.* 2006).

- Plasmacytoid DCs lin 1^- $CD11c^-$ $CD123^+$
- Myeloid DCs lin 1^- $CD11c^+$ $CD123^-$

DCs also express costimulatory molecules including CD80 and CD86, which are upregulated during DC activation. (These activation markers are described in chapter 4). DCs are crucial in the defence against pathogens. Invading pathogens are recognized by Toll-like receptors (TLRs) and receptors such as C-type lectins expressed on the surface of DCs. However, it is becoming evident that some pathogens, including viruses, such as HIV-1, and non-viral pathogens, such as *Mycobacterium tuberculosis,* subvert DC functions to escape immune surveillance by targeting the C-type lectin DC-SIGN (DC-specific intercellular adhesion molecule-grabbing nonintegrin) (Geijtenbeek TB *et al.* 2000). Notably, these pathogens misuse DC-SIGN by distinct mechanisms that either circumvent antigen processing or alter TLR-mediated signalling, skewing T-cell responses (Kwon DS *et al:* 2002). This implies that adaptation of pathogens to target DC-SIGN might support pathogen survival. In infectious diseases, the particular DC activation state is dependent on which microbe is encountered, which TLR are engaged, and whether the TLR are functioning normally (Kapsenberg ML: 2003; Pulendran B *et al.*2004b).
TLR signaling is essential for generating effective TH1 immunity against for example *M. tuberculosis.*

3.2 DCs and *Mycobacterium tuberculosis:*

Infection with *Mycobacterium tuberculosis* continues to be a major cause of mortality and morbidity throughout the world. Mycobacteria persist in macrophages within the granuloma in the organs of infected hosts. A study done by Demangel C *et al*. (1999) showed that BCG loaded DCs induced appropriate maturation of the DCs, *in vitro*, including stimulating IL-12 secretion. From the phagosomes, where they reside, they are believed to secrete proteins, also called "secretory antigens" (Beatty W *et al*. 2000). In this study they explain that these antigens contribute to the development of protective immunity by serving as targets for the immune system early in the infection where they are likely to be taken up by antigen-presenting cells (APCs). Among the most potent of these APCs are the different subsets of DCs that have the ability to stimulate inactive, naive, and memory T lymphocytes (Banchereau J *et al*. 2001b). Immature DCs are programmed for antigen capture and they express low levels of surface major histocompatibility complex [MHC] and costimulatory molecules. After contact with various stimuli—such as lipopolysaccharide (LPS), tumour necrosis factor alpha (TNF-α), CD40 ligand and certain antigens they undergo a process of maturation (Nelson EL *et al:* 1999). Recently, the 19-kDa lipoprotein of *Mycobacterium tuberculosis* has been shown to induce the maturation of DCs. During maturation, they up-regulate their MHC (class I and II) and costimulatory molecules (CD80, CD86, CD40, and CD54) and are very efficient T cell stimulators. Therefore, antigens that are able to induce maturation of DCs play a major role in defining the character of primary immune responses against the pathogen and, thereby, have an important role in determining the course of an infection

DCs are the major antigen-presenting cells in the induction of cellular responses to intracellular pathogens, such as mycobacteria. The interaction of DC with microbial antigens may be the controlling factor in the development of a TH1-orientated protective immunity. Demangel C (2000) explained mycobacteria-infected DC have an enhanced capacity to release pro-inflammatory cytokines and chemokines and are potent inducers of interferon-gamma-producing cells *in vivo*. DCs are likely to play a key role in anti-mycobacterial immunity and protection against tuberculosis. DCs exhibit the unique ability to ingest pathogens at the sites of infection, and to migrate to secondary lymphoid organs, such as the lymph nodes, to present pathogen-derived antigens to naive T cells. Several *in vitro* and *in vivo* studies, in mice and rats, have shown that DCs can be activated upon infection with various mycobacteria, including *M. tuberculosis* and the vaccine strain *M. bovis* BCG (Henderson RA *et al.*1997; Demangel C *et al.* 1999). Mycobacteria-activated DCs express surface presentation, co-stimulation and migratory molecules (Kim KD *et al*. 1999).

Mycobacterium tuberculosis, the cause of tuberculosis, remains a pathogenic organism capable of infecting a large number of individuals and of resisting the immune response of the infected host. The main constituents of this response are the antigen presenting cells such as DCs cells, macrophages and T lymphocytes. Studies of the interactions between *M. tuberculosis* and the antigen presenting cells has shown that DCs do not permit intracellular growth of *M. tuberculosis*, unlike that seen in macrophages. A hostile intracellular compartment creates a bacteriostatic environment. *M. tuberculosis* is internalised by binding to a C-type lectin receptor (DC-SIGN) (Tailleux L *et al*. 2003). This receptor recognises polysaccharide compounds on the surface of *M. tuberculosis*. This sugar-lectin bond may compensate for the bond between bacterial compounds and

Toll receptors, partially inhibiting the protective inflammatory reaction or compensating for an excessive inflammatory reaction. This bond encourages both the persistence of quiescent bacteria in the DCs and the reciprocal adaptation of the host and the bacteria over the course of time. Freshly isolated human lung DCs were found to express DC-SIGN. The intracellular behavior of *M. tuberculosis* inside DCs differs compared to macrophages, with a failure of replication. The intracellular compartment of the DC, disconnected from the exocytic and endocytic pathways, and is characterized by the absence of endoplasmic reticulum and Golgi features.

Animal studies done showed mycobacteria-infected DCs can stimulate mycobacterial antigen-specific T cells *in vitro* and give protection against mycobacteria. *In vivo*, it is likely that mycobacteria-infected DCs can stimulate the development of IFN-γ-secreting T cells, essentially through the secretion of IL-12. Giacomini E *et al.* (2001) showed that DCs in cultures exposed to *M. tuberculosis* up-regulated surface MHC class II and increased the DC maturation marker CD83, and CD40, CD54, CD58, CD80, and CD86. Henderson *et al.* (1997) found that DCs phagocytose *M. tuberculosis* efficiently, a process resulting in up-regulation of MHC class I and MHC class II. CD40, CD54, CD58, and CD80 were also increased in *M. tuberculosis* infected DCs. Jiao *et al.* (2002) showed in his study an increased number of DC following BCG administration. They next analyzed the expression of costimulatory molecules by these cells required for T cell activation. Fifteen to 20% of DC showed an increase of CD86 and CD83 from 4 to 48 h post infection. The increase of CD80 and CD40 occurred more slowly but was observed on a much larger set of DC at day 2.

3.3 DCs and HIV:

DCs play a critical role in the generation of an effective immune response against incoming pathogens. Bancehraeau J *et al.* (2000a) explained that DCs act as antigen-presenting cells , transporting processed antigens from peripheral tissue to draining lymph nodes. In the lymph nodes the DCs present the antigens to circulating T cells, initiating an antigen-specific T-cell response. Persisting HIV infection leads to the destruction of T cells. DCs are early targets for infection by HIV at peripheral sites. In early infection, HIV enters both DCs and macrophages/monocytes at mucosal sites by utilizing target cell surface CD4 and a chemokine receptor CCR 5 (Larsson M *et al.* 2004b). Infected monocytes stimulated with GM -CSF and IL-4 develop into DCs and the exposure to LPS stimulates the DCs to mature and migrate to secondary lymphoid tissue where they can infect $CD4^+$ T cells. Because of their important role in inducing the antiviral response, many viruses and bacterial pathogens target DC and T cells (Alcami A *et al*. 2000; Tortorella D *et al*. 2000).

Upon exposure of mucosal surface to HIV-1 during transmission, different cells are exposed to the virus. Among these are immature DC, Langerhans cells, and resting T cells. Immature DC, through viral engagement with C-type lectins, may be one of the first leukocytes to capture HIV-1with subsequent replication and crossing of mucosal surfaces, thereby transmitting virus to nearby $CD4^+$ T cells for amplification through DC and T-cell interaction. Recent studies have also demonstrated that DCs infected with HIV-1 selectively fail to mature, and are defective in interleukin 12 (IL-12) production.

mDCs are professional antigen-presenting cells for T cells, and include Langerhans cells, dermal DCs, and interstitial DCs. These cells have been associated with HIV-1 capture and sexual transmission, whereas pDCs play an important role in the innate immune responses to different types of viruses, including HIV-1. Groot F *et al.* (2006) found in his study that mDCs enhance HIV-1 infection through capture of the virus and subsequent transmission to T cells, and that differently matured mDC subsets have different HIV-1 transmission efficiencies. These differences were not due to soluble factors, viral capture differences, or the expression of integrins ICAM-1, -2, -3, or LFA-1 (Sanders RW *et al.* 2002).

DCs play a major role in HIV pathogenesis (Rowland Jones SL, 2000). Studies done by (Pacanowski J *et al*.2001; Chehimi J *et al.*:2002; Donaghy H *et al*.2003b) showed that the numbers of both mDCs and pDCs are substantially reduced in the blood of patients infected with HIV-1 and that the DCs are functionally impaired with respect to T-cell proliferation and cytokine production (Macatonia *et al*. 1990a; Pacanowski J *et al*. 2001; Donaghy H *et al*. 2003b). Epithelial DCs appear to be one of the first cells infected after sexual transmission and transfer the virus to CD4 lymphocytes, simultaneously activating these cells. Such transfer may occur locally in inflamed mucosa or after DCs have matured and migrated to local lymph nodes. Therefore, the mechanism of binding, internalization, infection and transfer of HIV to CD4 lymphocytes is of great interest.

Stebbing J *et al*. (2006) explained that mDCs enhance and pDCs inhibit HIV replication in T cells, a process mediated by different mechanisms for each DC subset: mDCs actively transmit HIV to T cells, and factors that are secreted during mDC maturation or after T-cell encounter probably do not influence this process. Secreted factors during pDC activation were completely responsible for the inhibition of HIV-1 infection of T cells. pDCs secrete high amounts of IFN-α, which inhibits HIV replication, but most interestingly, they also

secrete a small molecule less than 3 kDa in size that inhibits HIV replication. An interisting pathway that is well developed in DCs is the exogenous pathway for nonreplicating viral antigens to be presented on class I MHC products. This should allow DCs to stimulate $CD8^+$ T cells after uptake of antibody-coated HIV-1 and dying infected T cells.

Recently, the role of the C-type lectin DC-SIGN as a DC receptor for HIV has been intensively studied with *in vitro* monocyte-derived DCs. However, it is clear that other C-type lectin receptors such as Langerin on Langerhans cells and mannose receptor on dermal DCs are at least equally important for gp120 binding on epithelial DCs. Therefore, DC manipulation for maximal antigen presentation and TH1 cytokine production may form the basis of a new generation of vaccines, with improved efficacy against mycobacterial infections.

Interactions between HIV-1 and the cell surface

Exposure of DC to gp120 led to an upregulation of activation markers indicative of functional maturation (Manca F *et al.* 1994; McCarthy DA *et al.* 1997). Despite their phenotype, however, these cells retained antigen uptake capacity and showed an impaired ability to secrete cytokines and chemokines and to induce T-cell proliferation. HIV-1 enters cells by direct fusion with the plasma membrane or through receptor-mediated endocytosis. Uptake via nonspecific endocytosis is considered to be less significant. HIV-1 acquires surface glycoproteins including Env. Poon DTK *et al.* (2000) explains that Env is made up of the surface glycoprotein $gp120^{SU}$. The $gp120^{SU}$ is noncovalently bound to the $gp41^{TM}$ transmembrane protein. This complex binds to CD4 and various coreceptors, usually CXCR4 or CCR5 and allows for viral entry. Fusion is pH independent and facilitates the insertion of HIV-1 cores into the cytoplasm, which, depending upon the cell type, may lead to productive infection (See Figure 3.1-3.5).

In contrast, endocytosis can result in inactivation and concomitant degradation of virions within acidified endolysosomes and, in most cells, in nonproductive infection (Dezutter-Dambuyant C *et al.* 1991). In the case of mDCs, vesicular uptake of HIV-1 primarily occurs via CD4 (which acts as an endocytic receptor) and through CLR's such as DC-SIGN (McDonald D *et al.* 2003). Far less is known about how pDCs interact with HIV-1. pDCs express CD4, CXCR4, and CCR5, but not DC-specific ICAM-3–grabbing nonintegrin (DC-SIGN). Studies showed pDCs undergo profound activation in response to HIV-1 including secretion of IFN-α, which may protect pDCs and surrounding cells from HIV infection.

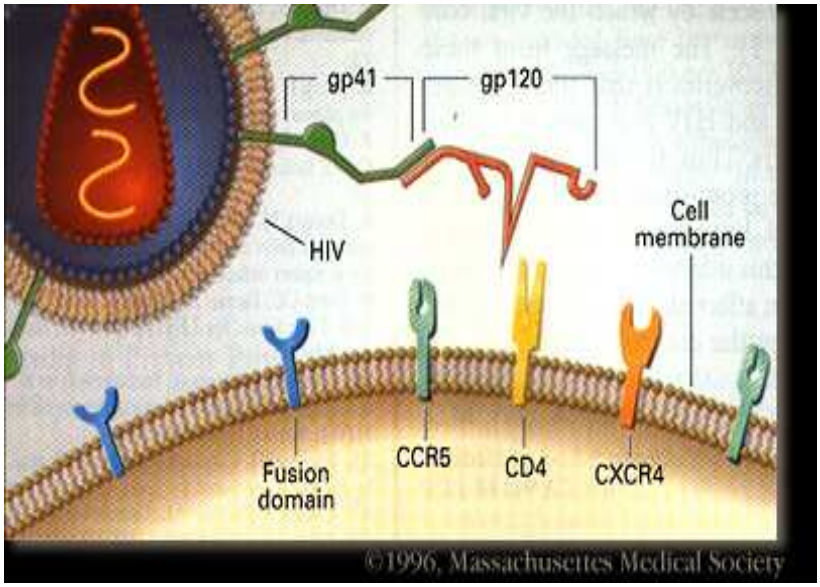

Figure 3.1: HIV-1 interacts with a cell-surface receptor, primarily CD4, and become more closely associated with the cell by interacting with other cell- surface molecules, such as CCR5 and CXCR4.

(Website:www.student.biology.arizona.edu)

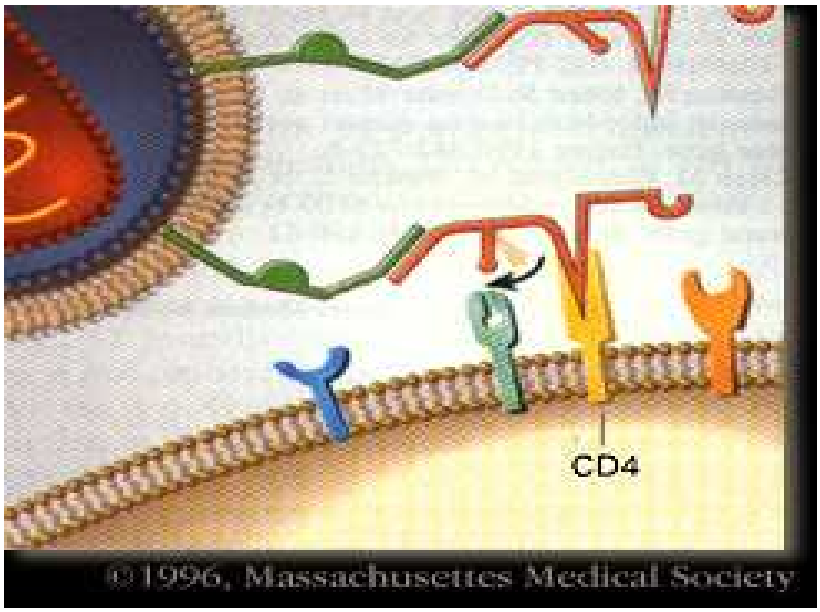

Figure 3.2: The CD4 molecule on the cell surface interact with the CD4 binding site on the HIV-1 gp120. Website: (www.student.biology.arizona.edu)

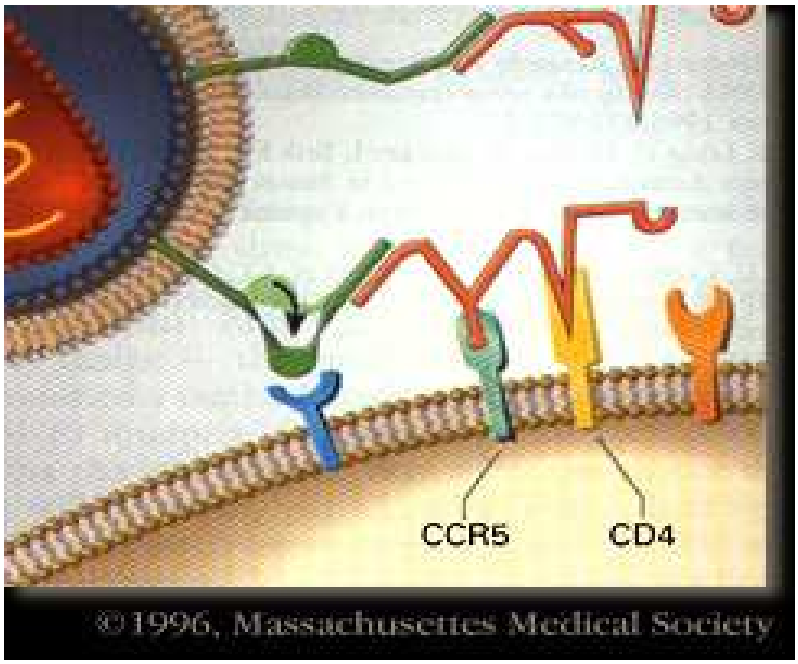

Figure 3.3 Changes in both the CD 4 receptor and the viral envelope allows the binding of gp120 to another cell surface receptor, such as CCR5. Website: (www.student.biology.arizona.edu)

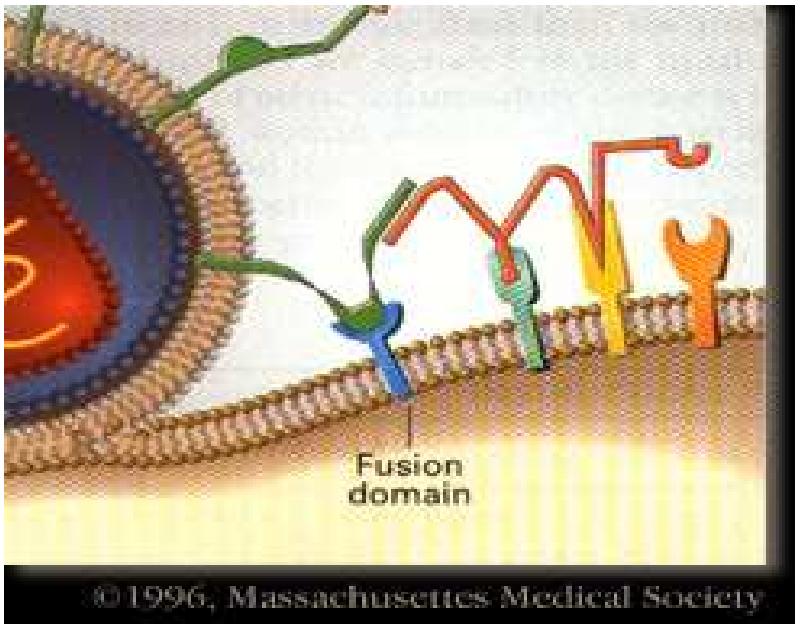

Figure 3.4: After the second attachment the viral envelope and the cell surface moves closer together and allow interaction between gp41 on the viral envelope and a fusion domain on the cell surface. HIV fuses with the cell (Website: www.student.biology.arizona.edu)

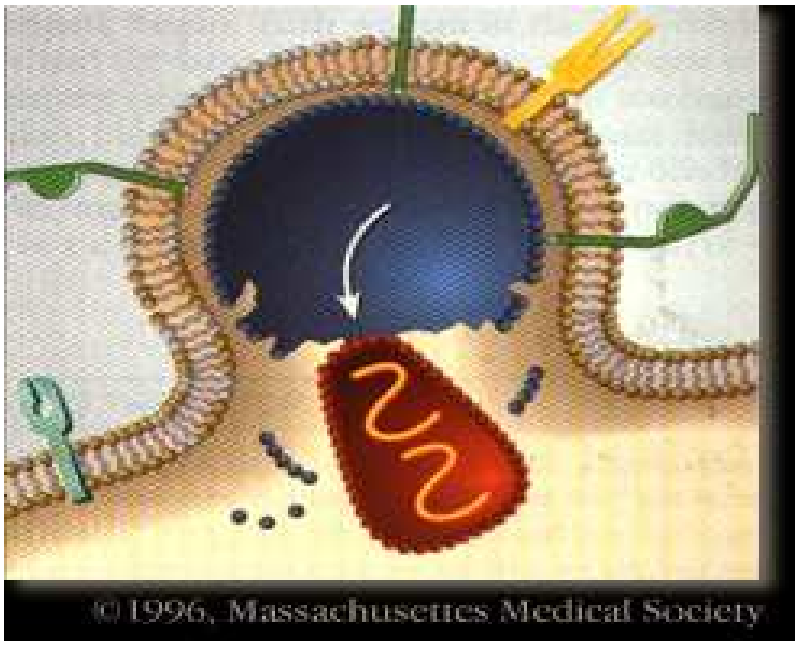

Figure 3.5: The viral nucleoid enters into the cell. The cycle of viral replication begins (Website: www.student.biology.arizona.edu)

C-type lectin receptors play a role in virus transfer to T cells, either with or without infection. Both these processes are important *in vitro*, and both may have a role *in vivo*, although the low-level infection of immature DCs may be more important as it leads to HIV strain selection and persistence of virus within DCs for at least 24 h, sufficient for these cells to transit to lymph nodes. The exact details of these processes are currently the subject of intense study.

Groot F *et al.* (2006) have shown that during HIV infection both mDCs and pDCs are reduced in number, that their function is impaired, and that through C-type lectins such as DC-specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN) they capture and transmit the virus at the DC–T-cell interface, the infectious synapse.

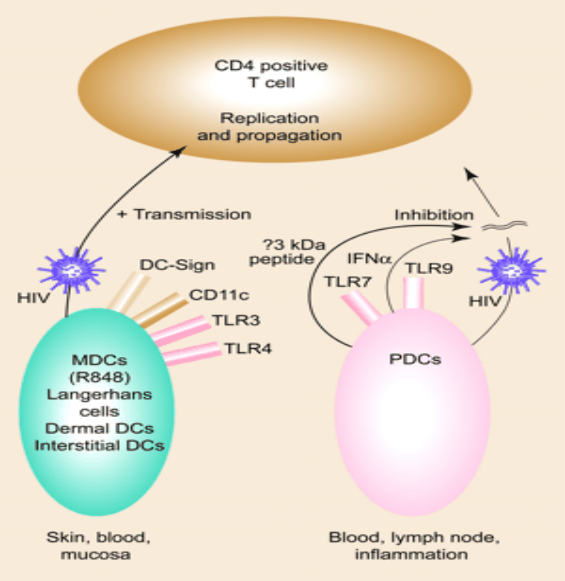

Figure 3.6 mDCs enhance transmission of HIV-1 across the infectious synapse; pDCs inhibit this process via IFN-α(Website:.http://bloodjournal.hematologylibrary.org/cgi/content/full/108/6/1785/FIG1)

A study done by Groot F *et al.* (2006c) compared differently matured mDCs and pDCs from peripheral blood for their influence on HIV-1 infection of T cells. The study showed

that mDCs strongly promoted HIV-1 infection whereas pDCs severely inhibit HIV-1 replication. The differential impact of mDCs and pDCs on HIV-1 infection may reflect the different location and function of these cells in the human body. The numbers of both mDCs and pDCs in blood of HIV-1–infected patients are reduced. (See Figure 3.6)

3.4 DCs that attack cancer:

DCs, located in most tissues of the body, capture and process antigens, which are then displayed as MHC-peptide complexes on the DC surface. This works well against foreign cells that enter the body, but cancer cells often evade the self/non-self detection system (Amigorena S *et al*. 1998). In most cases, tumour rejection is initiated by $CD8^+$ cytotoxic T lymphocytes (CTLs), which infiltrate solid tumours, recognize tumour antigens, and kill tumour cells (Ashley D *et al*. 1997; Boczkowski *et al*. 2000; Dallal *et al.* 2000).

Tumor antigens are presented by MHC I or MHC II molecules on the surface of tumor cells. These antigens can sometimes be presented by tumor cells and never by the normal ones. In this case, they are called tumor-specific antigens (TSAs) and typically result from a tumor specific mutation. More common are antigens that are presented by tumor cells and normal cells, and they are called tumor-associated antigens (TAAg). Cytotoxic T lymphocytes that recognized these antigens may be able to destroy the tumor cells before they proliferate or metastasize.

Ardavan *et al.* (2004) explains that immunotherapy in cancer builds a cancer specific immune response that will help the patient to fight cancer. By modifying DCs, researchers are able to trigger a special kind of autoimmune response that includes a T cell attack of the cancer cells (Colaco CA. 1999; Nouri – Shirazi M *et al*. 2000). Only mature DCs can present the tumour antigens to the T cells. When the DC begins to mature, it also starts

moving, or migrating toward a lymph node. The lymph nodes contain large numbers of lymphocytes (T cells). The neck, armpits, and groin areas all have clusters of nodes that lie close to the skin. Essential co-stimulatory molecules are upregulated on DCs as they migrate to secondary lymphoid organs (the spleen and lymph nodes) where they liaise with naïve T cells, inducing the activation and proliferation of Ag specific CTLs. The CTLs are the body's main defense against tumour cells. When the right CTL comes in contact with the DC, it will become activated and begin to proliferate, ready to kill any cancerous or pre-cancerous cell. The CTLs arrest in close contact to tumour cells, expressing their cognate antigen. In regions where most tumour cells are dead, CTLs resume migration, sometimes following collagen fibers or blood vessels. CTLs also infiltrate tumours in depth, but only when the tumour cells express the cognate CTL antigen. In tumours that do not express the cognate antigen, CTL infiltration is restricted to peripheral regions, and lymphocytes neither stop moving nor kill tumour cells (Fong L *et al*. 2000; Gabrilovich DI *et al*. 1996). Antigen expression by tumour cells therefore determines both CTL motility within the tumour and deep tumour infiltration.

Effective DC function in cancer involves several interlinked biological processes that occur in sequence (Vicari AP *et al.* 2004).

(a) TAAg presentation and recognition in tissues which involves proteolytic intracellular cleavage and peptide surface representation.

(b) DC activation and trafficking to regional tumour-draining lymph nodes (LNs), and interaction with $CD4^+$ T cells via the TCR and associated co-stimulatory molecules (CD40, CD80 and CD86), resulting in the generation of Ag-specific CTLs, and

(c) migration of CTLs to the tumour site and induction of cancer cell death.

3.5 Materials and Methods

3.5.1 Method for identification of DCs:

Source of blood samples:

Heparinized blood samples were collected from 10 healthy individuals, 10 HIV+ patients, 5 patients with HIV and TB and 5 patients with lymphoma. In the case of the different disease groups, routine blood samples were obtained from the NHLS laboratory at Tygerberg hospital. These samples had been drawn for routine cilincal management of the respective patients and the remaining volumes were going to be discarded. No patient demographics of any blood samples were recorded. All samples were coded. The samples were nonselected and randomly chosen. Two samples were used frompatients seen in the private sector: these patients gave consent for a project to measure immune markers in response to photo-therapy. The SAMA reference number for this study is WYE001/006 (6 Sept.06).The 10 normal individuals were healthy volunteers.

Blood were collected in EDTA and heparinized tubes. Monoclonal antibodies (obtained from Becton Dickinson) were added to 100µl of the individual's blood. Following an incubation of 15 minutes, red blood cells were lysed and the white blood cells were washed and fixed with paraformaldehyde. Prepared samples were acquired on a flow cytometer(BD™ FACSCalibur). CellQuest™ software was used for acquisition with a threshold set on FSC to exclude debris. DCs occur with low frequency in peripheral blood. To make sure enough DCs were acquired a minimum of 2000 events in Gate 2 were acquired. In the case of mDCs Gate 2 was set on CD11c APC and in the case of pDCs Gate 2 was set on HLA-DR PerCP. pDCs are CD11c negative but HLA-DR positive.

Preparation of working solutions:

Sterile Phosphate buffered saline (PBS)

- 1L Schott bottle was filled with double distilled water and autoclaved
- One bottle Dulbecco's phosphate buffered saline (obained from Sigma Aldrich) was added to the 1L of autoclaved water and stored at $4^{O}C$

FACS lysing solution (10x concentrate)

- Lysing solution is used for lysing red blood cells following direct immunofluorescence staining of human peripheral blood cells with monoclonal antibodies. It lyses erythrocytes under gentle hypotonic conditions while preserving the leucocytes.
- FACS lysing solution contains <15% formaldehyde and< 50% diethylene glycol
- Lysing solution was diluted 1:10 with distilled water (e.g. 10ml FACS lysing + 90ml (distilled water)
- The working solution can be stored at room temperature for up to 1 month

5% Fixative:

- Paraformaldehyde (obtained from MERCK) was diluted 1:20 with PBS (e.g. 5ml paraformaldehyde + 95ml PBS) and stored at room temperature for up to 1 month

Method:

The recommended amount of antibodies (See Table 3.1) and 100 µl heparinized whole blood were mixed together in Falcon Tubes. Tubes were incubated for 15 minutes at room temperature in the dark and 2ml of FACS Lysing Solution were added to each tube. Tubes were vortexed and incubated for 10 minutes at room temperature in the dark. Tubes were centrifuged at 1800rpm for 5 minutes and the supernatant was discarded. Tubes were vortexed gently to resuspend the pellet and 1 ml PBS was added to each tube. Tubes were centrifuged again at 1800rpm for 5 minutes, the supernatant discarded and the pellet of cells was resuspended in 500 µl of 5% paraformaldehyde. Tubes were analyzed on a FACS Calibur Flow cytometer.

Data Acquisition and Analysis:

CaliBRITE™ beads and FACSComp™ software were used to adjust the photomultiplier tube (PMT) voltages and fluorescence compensation, and to check the sensitivity of the instrument. Prepared samples were acquired on a flow cytometer. CellQuest™ software was used for acquisition with a threshold on FSC to exclude debris. DCs occur with low frequency in peripheral blood. To make sure enough DCs were acquired a minimum of 2000 events in Gate 2 were acquired. In the case of mDCs, Gate 2 was set on CD11c APC and in the case of pDCs, Gate 2 was set on HLA-DR PerCP. pDCs are $CD11c^-$, but HLA-DR^+. The same software was used for data analysis.

Table 3.1 Mixture of monoclonal antibodies used for the identification and maturation markers on DCs

TUBE NUMBER	Mixture of Monoclonal Antibody	Functional Subset
1	**20µl CD80** – FITC **20µl HLA DR** – PerCP **20µl CD123** – PE **5µl CD11c** – APC	Maturation status of pDCs/mDCs
2	**20µl CD83** – FITC **20µl HLA DR** – PerCP **20µl CD123** – PE **5µl CD11c** – APC	Maturation status of pDCs/mDCs
3	**20µl LIN 1** – FITC **20µl HLA DR** – PerCP **20µl CD123** – PE **5µl CD11c** – APC	Identification of pDCs/mDCs

3.6 Results:

Flow cytometric results:

Identification:

Fig 3.7 demonstrates the dot plots used for the identification of mDCs and pDCs. Primary gating strategy (FSC vs SSC) was used to yield gate 1 (R1) which include all lymphocytes and monocytes. Secondary gating on HLA R indicated all activated lymphocytes. CD11c and CD123 were used for the identification of mDCs and pDCs.

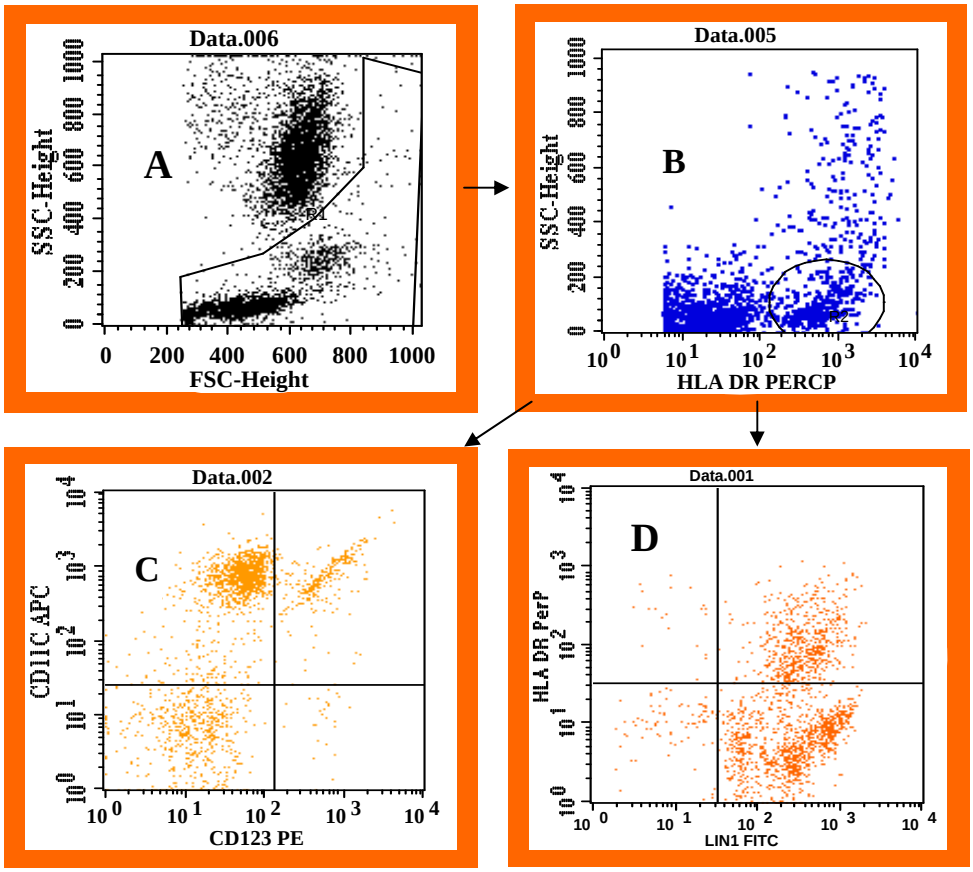

Figure 3.7: Example of DC identification. Dot plot A indicates the gating strategy (FSC vs SSC) used to yield gate 1 (R1) within which the expression of HLA-DR by all activated lymphocytes is measured. (Dot plot B). Dot plot C indicates the CD 123/CD11c expression by DCs . mDCs are $CD11c^{+}$/ $CD123^{-}$. pDCs are $CD11c^{-}$/ $CD123^{+}$. Dot plot D indicates different types of lymphocytes. DCs are lin 1^{-} while all the other lymphocytes are $lin1^{+}$.

Maturation markers:

Figure 3.8 demonstrated the dot plots used to determine the maturation status of mDCs and pDCs. Primary gating strategy (FSC vs SSC) was used to yield gate 1 (R1) which include all lymphocytes and monocytes. Secondary gating on HLA DR for pDCs and CD11c for mDCs was used to identify the DCs. In these gates the maturation status of the DCs were measured looking at CD80 and CD83.

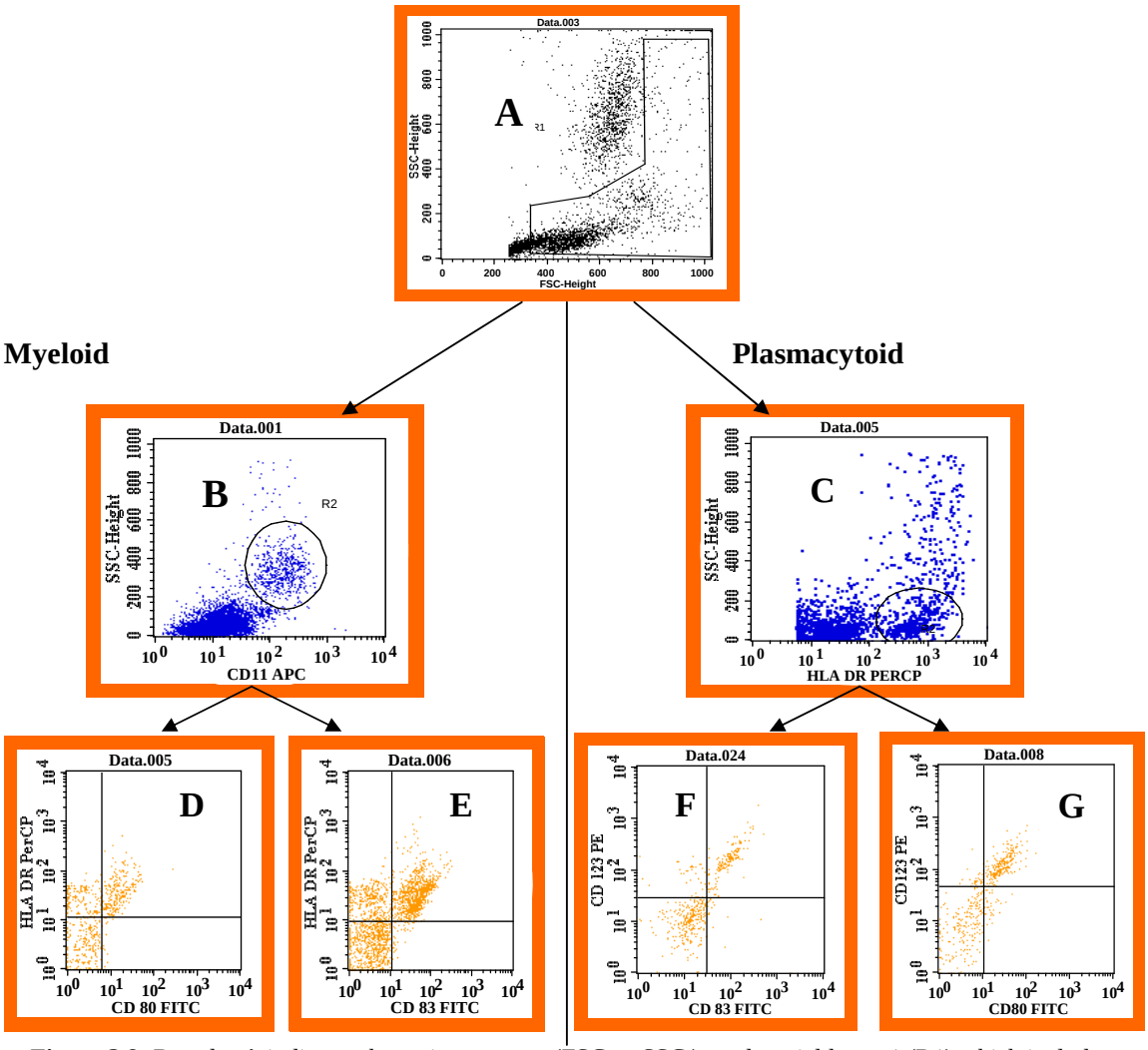

Figure 3.8: Dot plot A indicates the gating strategy (FSC vs SSC) used to yield gate 1 (R1) which include lymphocytes and monocytes. Dot plot B indicates a secondary gate on CD11c and Dot plot C indicates a secondary gate on HLA DR Dot plot D and E represent the mature mDCs which are $CD80^{+}$ HLA DR^{+} and $CD83^{+}$ HLA DR^{+}. Dot plots F and G represent the mature pDCs which are $CD80^{+}$ $CD123^{+}$ and $CD83^{+}$ CD123.

This method used is a simple four colour method for the identification of the major subsets of DCs in whole blood using commercially available reagents. To make sure enough DCs were acquired a minimum of 2000 events in Gate 2 were acquired. The same software (Cellquest^{TM}) was used for data analysis. In the case of mDCs, Gate 2 was set on CD11c APC and in the case of pDCs, Gate 2 was set on HLA-DR PerCP. pDCs are CD11c^{-}, but HLA-DR $^{+}$. The same software was used for data analysis.

Statistical analysis of data:

An unpaired t test was used for the analysis of data sets. All results were considered statistically significant when $p < 0.05$. All groups were compared to the normal group.Ranges were determined for mDCs and pDCs as well as the maturation markers, CD80 and CD83, for both pDCs and mDCs. (See Table 3.2)

Table 3.2 Normal reference ranges

Type of DC and maturation markers	**Normal range obtained from 10 healthy individuals (%)**
Myeloid DCs	1.7-19.41
CD80 (mDCs)	1.3 – 8.19
CD83(mDCs)	6.24 – 21.49
Plasmacytoid DCs	0.86-7.74
CD80 (pDCs)	0.0 -9.75
CD83(pDCs)	0.39-5.5

Since there is no published reference ranges for CD80 and CD83, we created these ranges above from the data obtained from the 10 normal individuals. Interestingly enough Della Bella S *et al.* 2006 analysed the number and phenotype of DCs in peripheral blood from 70 healthy subjects. Their results showed that the mean %mDCs was 16.50% and the %pDCs 7.2%.These data fits in with the ranges obtained in table 3.2 in this study.

Results of measurement of DCs in blood from patients with different diseases:

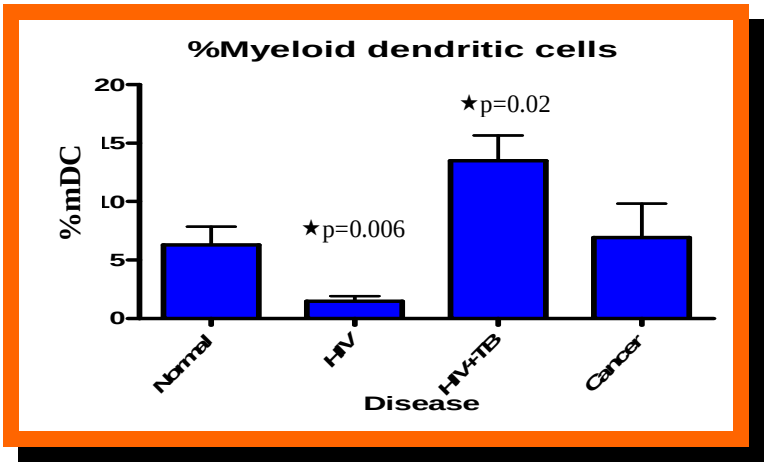

Figure 3.9: Myeloid DCs in peripheral blood from individuals with different diseases.

Significant columns are indicated with★

Figure 3.9 indicates the measurement of the % mDCs in different diseases. The average % mDCs in each group were 6.4% (Normal), 1.47% (HIV), 13.5% (HIV+TB) and 6.92 % (Cancer). The % mDCs were increased in the case of HIV with TB (n=5) and decreased in the case of HIV (n=10).

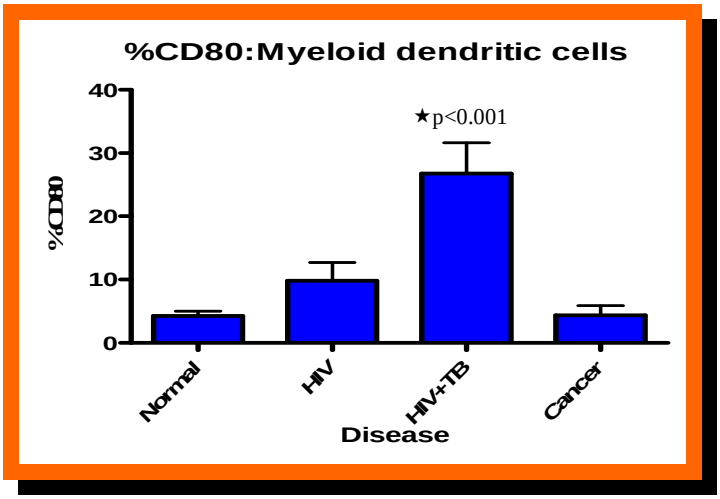

Figure 3.10 %CD80 expressed by myeloid DCs in peripheral blood from individuals with different diseases. Significant columns are indicated with★

Figure 3.10 indicates the measurement of CD80 expression by mDCs. The average expression of CD80 in each group was 6.4% (Normal), 9.8% (HIV) and 26.8% (HIV+TB) and 4.3% (Cancer). The expression of CD80 was increased by HIV+TB (n=5) ($p<0.001$). The HIV (n=10) group also showed a slight increase in the expression of CD80 (non significant). Percentage values were calculated from average values and compared to the normal samples (n=10).

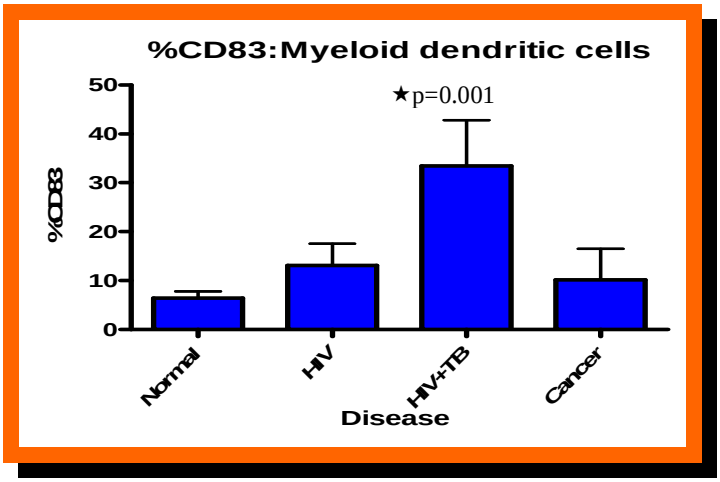

Figure 3.11 % CD83 expressed by myeloid DCs in peripheral blood from individuals with different diseases. Significant columns are indicated with★

Figure 3.11 indicates the regulation of CD83 expression by mDCs in different diseases. The average expression of CD83 in each group was 6.5% (Normal), 14.2 % (HIV), 26.8% (HIV+TB) and 10.5 % (Cancer). The expression of CD83 was upregulated by HIV+TB (n=5) (p=0.001) and the HIV group (n=10) (non significant) and cancer (n=5) (non significant) showed a slight increase in the expression of CD83.

Plasmacytoid DCs:

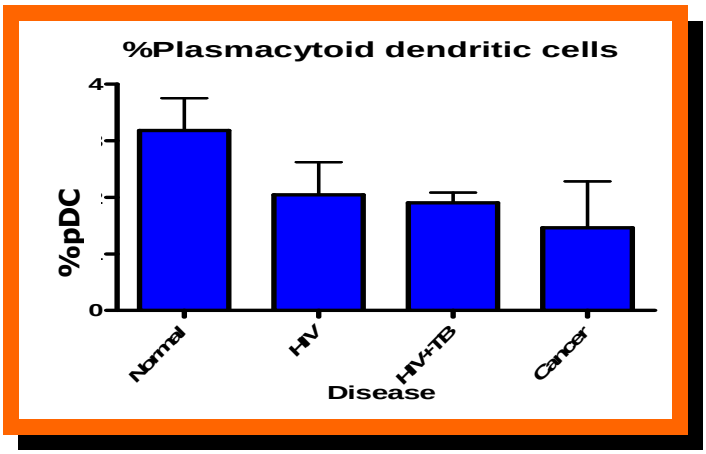

Figure 3.12 Plasmacytoid DCs in peripheral blood from individuals with different diseases.

Figure 3.12 indicates the % pDCs cells measured in the peripheral bloods of patients diagnosed with different diseases: the average % in each group were 3.6% (Normal), 2.6% (HIV), 1.98% (HIV+TB) and 1.53% (Cancer). The % pDCs cells was slightly decreased in the case of HIV (n=10), HIV+TB (n=5) and Cancer (n=5), but these were not significantly different when compared to the normal controls.

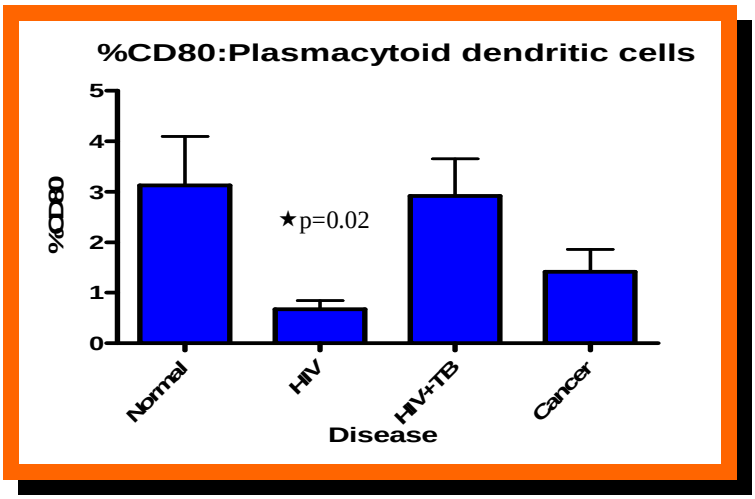

Figure 3.13 %CD80 expressed by plasmacytoid DCs in peripheral blood from individuals with different diseases. Significant columns are indicated with★

Figure 3.13 indicates the measurement of CD80 expression by pDCs .The average expression of CD80 in each group were 3.3% (Normal), 0.82% (HIV), 2.76% (HIV+TB) and 1.52% (Cancer). The expression of CD80 was significantly (p=0.02) decreased not only by HIV (p=0.02) (n=10), but also the cancer (n=5) (non significant) group showed a decrease in CD 80 expression.

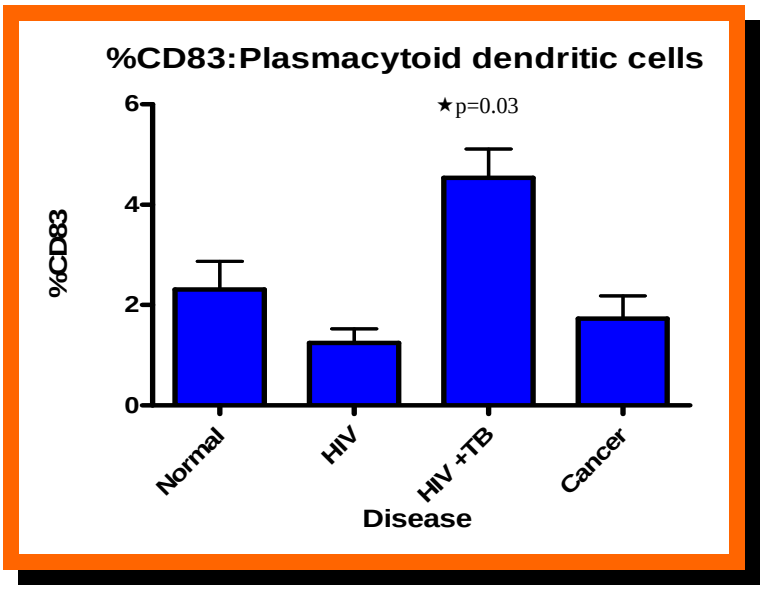

Figure 3.14 %CD83 expressed by plasmacytoid DCs in peripheral blood from individuals with different diseases. Significant columns are indicated with★

Figure 3.14 indicates the regulation of CD83 expression by plasmacytoid DCs in different diseases. The average expression of CD83 in each group were 2.23% (Normal), 1.36 % (HIV), 4.69 % (HIV+TB) and 1.86 % (Cancer).The expression of CD80 was significantly upregulated (p=0.03) by HIV+TB (n=5) and the HIV (n=10) (non significant) and cancer (n=5) (non significant) group showed a slight decrease in the expression of CD83.

3.7 Discussion

This study showed that both mDCs and pDCs can be identified in peripheral blood by making use of a quick and easy method. We identified both types of DCs in the peripheral blood of 10 normal healthy individuals and in the peripheral blood of patients presenting with different diseases. A normal reference range was determined after analyzing the data of the normal individuals (See Table 3.2). The data of the different disease groups were compared to the normal group. The first disease we look at was HIV. Several studies have shown that during HIV infection, both DC subsets are reduced in the blood. (Donaghy H *et al.* 2006c; Schmidt B *et al.* 2006b) showed in their study that pDCs and mDCs are reduced in AIDS patients. The results from this study also showed a decrease in the % mDCs ($p=0.006$) and % pDC (although non significant) in HIV infection. Meyers JH *et al.* (2007) showed in their study that the levels of pDCs present in blood from HIV-infected volunteers were significantly lower than those of HIV-negative individuals. They also suggest in this study that HIV-infected cells may also directly fuse with and kill pDCs, which could explain the decrease of pDCs in HIV. The maturation markers done on mDCs showed a slight increase in both the % CD80 and % CD83. Piguet V and Steinman RM (2007) explained in their study that HIV-infection of DCs does not lead to maturation, but in cases of high viral loads maturation of DCs can occur. This statement might explain the slight increase in maturation markers in the HIV group. In this study the viral loads were unknown, but it might have influenced the increase in maturation markers.

Fonteneau JF *et al.* (2004) demonstrated in his study that the mDC do not mature upon contact with HIV-1, but they do mature indirectly as a consequence of the production of

cytokines by HIV activated pDCs. The maturation status of the pDCs in this study was lower than the normal (% CD80: p=0.02). We also looked at the correlation between the CD4% and the %mDC or %pDC. The correlation was non significant. Further studies need to be done on HIV to make final conclusions. In this study we had no background or treatment status of the patients (n=10). A more specific study will show more accurate results. Blocking DC-SIGN-mediated capture of HIV represents a potential therapeutic antiviral strategy for HIV disease. An intriguing pathway that is well developed in DCs is the exogenous pathway for non replicating viral antigens to be presented on class I MHC products. This should allow DCs to stimulate CD8+ T cells after uptake of antibody-coated HIV-1 and dying infected T cells.

The second group of patients were only discovered when we recruited blood to test DC subsets in HIV: the results of these patients looked different and it was discovered that these patients were HIV+ and had clinical signs of active TB (diseased). Although a small group (n=5), the results were interesting. The % mDC (p=0.02) and % pDC (although non significant) showed an increase in the peripheral blood when compared to the healthy controls. The maturation markers % CD80 (p<0.001) and % CD83 (p=0.001) in mDCs were increased. In the case of pDCs maturation markers % CD83 (p=0.003) were increased and the % CD 80 were almost the same as that of the control group. The % CD80 in case of HIV and TB was higher than in the HIV only group, which showed that TB does play a role in the maturation of DCs. Several *in vitro* and *in vivo* studies, in mice and rats, have shown that DCs can be activated upon infection with various mycobacteria, including *M. tuberculosis* and the vaccine strain *M. bovis* BCG (Henderson RA *et al.*1997). Mycobacteria-activated DCs express surface presentation, co-stimulation and migratory molecules (Kim *et al.*1999). Animal studies done showed mycobacteria-infected DCs can

stimulate mycobacterial antigen-specific T cells *in vitro*, and confer protection against mycobacteria. *In vivo*, it is likely that mycobacteria-infected DCs can stimulate the development of IFN-γ-secreting T cells, essentially through the secretion of IL-12.

In the case of cancer we only recruited 5 patients with lymphoma. Lymphomas fall into 1 of 2 major categories: Hodgkin lymphoma/disease and other lymphomas (non-Hodgkin lymphomas/NHL). Hodgkin disease develops from a specific abnormal B lymphocyte lineage. NHL may derive from either abnormal B or T cells and are distinguished by unique genetic markers. Lymphoma can occur at any age, including childhood. Hodgkin disease is most common in 2 age groups: young adults aged 16-34 years and in older people aged 55 years and older. Non-Hodgkin lymphoma is more likely to occur in older people. The results of this study showed that the % mDCs and % pDCs in this group were in the same range as the normal group or slightly decreased. Esche C *et al*. (1999) and Timmerman JM *et al*. (1999) proved in their studies that cancers are able to escape from the immune system by several mechanisms, for example, by inducing apoptosis in DCs so that they are unavailable to generate a productive immune response.

The maturation markers done on mDCs showed a slight increase in the % CD83. The results were very similar to those of the normal group. The % CD80 showed a slight decrease compared to the normal group, but it was not significant. The results of the % pDC and the maturation markers done on the pDC showed a decrease. Smyth MJ *et al*. (2001) explains an important property of tumour cells is that they typically do not release danger signals so that even if immature DCs take up and process tumour antigens or apoptotic tumour cells, they may fail to mature. The study of DCs and DCs maturation in different diseases needs further investigation: more sample numbers and better defined patient groups.

Chapter 4

In vitro activation of dendritic cells using a Mixed killed Bacterial Vaccine (MBV)

Abstract:

This chapter describes a flow cytometric method to measure the activation and maturation status of DCs. In this study, we investigated the response of human DCs to MBV and LPS. Previous studies showed DCs could be activated using killed *Streptococcus pyogenes.* With this study in mind it was hypothesized that the MBV used in this study might modulate DC maturation. The results of this study showed that the MBV did indeed induce the maturation of both pDCs and mDCs as measured by increased surface expression of costimulatory molecules such as CD80 and CD83. Interactions between DCs and microbial pathogens are fundamental to the generation of innate and adaptive immune responses. Upon the activation and stimulation with bacteria or bacterial components such as lipopolysaccharide (LPS), immature DCs undergo a maturation process that involves expression of costimulatory molecules, HLA molecules, and cytokines and chemokines, thus providing critical signals for lymphocyte development and differentiation.

4.1 Introduction

DCs express costimulatory molecules including CD80 and CD86, (Chow A *et al*. 2002) which are up-regulated during DC activation. CD86 is a marker of early DC maturation, while CD80 only appears in mature DCs. Two other markers for mature DCs are CD83 and CMRF-44. CD83 also stains activated B cells and CMRF-44 will also stain

macrophages and monocytes. In this study CD83 and CD80 were used as activation markers and LPS and TNF-α were used to stimulate mDCs and pDCs to obtain positive controls. Cella M *et al*. (1997) explains that CD80 antigen acts as the ligand for both the CD28 and CTLA-4 glycoproteins, which are expressed on T lymphocytes. The CD80 antigen interacts with the CD28 and CTLA-4 glycoproteins to provide a stimulus for T-lymphocyte activation. The interaction of the CD80 and CD28 antigens mediates heterotypic cell adhesion and also results in augmentation of T-lymphocyte proliferation and cytokine production. The ligation of the CD80 and CD28 antigens also enhances cytotoxic effectors of cytotoxic T lymphocytes (CTL). The CD80 antigen is expressed on activated B lymphocytes and monocytes and is constitutively expressed on DCs. The CD80 antigen is also expressed on activated human peripheral blood T lymphocytes, $CD4^+$ and $CD8^+$ T-lymphocyte clones, and NK cell clones. CD3-activated T lymphocytes are able to induce normal or leukemic B lymphocytes to express the CD80 antigen and significantly higher levels of the CD54 antigen (Hellman P *et al.* 2007).

Along with CD86, these molecules provide the necessary stimuli to prime T cells against antigens presented by antigen-presenting cells. Bender A *et al*. (1996) states that CD83 is expressed on cultured DCs, interdigitating, follicular, and circulating DCs as well as some proliferating lymphocytes and human cell lines. CD83 serves as a useful marker for mature human blood DC identification. In this study LPS was used as stimulant to obtain a positive control for mDCs. LPS is a large molecule consisting of a lipid and a polysaccharide (carbohydrate) joined by a covalent bond and is a major component of the outer membrane of Gram-negative bacteria, contributing greatly to the structural integrity of the bacteria, and protecting the membrane from certain kinds of chemical attack (Verhasselt V *et al.* 1997). LPS is an endotoxin, and induces a potent response from

normal immune cells (de Smedt T *et al*. 1996). TNF-α was included in this experiment as a stimulant to obtain a positive control for pDCs (Nelson EL *et al.* 1999). TNF-α is produced by monocytes, macrophages and T and B cells. Yu O *et al.* (2003) confirmed that pDCs express CD80, CD86 and CD83 in the presence of TNF-α.

The vaccine used in this study is MBV. This has previously been described. Briefly, it contains a mixture of different bacteria, but no phenol. The concentration of the vaccine is 10^9 CFU /ml of each bacterial species listed: *Staphylococcus aureus, Streptococcus pneumoniae, Moraxella (Neisseria) catarrhalis, Klebsiella pneumoniae, Heamophilus influenza, Corynebacterium diphtheria, Streptococcus pyogenes, Pseudomonas aeruginosa, Proteus vulagaris, Proteus mirabilis, Candida krusei* and *Candida albicans*

4.2 Materials and methods:

4.2.1 Study design:

Seeing that the effect of the MBV on DCs is unknown, we had to determine which concentration of the vaccine works the best as well as to determine the kinetics of activation. Five milliliter Heparinized blood samples were collected from 2 healthy individuals. A dose response of the MBV was done to determine the optimal activation of the vaccine. Human mononuclear cells are often required in order to conduct specialized assays. The Peripheral Blood Mononuclear cells are therefore isolated from the remaining blood elements by density gradient centrifugation. The isolated cells were incubated with different concentrations of the MBV and incubated for 8, 12 and 24 hours. Cells were centrifuged and the supernatants removed. The supernatants were stored at -20°C for cytokine detection as described in chapter 6. Monoclonal antibodies to CD80 or CD83 were added to the cells to determine the activation and maturation status of the DCs. Prepared samples were acquired on a flow cytometer (BD™ FACSCalibur). CellQuest™

software was used for acquisition with a threshold on FSC to exclude debris. Since DCs cells occur with low frequency in peripheral blood, we acquired a minimum of 2000 events in Gate 2 in order to ensure that sufficient events were analysed. In the case of mDCs, Gate 2 was set on CD11c APC and in the case of pDCs, Gate 2 was set on HLA-DR PerCP. pDCs are $CD11c^-$, but HLA-DR^+. The same software was used for data analysis.

4.2.2 Preparation of reagents

RPMI 1640 growth medium (GIBCO® 61870) (Obtained from Sigma Aldrich)

Fetal Bovine Serum (Obtained from Adcock Ingram) (GIBCO®) (decomplemented at 56°C for 30 minutes in waterbath)

Histopaque-1077 solution (Obtained from Sigma Aldrich)

Distilled or deionized water

Sterile Phosphate buffered saline (PBS):

- 1L Schott bottle was filled with double distilled water and autoclaved
- One bottle Dulbecco's phosphate buffered saline (obtained from Sigma Aldrich) was added to the 1L of autoclaved water and stored at 4°C

5% Fixative:

- Formaldehyde (obtained from MERCK) was diluted 1:20 with PBS (e.g. 5ml formaldehyde + 95ml PBS) and stored at room temperature for up to 1 month

LPS:

One milliliter sterile PBS was added to the stock of 1mg LPS (Obtained from Sigma Aldrich). We made a 1/10 dilution of the stock solution (50µl LPS+450µl RPMI). 20µl of this dilution was added to each tube for LPS.

TNF-α:

A recombinant stock solution 10 ng/ml TNF-α (Obtained from R&D Systems) was diluted 1/10 (50µl TNF-α+450µl RPMI).Twenty mikroliter of this dilution was added to each tube for TNF-α activation.

Preparation of MBV:

The MBV used in this study was kindly provided by Dr. Brigitte Riedelsheimer, Dusseldorf, Germany. This preparation is used clinically by oncologists in Germany to induce a non-specific immune response as part of their therapeutic approach to cancer patients. Different concentrations of the MBV were tested to determine the optimal dose for the *in vitro* activation and maturation of DCs: please refer to Table 4.1 for the preparation of the different concentrations of the MBV

PBMC preparation

Principle of density gradient centrifugation:

The density of human mononuclear cells is 1.077 g/L. When diluted blood is layered onto a complex carbohydrate solution having the exact density as that of the cells, followed by centrifugation, the cells with the identical density will be “trapped” at the interface between the blood and the carbohydrate solution and not move further into the solution. All other blood elements with higher densities will travel through the interface and settle at their respective densities (including red blood cells, granulocytes, etc). The cells of interest can be easily recovered at the interface, washed and used in subsequent assays or stored for later usage (See Figure 4.1)

Method for PBMC preparation:

The procedure should be performed in a biosafety cabinet under sterile conditions. Three 15ml sterile conical tubes per sample were labeled as “Ficoll”, “PBS”, “Lymphocytes” respectively. The Histopaque-1077 (Ficoll) was allowed to reach room temperature and 4ml were added to the 15ml sterile conical tube labelled as “Ficoll”. Five milliliter Heparinized whole blood was mixed by inverting the tube several times. The blood was transferred to another sterile conical tube labelled as “PBS” and double diluted in the conical tube with sterile PBS by mixing it gently. The diluted blood was carefully layered onto the Ficoll solution and centrifuged at 1800rpm at 4^{O}C for 25 minutes. After the centrifugation, the mononuclear cells formed a visible, clear interface between the Ficoll and the plasma.The plasma layer was removed and the mononuclear cell layer was transferred to a sterile 15 ml conical tube labelled as “Lymphocytes” using a sterile Pasteur pipette. The tube was filled with sterile PBS, capped and centrifuged for 15 minutes at 1500rpm.The supernatant was removed and discarded. Tubes were vortexed and the tube filled with sterile PBS and centrifuged again at 1800rpm for 10 minutes. After the

centrifugation, the supernatant was removed and discarded. Tubes were vortexed and 1 ml of RPMI growth medium containing 10% Fetal Calf Serum was added to each tube. Twenty microliter of the cell suspension was added to 180 µl of Trypan blue and 10µl of the trypan blue/cell mixture was placed beneath the cover slip on a Haemocytometer. The lymphocyte count was done under a microscope to determine the concentration of the cells. The cells were made up to a final concentration of 1 x 10^6cells/ml.

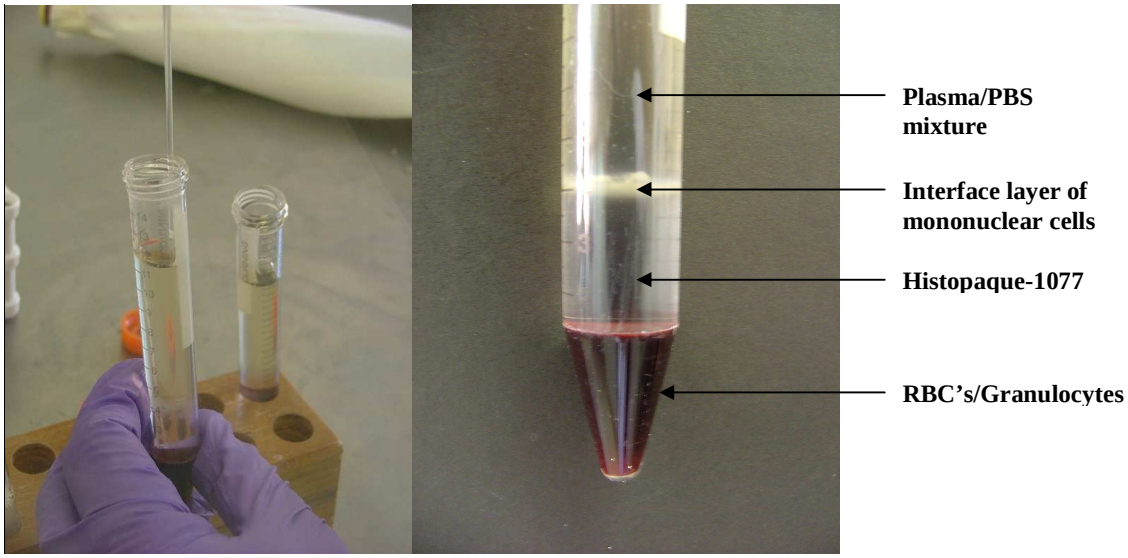

Figure 4.1 These photos show the different layers that forms after loading the diluted blood on Histopaque-1077 and centrifuging. (Taken 22 March 2007 10:00)

4.3 Dose response of MBV

Three sets of 7 Falcon tubes (Obtained from BD Biosciences) were labelled and the following added to each tube (See Table 4.1)

Table 4.1:

Tube number and contents	Cells at concentration of $1x10^6$cells/ml	Volume of MBV/LPS/TNF-α	Volume of RPMI
1 : Unstim	500µl	0	500µl RPMI
2 : LPS	500µl	20µl LPS (2µg/ml)	480µl RPMI
3. TNF-α	500µl	20µl TNF-α (0.2µg/ml	480µl RPMI
4: 1/10	500µl	50µl MBV	450µl RPMI
5: 1/50	500µl	10µl MBV	490µl RPMI
6: 1/100	500µl	5µl MBV	495µl RPMI
7: 1/200	500µl	2.5µl MBV	497.5µl RPMI

One set was incubated for 8 hours, one for 12 hours and one for 24 hours. After the desired incubation periods, the cells were centrifuged at 1800rpm for 5 min. The supernatant was aspirated and put in cryovials and kept frozen at -20°C: these were used to determine cytokine production as described in chapter 6. One ml PBS was added to the cells in each tube. The cells were vortexed and used for the measuring of activation/maturation markers following the staining of the surface activation markers as described below.

The recommended amount of antibodies (same as used in chapter 3 to identify the DCs) and 100 µl of the cultured PBMC's were mixed together in two Falcon Tubes. The LIN 1

cocktail was not included in this study, because of lack of funds. Tubes were incubated for 15 minutes at room temperature in the dark and 2ml of FACS Lysing Solution were added to each tube. Tubes were Vortexed and incubated for10 minutes at room temperature in the dark. Tubes were centrifuged at 1800 rpm for 5 minutes and the supernatant was discarded. Tubes were Vortexed gently to resuspend the pellet and 1 ml PBS was added to each tube. Tubes were centrifuged again at 1800rpm for 5 minutes, the supernatant discarded the pellet of cells was resuspended in 500 µl of 5% paraformaldehyde. Tubes were analyzed on a FACS Calibur Flow cytometer.

4.4 Data Acquisition and Analysis:

CaliBRITE™ beads and FACSComp™ software were used to adjust the photomultiplier tube (PMT) voltages and fluorescence compensation, and to check the sensitivity of the instrument. Prepared samples were acquired using the CellQuest™ software with a threshold set on FSC to exclude debris.

4.5 Results:

4.5.1 Flow cytometric analysis of mDCs post MBV activation:

Data analysis showed that the MBV did induce the expression of CD80 and CD83 on both mDCs and pDCs and does play an important role in DC maturation. An unexpectedly high background was found of the unstimulated sample: we detected relatively high $CD80^+$ and $CD83^+$ events in the unstimulated samples although the stimulated samples showed even higher positive events. The only explanation that we can propose is that due to the fact that we used PBMC's to measure the maturation of DCs, these cells could have possibly been activated indirectly during the sample preparation. It is difficult to study DC in fresh peripheral blood since these cells are far outnumbered by lymphocytes and monocytes and it

is known that monocytes can become activated by the plastic tube during cell separation (plastic adherence) and secrete cytokines that can induce the maturation of DCs. What we were seeing in the unstimulated samples could in fact be a representation of indirect DCs activation. Measurement of cytokine release (as reported in chapter 6) seems to confirm *in vitro* activation of these cultures. The results in chapter 6 showed an increased secretion of cytokines in the Unstimulated cultures. This is however speculative.Test done at local laboratories showed that less than one in three batches Heparin Vacuum Tubes are not contaminated with LPS which causes DC maturation (Dr. E. Pool, personnal communication). This could be an explanation why the unstimulated values for the dose response could be higher than the unstimulated values tested for the 10 normal individuals. We used two different batches during the period of blood draw.

An example of the dose-response of the DCs to the MBV is shown in Figure 4.2. It is clearly visible that although the unstimulated cultures exhibited raised $CD80^+$ cells, those cultures which had received the stimulus showed even higher values, and this data was statistically significant as indicated. Tkachenko N *et al.* (2005) tested different culture media for the generation of DCs and discovered that the growth and maturation of DCs in culture can be influenced by a variety of factors. In this study they claim that media supplemented with FCS can influence the DC maturation. FCS contains endotoxin, for example LPS, which can activate the DCs, even though the concentrations are very low.

Another possible explanation for the high percentages of activated DCs detected in the unstimulated preparations is that these cells could have become “enriched” during the culture period. It is plausible that during the culture, cells such as lymphocytes and monocytes died leaving increased (relative to the other cell populations) numbers of DCs

in the gated area). Since the purity of the gated cells at the end of the culture period was not verified using specific monoclonal antibodies to lymphocytes and monocytes, this cannot be stated with certainty. This rationale is based on the fact that during lymphocyte cultures, there is a natural enrichment which takes place due to the selection of the activation stimulus such as the mitogen or antigen used.

It must also be brought to the attention of the reader that the culture of the DCs with the MBV induced various amounts of debris: in the 12 and 24 hours cultures, the primary gating proved difficult at times and although it may appear that the 12 or 24 hours induced the highest expression of the activation markers, it was decided to analyse only the 8 hour data: this showed that the 1/50 dilution of this stimulus induced the highest CD80 and CD83 expression on the DCs. In parallel, LPS which was used as a positive control proved to be able to induce very high levels of the activation markers (Figure 4.4). The results for the maturation markers obtained during the dose reponse were higher than the results obtained from the 10 healthy volunteers. The only explanation for this is the group size. The dose response was done on only two individuals.

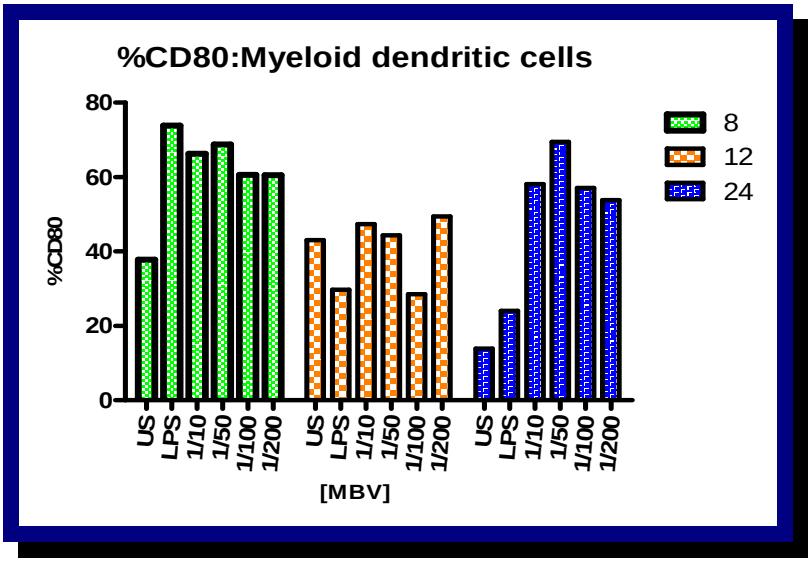

Figure 4.2 Representative data of the dose response of MBV to induce the expression of CD80 by myeloid DCs after 8, 12 and 24 hours incubation.

Statistical analysis of dose reponse data:

For the analysis of the dose response data we used a Paired T test. All results were considered statistically significant when $p < 0.05$. All groups were compared to the unstimulated group to see the effect of the mixed killed bacterial vaccine.

Table 4.2

Statistical analysis of 8 hour dose response: CD80 mDCs	
Unstim vs LPS	p =0.013
Unstim vs 1/10	p =0.012
Unstim vs 1/50	p<0.05
Unstim vs 1/100	p<0.05

To confirm the data of the dose response blood was drawn from 10 normal healthy individuals. Samples were treated the same as in the dose response method explained above. We decided to use the 1/50 dilution of the MBV. Cells were incubated for 8 hours with medium, TNF-α, LPS and MBV. TNF-α was included in this experiment as a positive control for pDCs and LPS as a positive control for mDCs. The data from the individual groups were analysed by the Wilcoxon matched paired test and the results were considered statistically significant when $p < 0.05$. All groups were compared to the unstimulated group to see the effect of the MBV: the presence of MBV significantly influenced the maturation status of DCs *in vitro.* The MBV showed a very significant increase in both the % CD83 ($p=0.002$) and % CD80 ($p=0.002$) expressed by mDCs. It also appears that the % mDCs increased significantly ($p=0.04$) but as explained earlier, this was possibly due to the "enrichment" of these cells during the culture period (Figure 4.3)

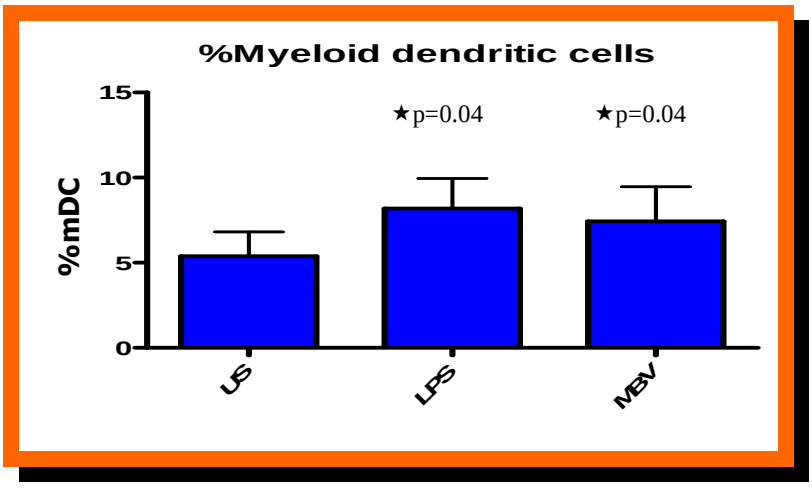

Figure 4.3 Data from healthy individuals (n=10) stimulated with MBV to measure the percentage myeloid DCs after 8 hour incubation. Significant values are indicated with★

From Figure 4.3 above, the % mDCs in each group were 5.7% in the unstimulated cultures, 8.2% (LPS stimulated) and 7.4% (MBV stimulated). This increase in the % mDCs may be due to enrichment of Gate 2 (as explained earlier). This data just confirms the results of the dose response done above: 1/50 dilution of the MBV was used and the cells were incubated for 8 hours after which they were stained for the cell surface expression of the activation markers.As shown in Figure 4.4, mDCs incubated with the MBV were induced to express high levels of the CD80 antigen.

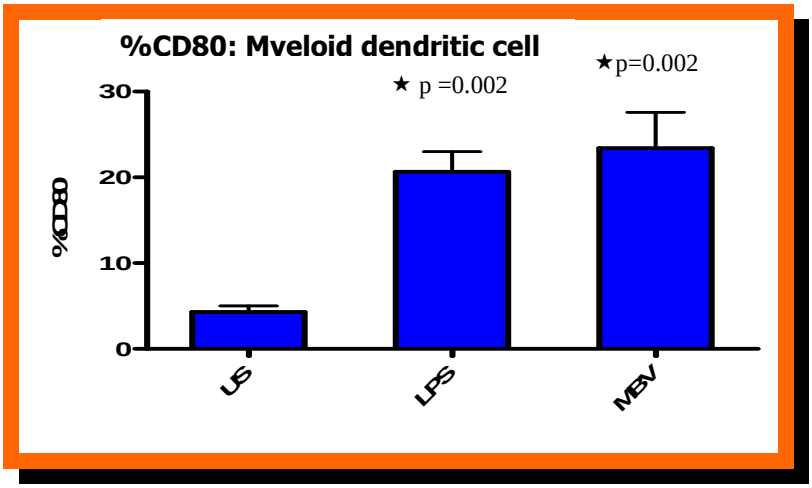

Figure 4.4 Data from healthy individuals (n=10) stimulated with MBV to measure the expression of CD80 by myeloid cells after 8 hour incubation. Significant values are indicated with★

The expression of CD80 in each group was 4.3% (unstimulated cells), 20.6% (in cultures incubated with LPS) and 23.4% (MBV-induced expression). The expression of CD80 was up-regulated not only by LPS (p=0.002) but also by the MBV (p=0.002) when this was compared to the unstimulated cultures. It was interesting to note that the MBV group of cultures showed a higher %CD80 expression than the LPS group: this could be due to the mixture of bacteria used rather than a single preparation of endotoxin.

Similarly, when the expression of CD83 activation marker was analysed, the MBV induced high levels of this marker (Figure 4.5). After incubation at 37°C for 8 h, cells were harvested and the expression of cell surface antigens was analyzed by FACS analysis. The average expression of CD83 in each group was 13.5% (US), 27.4% (LPS) and 28.9% (MBV). The expression of CD83 was up-regulated not only by LPS (p=0.002), but also by the MBV (p=0.002).

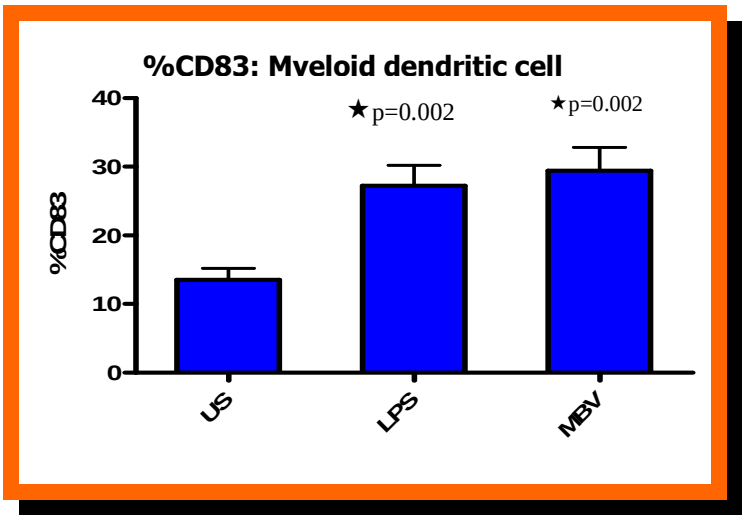

Figure 4.5 Data from healthy individuals (n=10) stimulated with MBV to measure the expression of CD83 by myeloid cells after 8 hour incubation. Percentage values were referenced against the unstimulated samples. Significant values are indicated with ★

4.5.2 Flow cytometric analysis of pDCs post MBV activation

By using the different set of monoclonal antibodies which identified the pDCs, it was possible to show that the presence of MBV significantly influenced the maturation status of pDCs *in vitro.* The MBV induced a very significant increase in both the % CD83 (p=0.004) and % CD80 (p=0.004) expression by pDCs. Once again, we observed a relative increase in the pDCs after the culture periods (Figure 4.6) and the same explanation as provided above refers: this could be due to an *in vitro* "enrichment" during the culture

period and it seems as if more pDCs are obtained after the incubation. This is not feasible due to the fact that these cells do not proliferate. From this figure, it appears that the 3.2% pDCs in the unstimulated almost doubled in the presence of TNF-α (5.4%) or MBV (6.9%) and these were statistically significant (p=0.01).

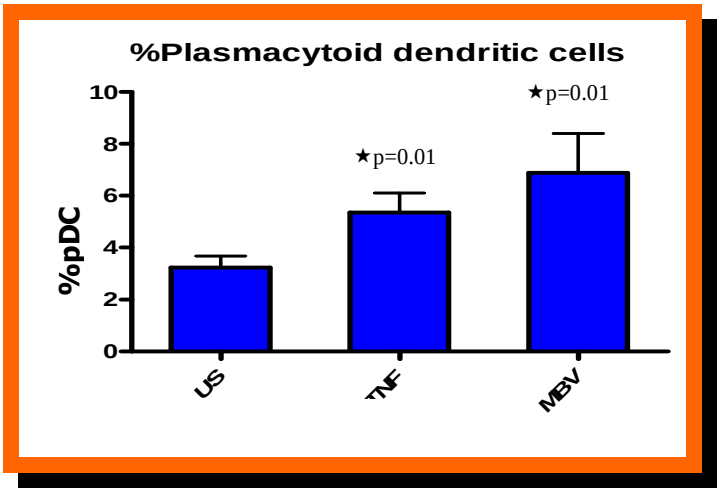

Figure 4.6 Data from healthy individuals (n=10) stimulated with MBV to measure the %plasmacytoid DCs after 8 hour incubation. Significant values are indicated with★

Despite the above, the analysis of the cells following the incubation using the different stimuli revealed an increased/upregulated expression of the markers indicating maturation. After incubation at 37°C for 8h, cells were harvested, and the expression of cell surface antigens was analyzed by FACS analysis. From Figure 4.7, CD80 expression on TNF-α cultured cells was 5.6% (p =0.03) and those incubated in the presence of the MBV tested 7.1% positive (p=0.004) compared to the unstimulated cultures (2.6%).

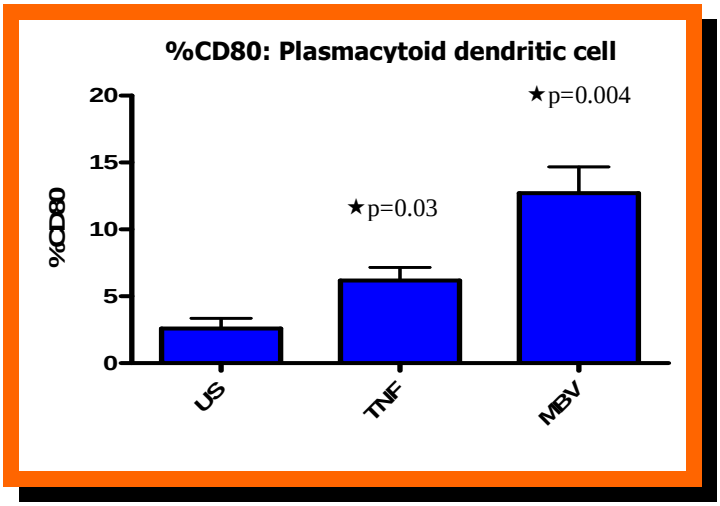

Figure 4.7 Data from healthy individuals (n=10) stimulated with MBV to measure the expression of CD80 by plasmacytoid DCs after 8 hour incubation.

There was a similar trend for the analysis of CD83 expression (Figure 4.8):

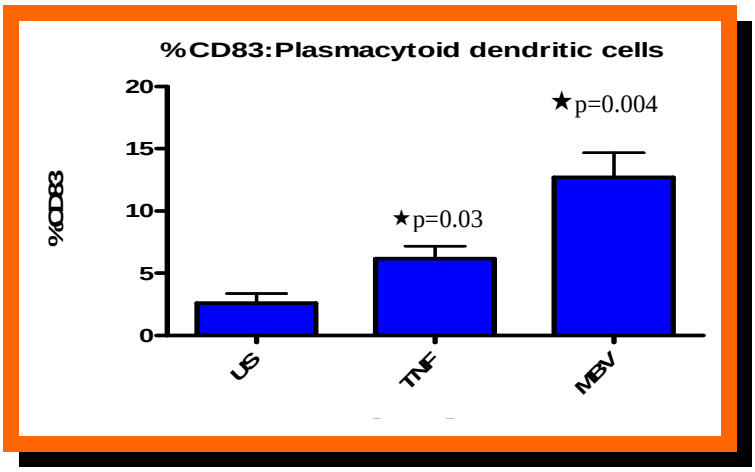

Figure 4.8 Data from healthy individuals (n=10) stimulated with MBV to measure the expression of CD83 by plasmacytoid DCs after 8 hour incubation. Significant values are indicated with★

After incubation at 37°C for 8 h, cells were harvested and the expression of cell surface CD83 expression was analyzed by FACS analysis. The average expression of CD83 in each group was 2.6% (US), 6.2% (TNF-α incubated cells) and 12.7% (MBV cultures). The expression of CD83 was up-regulated not only by TNF-α (p=0.03), but also by the

MBV (p=0.004). The MBV group showed a higher %CD80 expression than the TNF-α group.

4.6 Discussion

Mature DCs arise from immature precursors which are the cells that exhibit endocytic activity (Trombetta ES *et al*.2003). Maturation is a terminal differentiation process that transforms DCs from cells specialized for antigen capture into cells specialized for T-cell stimulation, (Chapuis F *et al*.1997; Rescigno M *et al*.1998) accompanied by changes in the expression of numerous cell-surface antigens that reflect the changing functional role of the cells (Reis e Sousa C *et al*. 2004b). Maturation is characterized by reduced phagocytic uptake, the development of cytoplasmic extensions or "veils", migration to lymphoid tissues, and enhanced T-cell activation potential (Romani N *et al*. 1996b Rovere P *et al*.1998).

It is clear that the maturation of DCs is crucial for the initiation of immunity. The maturation of DCs is completed only upon interaction with T cells. It is characterized by loss of phagocytic capacity and expression of many other accessory molecules that interact with receptors on T cells to enhance adhesion and signaling (co-stimulation); for example CD80, CD86 and CD83. Expression of one or both of the costimulatory molecules CD80 and CD86 on the DCs are essential for the effective activation of T lymphocytes, and, for IL-2 production. These co-stimulatory molecules bind the CD28 molecules on T lymphocytes

The activation and maturation of DCs by bacterial products has been proven by (Nakahara S *et al*. 2003; Reis de Sousa *C et al*. 2003a). With this study in mind it was hypothesized

that the MBV used in this study might modulate DC maturation. This study determined the effects of the mixed killed bacteria at a number of concentrations on the maturation status of both mDCs and pDCs. The first part of the results of this study demonstrated the dose response done to determine the concentration that plays a significant role in the maturation of DCs and the incubation time needed for this. The second part demonstrated the data generated to confirm the data of the dose response.

After incubating the DCs with different concentrations of mixed killed bacteria, the data after 8 hours showed the best results to measure the maturation status of the DCs. In a study done by Camporeale A *et al.* (2003) they also pulse DCs with a CTL epitope and showed that DCS exposed to LPS for 8 hours had a more powerful TH1 response than untreated DCs, or DCs exposed to LPS for 48 hours. This study showed 8 hour DCs were the most potent protective and therapeutic vaccine against B16 melanoma. The dose response also showed the scatter for primary and secondary gating after 8 hours was integrable and therefore we only used the 8 hour data. We found increases in the expression of the maturation markers CD80 and CD83 on DCs in response to stimulation with the MBV, LPS and TNF-α.

Benvenuti F *et al.* (2004) showed in their study that maturation of DCs change the physical interaction with naïve CD 4^+ T cells. Immature DCs create very short contact of low stability. In contrast, mature DCs created longer contacts. The mixed killed bacteria had significantly increased the %CD83 ($p = 0.004$) in the case of mDCs as well as pDCs ($p=0.002$). The vaccine also increased the %CD80 expression in myeloid ($p=0.002$) as well as pDCs ($p=0.004$). We also observed that, in both DC populations, CD83 expression

seemed higher than the CD80 expression: whether this has any significance remains unknown to us at present.

With the results of the study in mind we hypothesized that the MBV could be used therapeutically - however this needs to be tested under clinical conditions. We know that the same MBV is used clinically by oncologists in Germany and that interesting clinical responses are observed (Dr. B. Riedelsheimer, personnal communication). It would however be important to test the MBV under clinical conditions in South Africa. Nevertheless, our hypothesis is based on the fact that the response of pDCs to the MBV provides a key mechanism to control the HIV infection, because a decline in pDCs in chronic HIV-1 infection is associated with high viral loads and opportunistic infections. Patterson S *et al.* (2001c) explains that pDCs play a big role in the antiviral immune responses. They recognize viral components, leading to type I interferon production, and affect adaptive defense strategies designed to eliminate viral pathogens. Perhaps our data will shed some light on the potential use of this vaccine as a form of immunotherapy.

Chapter 5

In vitro activation of dendritic cells using Coley's Toxin

Abstract:

This chapter describes a flow cytometric method to determine the activation and maturation status of DCs post activation using Coley's Toxin. A key aspect that Coley found to be necessary for tumour regression was the induction of a mild to moderate fever. Researchers proved that elevation in body temperature causes maturation of DCs in the skin and other tissues. Coley's Toxin used in this study is a mixture consisting of peptides of heat killed bacteria of species *Streptococcus pyogenes* as well as *Serratia liquefaciens.* One of the biologically active ingredients in Coley toxins is LPS. *In vivo* LPS induces a fever for three to four hours that enhances lymphocyte activity and boosts the expression of TNF-α, IL-6 and IL-1β. The elevation of cytokine levels such as TNF-α contributes to DC maturation.With these results in mind we hypothesized that the Coley's toxin would enhance the maturation and activation status of the DCs in peripheral blood cells when incubated *in vitro* The results of this study showed that Coley's Toxin did indeed induce the maturation of both pDCs and mDCs as measured by increased surface expression of costimulatory molecules such as CD80 and CD83.

5.1 Introduction:

Mature DCs arise from immature precursors which are the cells that exhibit endocytic activity (Trombetta ES *et al.* 2003). Maturation is a terminal differentiation process that transforms DCs from cells specialized for antigen capture into cells specialized for T-cell stimulation (Chapuis F *et al.*1997; Rescigno M *et al*. 1998) accompanied by changes in the

expression of numerous cell-surface antigens that reflect the changing functional role of the cells (Reis e Sousa C *et al*. 2004b). Maturation is characterized by reduced phagocytic uptake, the development of cytoplasmic extensions or “veils”, migration to lymphoid tissues, and enhanced T-cell activation potential (Rovere P *et al*. 1998; Romani N *et al*.1996b). The vaccine used in this chapter is known as Coley’s Toxin (obtained from Dr Brigitte Riedelsheimer) and it consist of peptides from *Streptococcus pyogenes* as well as *Serratia liquefaciens.*

From 1891 until his death in 1936 Coley studied malignancies and the effects of inoculation with live bacterial cultures. Coley developed a vaccine that consist of extracts of killed Gram-positive *Streptococcus pyogenes* and Gram-negative *Serratia marcescens,* which became known as Coley's toxin. Wieman B *et al*. (1994) explained that the major effect of Coley’s Toxin comes primarily from LPS (an endotoxin) of Gram-negative bacteria. Different studies showed different effects of fever on the immune response and on cytokine expression and function. For instance, Van Oss CJ *et al*. (1980) showed in their study that human polymorphonuclear cells (PMN) phagocytose better in the presence of fever but that PMN chemotaxis is not enhanced. They also confirmed in their study that macrophage functions are enhanced in the case of fever, including the expression of Fc receptors, phagocytosis, pinocytosis and killing of intracellular bacteria. However, like PMNs, macrophages have markedly reduced function at temperatures > 41°C. The cytotoxic activity of human natural killer (NK) cells has been shown to be reduced in the case of fever. Basu S *et al*. (2003b) proved in their study that elevation in body temperature causes maturation of DCs in the skin and other tissues. Veckman V and Julkunen I (2007) showed in their study that *Streptococcus pyogenes* activates the maturation of both pDCs

and mDCs. With these facts in mind we hypothesized that the Coley's toxin would enhance the maturation and activity of the DCs.

Cancer studies have shown that there are high concentrations of immune cells within the tumour. Unfortunately, these immune cells are not receiving the proper signals to fight the cancer. Thus, the cancer continues to grow and spread despite the presence of many immune cells. In studies done by Coley the results showed that when his vaccine caused a fever, the cancer would start to shrink and the number of peripheral immune cells would increase. He also found that even though the tumour would start to shrink when the vaccine caused a fever, if he stopped injecting the vaccine too early, the cancer would start to grow again. Therefore, the immune cells could be stimulated to fight the cancer, but only for a limited period of time.

5.2 Materials and Methods:

5.2.1 Study design:

Heparinized Blood samples were collected from 2 healthy individuals. A dose response of Coley's Toxin was done to determine the optimal activation of the vaccine. Human mononuclear cells are often required in order to conduct specialized assays. The peripheral blood mononuclear cells are therefore isolated from the remaining blood elements by density gradient centrifugation. The isolated cells were incubated with different concentrations of the MBV and incubated for 8, 12 and 24 hours. Cells were centrifuged and the supernatants removed. The supernatants were stored at -20°C for cytokine detection described in chapter 6. Monoclonal antibodies to CD80 and CD83 were added to the cells to determine the activation and maturation status of the DCs. Prepared samples were aquired on a flow cytometer (BD™ FACSCalibur). CellQuest™ software was used for acquisition with a threshold set on FSC to exclude debris. DCs occur with low

frequency in peripheral blood. To make sure enough DCs were acquired a minimum of 2000 events in Gate 2 were acquired. In the case of mDCs, Gate 2 was set on CD11c APC and in the case of pDCs, Gate 2 was set on HLA-DR PerCP. pDCs are $CD11c^-$, but HLA-DR^+. The same software was used for data analysis.

5.2.2 Reagents preparation:

RPMI 1640 growth medium (GIBCO® 61870) (Obtained from Sigma Aldrich)

Fetal Bovine Serum (Obtained from Adcock Ingram) (GIBCO®) (decomplemented at 56°C for 30 minutes in waterbath)

Histopaque-1077 solution (Obtained from Sigma Aldrich)

Distilled or deionized water

Sterile Phosphate buffered saline (PBS)

- 1L Schott bottle was filled with double distilled water and autoclaved
- One bottle Dulbecco's phosphate buffered saline(obtained from Sigma Aldrich) was added to the 1L of autoclaved water and stored at 4°C

5% Fixative:

- Paraformaldehyde (obtained from MERCK) was diluted 1:20 with PBS (e.g. 5ml paraformaldehyde + 95ml PBS) and stored at room temperature for up to 1 month

LPS:

1 ml Sterile PBS was added to the stock of 1mg LPS (Obtained from Sigma Aldrich). We made a 1/10 dilution of the stock solution (50µl LPS+450µl RPMI). 20µl of this dilution was added to each tube which was to be activated using the LPS.

TNF-α:

A recombinant stock solution 10 ng/ml TNF-α (Obtained from R&D Systems) were diluted 1/10 (50µl TNF-α + 450µl RPMI). Twenty microliter of this dilution was added to each tube for TNF-α activation.

5.3 Preparation of Coley's Toxin and determination of optimal dose:

The Coley's toxin used in this study was a gift from Dr Brigitte Riedelsheimer, Dusseldorf, Germany. It is used for fever therapy in cancer patients. Different concentrations of the Coley's Toxin were tested to determine the optimal dose for activation and maturation of DCs: refer to Table 5.1. The optimal dose was determined as that which induced the highest expression of the activation markers CD80 or CD83.

The maturation of DCs *in vitro* was achieved by adding different concentrations of the toxin to blood obtained from two healthy donors. These cells were incubated for 8, 12 and 24 hours and analyzed. LPS was added as a positive control for mDCs and TNF-α as positive control for pDCs. Three sets of Falcon tubes (BD™ Biosciences) were labelled and the following added to each tube (See Table 5.1):

Table 5.1:

Tube number and contents	Cells at concentration of $1x10^6$cells/ml	Volume of Coley's/LPS/ TNF-α	Volume of RPMI
1 : Unstim	500µl	0	500µl RPMI
2 : LPS	500µl	20µl LPS (2µg/ml)	480µl RPMI
3. TNF-α	500µl	20µl TNF-α (0.2µg/ml)	480µl RPMI
4: 1/10	500µl	50µl Coley's	450µl RPMI
5: 1/50	500µl	10µl Coley's	490µl RPMI
6: 1/100	500µl	5µl Coley's	495µl RPMI
7: 1/200	500µl	2.5µl Coley's	497.5µl RPMI

One set was incubated for 8 hours, one for 12 hours and the last set for 24 hours. After each incubation period the cells were centrifuged at 1800rpm for 5 min. The supernatant was aspirated and put in cryovials to determine cytokine production described in chapter 6. One ml PBS was added to the cells in each tube. The cells were vortexed and used for the analysis of activation/maturation markers.

The recommended amount of antibodies (as indicated in chapter 3) and 100 µl PBMCs (0.1×10^6 PBMCs) were mixed together in two Falcon Tubes. Tubes were incubated for 15 minutes at room temperature in the dark and 2ml of FACS Lysing Solution were added to each tube. Tubes were vortexed and incubated for 10 minutes at room temperature in the dark. The tubes were centrifuged at 1800rpm for 5 minutes and the supernatant was discarded. Tubes were vortexed gently to resuspend the pellet and 1 ml PBS was added to

each tube. Tubes were centrifuged again at 1800rpm for 5 minutes, the supernatant was discarded and the pellet of cells was resuspended in 500 µl of 5% paraformaldehyde. Tubes were analyzed on a FACS Calibur Flow cytometer.

5.4 Data Acquisition and Analysis:

CaliBRITE™ beads and FACSComp™ software were used to adjust the photomultiplier tube (PMT) voltages and fluorescence compensation, and to check the sensitivity of the instrument. CellQuest™ software was used for acquisition with a threshold on FSC to exclude debris. Since DCs occur with low frequency in peripheral blood, we acquired a minimum of 2000 events in Gate 2 to ensure sufficient events were analysed. In the case of mDCs, Gate 2 was set on CD11c APC and in the case of pDCs, Gate 2 was set on HLA-DR PerCP.pDCs are $CD11c^-$, but $HLA\text{-}DR^+$. The same software was used for data analysis.

5.5 Results:

Once again, we observed a high background measurement of CD80 and CD83 expression in the unstimulated cultures. The same explanations as provided in Chapter 4 apply (see Figure 5.1 as an example of the dose response of the cells to Coley's toxin used). Once again, the 1/50 dilution used for 8 hours induced reproducible results without too much debris in the cell cultures. Very similar and reproducible results were obtained for the expression of CD83 marker using the same dose of the toxin (data not shown). For this reason, further experiments were conducted using the 1/50 dilution of the toxin provided and an incubation period of 8 hours used for optimal stimulation. This applied to both mDCs as well as pDCs. The results for the maturation markers obtained during the dose reponse were higher than the results obtained from the 10 healthy volunteers. The only

explanation for this is the group size and the fact that we changed batches of heparin tubes.The dose response was done on only two individuals and a different batch of heparin tubes were used for the blood draw.

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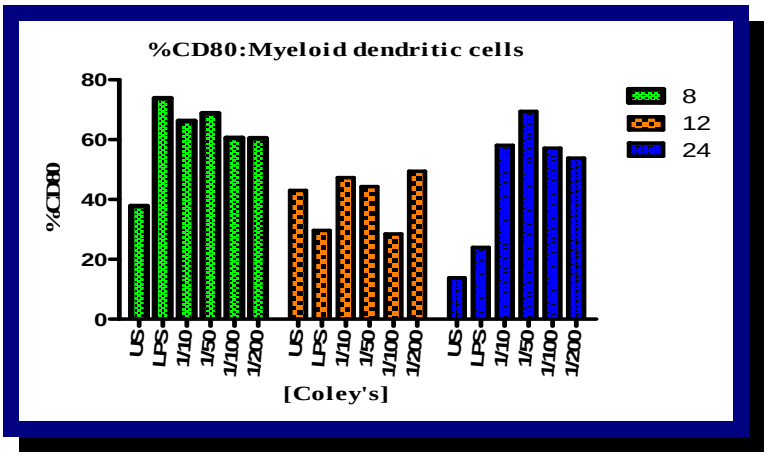

Figure 5.1 Dose response of Coley's Toxin to measure the expression of CD80 by mDCs after 8, 12 and 24 hours incubation

Statistical analysis of dose reponse data:

For the analysis of the dose response data we used a Paired T test. All results were considered statistically significant when $p < 0.05$. All groups were compared to the unstimulated group to see the effect of the mixed killed bacterial vaccine.

5.5.1 Flow cytometric analysis of mDCs post Coley's Toxin activation:

The assay was applied to blood samples obtained from 10 healthy controls. Samples were treated the same as in the dose response method explained above: The 1/50 dilution of Coley's toxin was used and the cells were incubated for 8 hours in the presence of medium (unstimulated) or LPS or TNF-α or Coley's Toxin. TNF-α was included in this experiment

as a positive control for pDCs and LPS as a positive control for mDCs. The individual groups were analyzed by the Wilcoxon matched paired test and all results were considered statistically significant when $p< 0.05$. All groups were compared to the unstimulated group to see the effect of Coley's Toxin.

The presence of Coley's Toxin significantly influenced the maturation status of DCs *in vitro.* Coley's Toxin induced a very significant increase in both the %CD83 (p=0.002) and %CD80 (p=0.002) expression by mDCs. Once again, we observed a relative increase in the percentage mDCs in the cultures post activation (Figure 5.2). Once again, the only explanation that we can offer to explain this phenomenon may be due to the "enrichment" of these cells during the culture period or the culture medium used: the unstimulated cultures displayed 5.8 % cells staining with the antibodies specific for mDCs, 8.2 % for the LPS cultures and 8.0 % for the Coley's toxin incubated cultures (Figure 5.2).

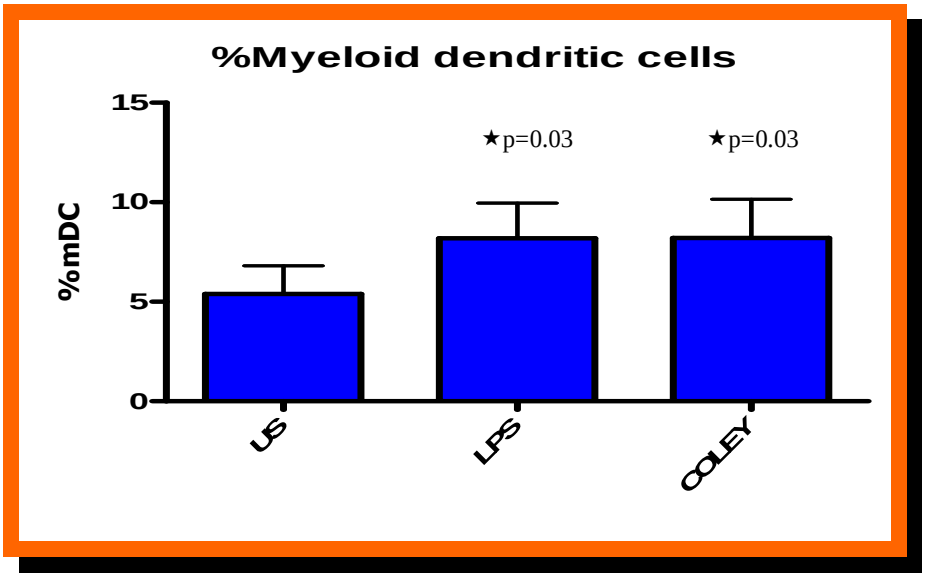

Figure 5.2: Data from healthy individuals (n=10) stimulated with Coley's Toxin to measure the % mDCs after 8 hours incubation. Significant columns are indicated with★

The analysis of the activation markers CD80 and CD83 in the mDCs showed a significant increase of these markers following the incubation with the stimuli (Figure 5.3 and Figure 5.4). After incubation at 37°C for 8 h, cells were harvested, and the expression of cell surface antigens was analyzed by FACS analysis. The expression of CD80 in each group were 4.3% (US), 20.6% (LPS) and 22.3% (Coley's).The expression of CD80 was increased, not only by LPS (p=0.002), but also by Coley's (p=0.002).

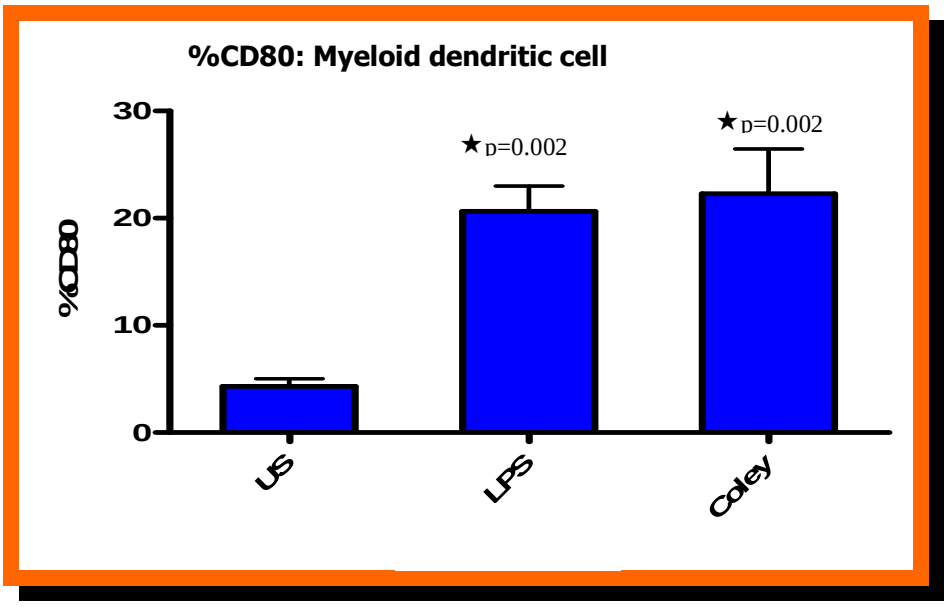

Figure 5.3 Data from healthy individuals (n=10) stimulated with Coley's Toxin to measure the expression of CD80 by mDCs after 8 hours incubation. Significant columns are indicated with★

Very similar results were observed when the CD83 data was analysed: Figure 5.4 indicates the increase of CD83 expression by mDCs incubated with medium alone (US) cells plus LPS or cells plus Coley's Toxin. The average expression of CD83 in each group was 13.5% (US), 27.2% (LPS) and 26.8% (Coley's). The expression of CD83 was significantly increased not only by LPS (p=0.002), but also by Coley's (p=0.002).

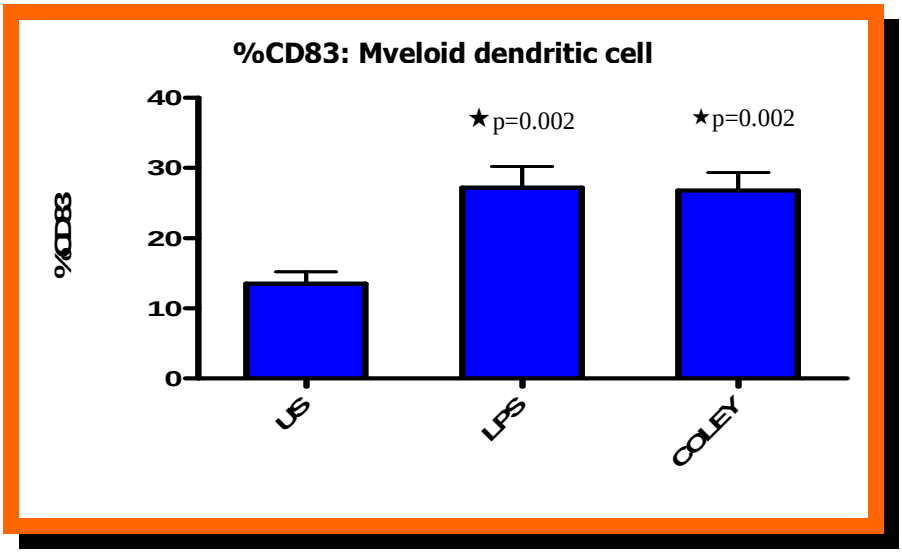

Figure 5.4 Data from healthy individuals (n=10) stimulated with Coley's Toxin to measure the expression of CD83 by mDCs after 8 hours incubation. Significant columns are indicatedl with★

5.5.2 Flow cytometric analysis of pDCs post Coley's Toxin activation:

The presence of Coley's Toxin significantly influenced the maturation status of DCs *in vitro.* Coley's Toxin showed a very significant increase in the %CD83 and a less impressive increase in % CD80 expression by pDCs. The % pDCs also increased significantly (p=0.01) when analysed after the incubation period (Figure 5.5): after incubation at 37°C for 8h, the percentage pDCs in each group were 3.6% (US), 6.6% (TNF-α) and 6.6% (Coley's). This significant increase of % pDCs in the TNF (p=0.01) and Coley's (p=0.01) is possibly due to enrichment of the cells analysed in Gate 2 as previously explained.

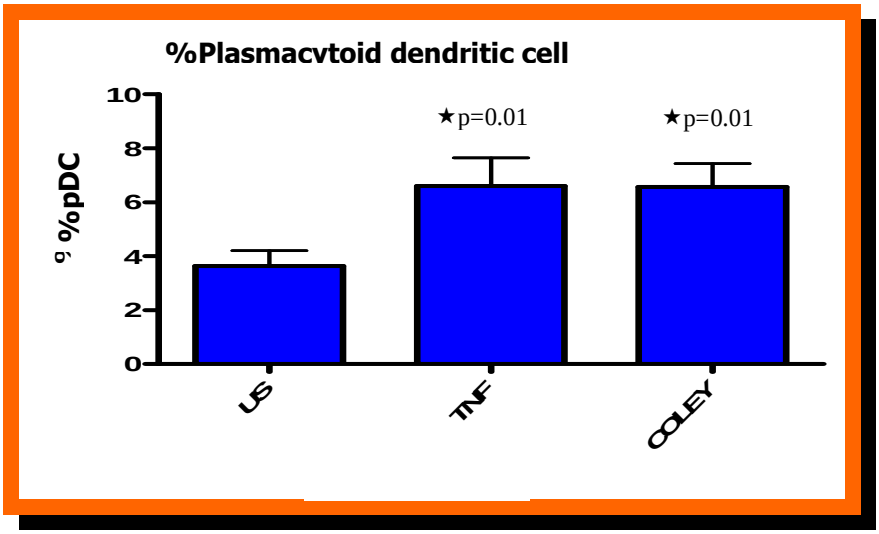

Figure 5.5 Data from healthy individuals (n=10) stimulated with Coley's Toxin to measure the % pDCs after 8 hour incubation. Significant columns are indicated with★

The expression of the activation markers as measured by CD80 or CD83 staining using the monoclonal antibodies was once again very similar to that measured in mDCs. However, the pDCs post activation with TNF-α or the Coley's toxin did not show statistically significant up-regulation of the CD80 antigen. In Figure 5.6, the up-regulation of CD83 expression by pDCs incubated with medium alone (US) or cells plus TNF-α or cells plus Coley's Toxin is shown. The average expression of CD83 in each group was 2.3% (US), 6.2% (TNF-α) and 10.1% (Coley's). This increased expression of CD83 was significant for both the TNF-α ($p=0.01$) as well as for the Coley's toxin ($p=0.002$) (Figure 5.6).

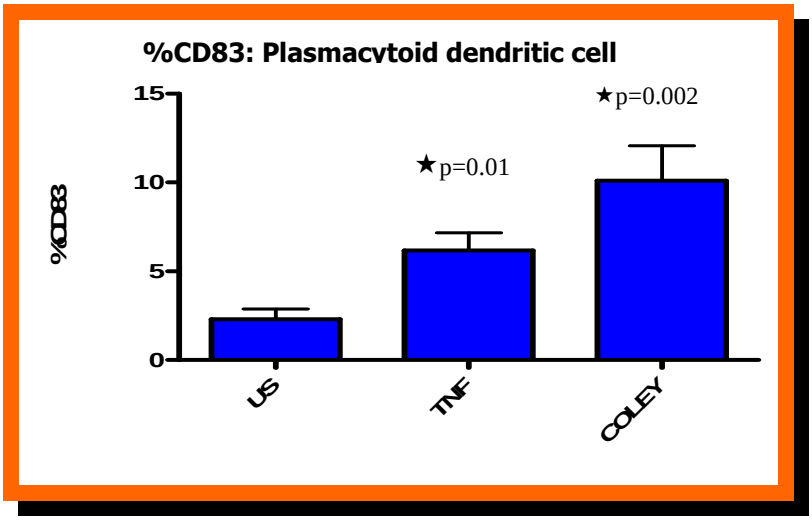

Figure 5.6 Data from healthy individuals (n=10) stimulated with Coley's Toxin to measure the expression of CD83 by pDCs after 8 hours incubation. Significant columns are indicated with★

On the other hand, the same culture conditions did not induce any significant enhanced expression of the CD80 marker (Figure 5.7). We did expect an increase in expression of %CD80. This figure clearly shows that CD80 expression was not statistically significantly ($p=0.06$) different between the medium alone 3.0% or TNF-α incubated cells (4.0%) or the cultures that had received the Coley's toxin (5.1%). In Figure 4.7 the TNF-α did show a significantly increase of the % CD80. The unstim value in the experiment with MBV was lower.The differential expression of the CD80 on pDCs post activation remains unclear at present. Duramad O *et al.* (2003) explained in their study that IL-10 is a potent anti-inflammatory cytokine that can be produced by different cell types. IL-10 can inhibit both TH1 and TH2 responses by affecting APC function and DC maturation. They also claim that IL-10 inhibits the activation of pDCs and induces apoptosis of pDCs. The IL-10 can be secreted by the other cells in the PBMC preparation, but recently Tomoki I *et al.* (2007) discovered that pDCs prime IL-10 producing T-regulatory cells. Only pDCs express inducible costimulator ligand

(ICOS-L). The ICOS-L expression allows pDCs to induce the differentiation of naïve CD4 T cells to produce IL-10. These cells do not produce the TH2 cytokines IL-4, IL-5 and IL-13.These IL-10 producing cells are called T regulatory cells.

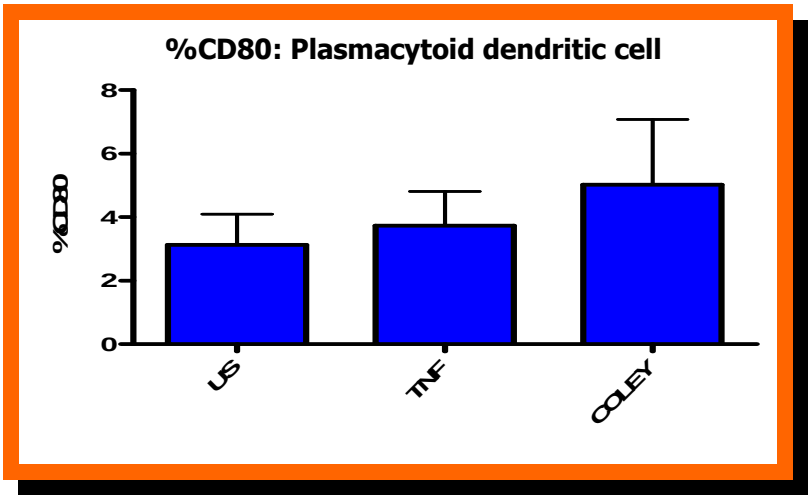

Figure 5.7 Data from healthy individuals (n=10) stimulated with Coley's Toxin to measure the expression of CD80 by pDCs after 8 hours incubation.

5.6 Discussion

Mature DCs arise from immature precursors which are the cells that exhibit endocytic activity (Trombetta ES *et al.* 2003). Maturation is a terminal differentiation process that transforms DCs from cells specialized for antigen capture into cells specialized for T-cell stimulation (Chapuis F *et al.* 1997; Rescigno M *et al.* 1998), accompanied by changes in the expression of numerous cell-surface antigens that reflect the changing functional role of the cells (Reis e Sousa C *et al.* 2004b). Maturation is characterized by reduced phagocytic uptake, the development of cytoplasmic extensions or "veils", migration to lymphoid tissues, and enhanced T-cell activation potential (Rovere P *et al.*1998; Romani N *et al.* 1996b).

The activation and maturation of DCs by bacterial products has been proven by Nakahara S *et al.* 2003; Reis de Sousa *C et al.* 2003a. With this study in mind it was hypothesized that Coley's Toxin used in this study might modulate DC maturation. This study determined the effects Coley's Toxin at a number of concentrations on the maturation status of both myeloid and pDCs. The first part of the results of this study demonstrated the dose response done to determine the concentration that plays a significant role in the maturation of DCs and the incubation time needed for this. The second part demonstrated the data generated to confirm the data of the dose response.

The activation of DCs by bacterial products (*S. pyogenes*) has been proven by Nakahara S *et al.* (2003). *S. pyogenes* infections are sometimes accompanied by the release of toxins from the bacteria. These toxins produced many of the symptoms of bacterial infections, such as fever. A key aspect that Coley found to be necessary for tumour regression was the induction of a mild to moderate fever. Basu S (2003) states that even brief exposure to

increased body temperature can cause maturation of DC in skin and other tissues. The increase in the levels of cytokines such as TNF-α related with fever, also plays a role in DC maturation.

We found increases in the expression of the maturation markers CD80 and CD83 on DCs in response to stimulation with Coley's Toxin, LPS and TNF-α. Coley's toxin had significantly up-regulated the expression of CD83 ($p = 0.002$) in the case of mDCs as well as pDCs ($p=0.002$). The vaccine also up-regulated the %CD80 in myeloid ($p=0.002$) but not on pDCs. We also observed that, in both DC populations, CD83 expression was more pronounced than the CD80 increase. With the results of the study in mind we hypothesized that Coley's Toxin can play an important role in the treatment of infectious diseases like HIV. The response of pDCs to Coley's Toxin provides a key mechanism to control the HIV infection, because a decline in pDCs in chronic HIV-1 infection is associated with high viral loads and opportunistic infections. Patterson S *et al* (2001c) explains that pDCs cells play a big role in the antiviral immune responses. They recognize viral components, leading to type I interferon (IFN) production, and affect adaptive defense strategies designed to eliminate the virus.

Together, these findings are consistent with the known role of mDC in T-cell priming and an immune regulatory role for pDCs. Thus, pDCs migrate rapidly from peripheral blood to lymph nodes following activation, where they can influence mDCs and T cells. We suggest that the potent and simultaneous activation of both DC subsets by Coley's Toxin makes this a useful reagent for screening DC function in clinical specimens by the assay described above, as well as a potentially useful immune adjuvant for clinical use.

Chapter 6

Cytokine profile of *in vitro* activated and matured dendritic cells

Abstract:

This chapter describes a flow cytometric method for the quantitation of cytokines secreted by DCs activated by Coley's Toxin and Mixed Killed bacteria. The method used is known as multiplexing, where the simultaneous assay are used to detect many analytes in a single sample. The BD™ Cytometric Bead Array (CBA) flex set was used to quantitatively measure different cytokines: these included Interleukin-6 (IL-6), Interleukin-4 (IL-4), Interleukin-10 (IL-10), Tumor Necrosis Factor- α (TNF-α), Interferon-gamma (IFN-γ) and Interleukin-12p70 (IL-12p70) protein levels in a single sample. The results of this study showed that both Coley's Toxin and the MBV induced cytokine profiles capable of leading to both TH1 and TH2 responses.

6.1 Introduction

Cytokines are small soluble factors released by cells to communicate and influence the functions of other cells through the interaction with different surface receptors. According to Baggiolini M (1998), the main characteristics of cytokines are

- Cytokines have many different effects to different cells
- Cytokines posses autocrine functions (feedback mechanism) thereby acting directly on the cell that released it.
- Cytokines have a paracrine effect on the cells immediately around them
- Cytokines can act like hormones and have endocrine effects on cells and organs
- They may induce the release of other cytokines

- Cytokines may act synergistically to achieve a greater effect than the sum of their individual actions.

Maturation of DCs can be influenced by a variety of cytokines including IL-1, GM-CSF, and TNF-α . To optimally induce DC maturation, especially in humans, these cytokines have been used at various timings, concentrations, and durations and in various combinations. TNF-α might be one of the most commonly used cytokines for this purpose. However, it has recently been reported that TNF-α alone cannot induce full maturation of DCs. Nakahara S *et al.* (2003) showed in their study that multiple cytokines are needed to induce full maturation of DCs. They also state that in addition to these cytokines, bacteria and molecules like LPS have been shown to stimulate DC maturation. Although cytokines activate the maturation of DCs, literature shows that IL-10 effectively inhibits the maturation of DCs (Spight D *et al:* 2004; Wallet MA *et al.*2005). Thus, it is now evident that multiple cytokines should be used for this purpose. In addition to these cytokines, bacteria and related molecules including LPS (cell wall component of Gram-negative bacteria) have been shown to induce DC maturation (Rescigno M *et al.* 1999)

DCs form intimate contact with T cells and these interactions induce the release of soluble cytokines by both cell types. T cell cytokine responses dominated by the production of high levels of IFN-γ and little IL-4 by CD4 T cells are called TH1 cell responses and are known to be induced by antigen presenting cells producing IL-12. In contrast, T cell responses dominated by IL-4 production with little IFN-γ are called TH 2 T cell responses. A variety of external stimuli can be applied to DC to regulate their production of IL-12 and the production of IFN-γ or IL-4 in the T cells with which they interact. Recent studies suggested that distinct types of DC may regulate distinct types of

T cell responses: for instance, pDCs appear to give rise to TH2 T cell cytokine responses, whereas monocyte-derived DC, which represent a typical mDC, give rise to TH1 T cell responses. Veckman V *et al*: (2004) proved in their study that *S. pyogenes* does induce the maturation of monocyte derived DCs. The stimulation with *S. pyogenes* resulted in the expression of CD80, CD83 and CD86. Their study also showed a TH1 cytokine and chemokine response, after the DCs were stimulated with *S.pyogenes.* Duramad O *et al.* (2003) explained in their study that IL-10 is a potent anti-inflammatory cytokine that can be produced by different cell types. IL-10 can inhibit both TH1 and TH2 responses by affecting APC function and DC maturation. They also claim that IL- 10 inhibits the activation of pDCs and induces apoptosis of pDCs. The IL-10 can be secreted by the other cells in the PBMC preparation, but recently Ito T *et al.* (2007) discovered that pDCs prime IL-10 producing T-regulatory cells. Only pDCs express inducible costimulator ligand (ICOS-L). The ICOS-L expression allows pDCs to induce the differentiation of naïve CD4 T cells to produce IL-10. These cells do not produce the TH2 cytokines IL-4, IL-5 and IL-13. These IL-10 producing cells are called T regulatory cells

We examined the secretion of TH1-type cytokines (IL-12p70, TNF-α and IFN-γ) and TH2-type cytokines (IL-4, IL-6 and IL-10) from DCs after stimulation with TNF-α, LPS, Coley's Toxin and Mixed Killed Bacteria. The method used for this assay is known as multiplexing, which means that simultaneous assay are used to detect many analytes in a single sample. The BD™ Cytometric Bead Array (CBA) Flex set is used to detect a series of multiple soluble analytes. In this study we used the BD CBA Human Soluble Protein Flex set: it is a particle-based immunoassay and each bead provides a capture surface for a specific protein (resembles an individually coated well in an ELISA plate). Only a small

volume sample is needed for this assay and the value of an unknown can be obtained in substantially less time compared to an ELISA. The BD™ CBA Flex set can be used to quantitatively measure IL-6, IL-4, IL-10, TNF-α, IFN-γ and IL-12p70 protein levels in a single sample.

6.2 Preliminary assays:

The method for Flexsets is a new method developed by BD Biosciences. In a preliminary set of experiments, we were provided 7 flexsets in order to test the method before we assayed samples from the dose responses as well as the samples from the 10 healthy individuals. For this we used Flexsets supplied by BD Biosciences free of charge: these consisted of IL-2, IL-6, IFN-γ , IL -8, IL-1β and IL-4.

Once the method had been set up and validated, we modified the flexsets used for this study and this consisted of IL-12 p70, TNF, IL-10, IFN-γ, IL-6, IL-4 and IL-2. We replaced the IL-8 and IL-1β flexset of the preliminary test with IL-12p70, IFN -γ and added IL-10 as well to determine if the *in vitro* activated DCs elicited a TH1 or a TH2 response. The results of the preliminary tests actually showed very high levels of IL-1β and IL-8 which indicates an inflammatory response: both the Coley's Toxin and the MBV stimulated cultures secreted IL-1β and IL-8 at high levels (>5000 pg/ml). These samples were not diluted and re-assayed to fit on the standard curve and to determine the real value.

6.3 Samples analysed:

Since the flow cytometric analysis of surface expression of the activation markers CD80 and CD83 indicated that the 8 hour incubation with 1/50 dilutions of both the MBV and the Coley's toxin induced maximal upregulation, we concentrated on the supernatants generated from these cultures to assay the cytokines. These had been generated and stored at -20^{o}C until assayed.

Blood was drawn from 10 normal healthy individuals and these samples were treated in the same manner as described in the previous chapters: cells were incubated for 8 hours with medium, TNF-α, LPS, the optimal concentration of MBV and Coley's Toxin. The data was analysed by the Wilcoxon matched paired test. All results were considered statistically significant when $p < 0.05$ compared to the unstimulated cultures.

6.4 Results

The high background value of the unstimulated sample as observed in the surface expression of the activation markers were confirmed once again: the supernatants of these cultures revealed high levels of the cytokines although the stimulated cultures exhibited even higher levels, especially those having received the bacterial products (either the MBV or the Coley's toxin). Tkachenko N *et al.* (2005) tested different culture media for the generation of DCs and discovered that the growth and maturation of DCs in culture can be influenced by a variety of factors. In this study they claim that media supplemented with FCS can influence the DC maturation. FCS contains endotoxin, for example LPS, which can activate the DCs, even though the concentrations are very low.

As can be seen in the following tables, each cytokine measured for each donor has been tabulated with the corresponding stimulus used: The means and standard deviation of these results were not calculated due to the variation in results.

Table 6.1(a) IL-6 (pg/ml) released upon stimulation with various activators:

Individual	**Unstim**	**LPS★**	**TNF★**	**Coley's★**	**MBV★**
1	87.44	>5000	>5000	>5000	>5000
2	116.04	2220.41	2371.74	2495.59	1899.17
3	81.78	3128.30	2324.04	4282.47	1618.38
4	63.57	>5000	2560.11	1997.07	845.12
5	231.58	3260.72	>5000	2910.21	836.91
6	1487.25	>5000	>5000	1539.84	>5000
7	114.85	1562.99	2970.81	2277.35	3294.75
8	90.40	>5000	>5000	>5000	>5000
9	3773.60	>5000	1997.07	2470.28	1327.17
10	292.65	>5000	>5000	4420.94	>5000

Table 6.1(b)

Statistical analysis : IL- 6	
Unstim vs LPS	p= 0.002
Unstim vs TNF-α	p= 0.004
Unstim vs Coley's	p= 0.006
Unstin vs MBV	p= 0.02

From Table 6.1 above, it can be seen that the individual responses to the stimuli varied from one individual to the next and that the LPS and/or TNF-α induced much higher levels of IL-6 when compared to the experimental products, namely, the MBV or the Coley's toxin.

Table 6.2(a): TNF-α (pg/ml) secretion profile of the cultures:

Individual	**Unstim**	**LPS★**	**TNF★**	**Coley's★**	**MBV★**
1	8.1	460.31	690.39	449.54	1784.67
2	87.44	243.46	363.29	426.72	915.33
3	111.24	426.72	430.78	370.24	1156.33
4	87.44	817.87	491.90	266.70	735.06
5	117.30	403.16	246.38	253.00	333.58
6	218.91	1234.84	829.93	1572.62	1228.63
7	88.43	273.15	370.24	595.03	1015.40
8	28.43	501.33	842.16	802.10	3454.19
9	556.60	985.64	478.10	813.91	1228.58
10	200.53	809.94	842.16	884.39	1842.65

Table 6.2(b)

Statistical analysis : TNF-α	
Unstim vs LPS	p= 0.002
Unstim vs TNF-α	p= 0.004
Unstim vs Coley's	p= 0.006
Unstin vs MBV	p= 0.02

The data presented in Table 6.2 above indicates the concentrations of TNF-α secreted by PBMC's incubated with either medium alone (US) or cells plus LPS, TNF-α, MBV or Coley's Toxin. The data from each individual were compared to the individual's unstim value using the Wilcoxon test and all the data were statistically significant. The MBV induced higher levels of TNF-α than the LPS.

Table 6.3(a) IL-10 (pg/ml) secretion post-activation using various stimuli:

Individual	**Unstim**	**LPS★**	**TNF★**	**Coley's★**	**MBV★**
1	0	47	139.16	84.66	81.87
2	0	14.96	0	16.39	20.17
3	0	14.96	0	27.29	17.87
4	0	142.01	14.61	20.56	13.41
5	0	33.56	10.35	38.83	9.00
6	0	59.05	26.89	6.66	32.06
7	0	19.97	21.36	42.21	47.00
8	0	53.13	12.73	48.25	88.49
9	3.67	70.60	6.82	39.38	23.42
10	0	35.09	16.76	34.58	40.22

Table 6.3(b)

Statistical analysis : IL- 10	
Unstim vs LPS	p= 0.002
Unstim vs TNF-α	p= 0.008
Unstim vs Coley's	p= 0.002
Unstin vs MBV	p= 0.002

Table 6.3 above shows the measured levels of IL-10 secreted by PBMC's incubated with either medium alone (US) or cells plus LPS or TNF-α or the MBV or the Coley's Toxin. Each individual served as his/her own control with the stimulated values compared to the unstimulated value. Significant induction of IL-10 release was noted for all cultures with the levels secreted in the presence of Coley's toxin and the MBV being less than that induced by LPS group (but higher than the TNF-α cultures).

Table 6.4(a) IL-2 (pg/ml) induced secretion by various stimuli:

Individual	**Unstim**	**LPS**	**TNF**	**Coley's★**	**MBV**
1	16.15	13.47	23.23	8.51	6.68
2	6.7	34.49	16.15	10.46	0
3	8.51	0	0	8.5	13.47
4	0	8.81	8.51	8.51	0
5	0	6.68	13.11	23.23	8.51
6	16.51	8.51	0	29.54	25.39
7	3.92	0	8.51	8.51	18.64
8	16.15	0	8.71	33.52	16.15
9	12.01	8.51	23.23	18.64	6.51
10	8.51	8.51	0	0	8.51

Table 6.4 (b)

Statistical analysis : IL- 2	
Unstim vs Coley's	p= 0.04

Since IL-2 is a predominant T cell derived cytokine, it was decided to measure this cytokine in the supernatants of the cell cultures in order to determine whether the MBV and/or the Coley's toxin were general polyclonal activators or many cell types or whether they were more specifically targeted to the monocytes and DCs. It can be seen in the above table (Table 6.4) that only the Coley's Toxin induced statistically significant amounts of IL-2 to be released from the cell cultures. The LPS, TNF-α and the MBV cultures (although the stimulated cultures may have shown some enhanced secretion) did not show significantly increased levels of the cytokine when compared to the corresponding unstimulated value. This is not unexpected since the stimuli used are predominantly inducers of inflammatory cytokine release rather than T cell derived, immune regulatory cytokine inducers.

Table 6.5(a) IFN-γ (pg/ml) secretion by PBMC's activated using various stimuli:

Individual	Unstim	LPS	TNF	COLEY★	MBV★
1	0	0	0	110	359
2	0	0	0	16	0
3	2	0	0	17	13
4	0	0	0	0	0
5	0	0	0	44	47
6	0	0	0	176	84
7	4	0	0	18	14
8	0	0	0	25	11
9	0	0	0	9	29
10	0	0	0	8	0

Table 6.5 (b)

Statistical analysis: IFN-γ	
Unstim vs Coley's	p=0.002
Unstim vs MBV	p= 0.008

In Table 6.5 above, the results of secreted IFN-γ by PBMC's incubated with either medium alone (US) or cells plus LPS, or TNF-α oR the MBV or the Coley's toxin are presented. Once again, since IFN-γ is secreted predominantly by activated T cells and NK cells, it was not surprising that the LPS and TNF-α did not induce the release of this cytokine from the PBMCs *in vitro*. It was however interesting that both the MBV and the Coley's toxin were able to induce the cytokine under the same culture conditions. This implies that these bacterial products target T cells specific for these antigens and induce the activation of the T cells (and possibly the NK cells in the cell preparation). DCs are not known to release this cytokine: it has been described that IFN-α is one of the cytokines released from mature DCs amongst others so the origin of this factor could not be from T cells and/or NK cells.

The assay for the IL-12p70 measurements in the supernatants obtained after 8 hours of stimulation revealed no measurable levels of this cytokines in any of the cell cultures, irrespective of the stimulus used (data not shown). This surprising observation could be explained in one of two ways: either the time period for optimal IL-12p70 release was too short compared to other cytokines or the dose of the stimuli used was not optimal. Since the analyses of only the 8 hour cultures were conducted for all the cytokines (as reported above), it was decided to assay the supernatants obtained from the dose-response

experiments whereby 2 individuals were used and the supernatants collected over various time periods. The data of the 12 hour cultures are shown hereunder (Table 6.8).

Table 6.6(a) IL-12 p70 (pg/ml) secretion in response to Coley's toxin (12 hours culture):

Individual	**Unstim**	**1/10★**	**1/50★**	**1/100★**	**1/200★**
1	27.53	59.63	57.63	52.36	58.63
2	37.92	69.61	60.07	70.50	69.79

Table 6.6(b)

Statistical analysis: IL- 12 p70	
Unstim vs 1/10	p= 0.004
Unstim vs 1/50	p= 0.048
Unstim vs 1/100	p= 0.043
Unstim vs 1/200	p=0.008

It is very evident from the results presented in Table 6.6 above that the absence of IL-12p70 in the 8 hour culture supernatants was indeed due to the culture period being too short. Irrespective of the dose of the toxin used, the cytokine was measurable in these cultures and the levels were statistically significantly raised when compared to the unstimulated values. At this stage, due to financial constraints, we were not able to repeat the assay for IL-12p70 in the supernatants obtained from the same 10 individuals reported above: we did however assume that the 12 hour supernatants would have tested positive for this cytokine. Although limited in data size, we would like to postulate that Coley's toxin is possibly capable of inducing the *in vitro* maturation of immature DCs (iDCs) and to produce significant amounts of IL-12p70, a T-helper 1-type promoting cytokine. The same

may also apply to the other stimuli although we do not have the full data set to substantiate this claim.

6.5 Discussion

The soluble cytokine profile secreted by DCs varies with the different stages of DC development and maturation. A wide variety of cytokines may be expressed by mature DCs including IL-12, IL-1α, IL-1ß, IL-8, IL-15, IL-18, IFN-α, IFN-ß, IFN-γ, IL-4, IL-10, IL-6, IL-17, IL-16 and TNF-α (Aiba S,1998 and Hellman P *et al. 2*007). The cytokine patterns released by mature DCs determine their TH1/TH2 polarizing capacities and explain whether the outcome of an immune response is predominantly humoral or cell mediated in nature.

In this study, we instigated the secretion of TH1-type cytokines (IL-12, IFN-γ and TNF-α) and TH2-type cytokines (IL-4, IL-6, and IL-10) from DCs after stimulation with TNF-α, LPS, MBV and Coley's Toxin. After incubation at 37°C for 8h in a CO_2 incubator, supernatants were collected and tested for cytokine concentration using the BD CBA Flex set. LPS-treated DCs produced low amounts of both IL-12 and IL-10

In this study induction of high levels of IFN-γ production suggests that soon after bacterial encounter, DC produce TNF-α and IL-1β which can activate neighboring non-infected DCs as well as macrophages. Subsequently, those DCs produce IL-6 and IL-12 for the activation of B and T cells respectively (Rescigno M *et al*. 2000b). The PBMC's incubated with Coley's Toxin and MBV demonstrated a mixed THl/TH2 profile with secretion of IL-2, IL-4, IL-6, IL-10,TNF-α, IFN-γ and IL-12

The data reported here indicated that the PBMC's from the initial two individuals, used for the dose-resonse experiments, incubated with the stimuli did secrete IL-12 after incubation with Coley's Toxin and promote a rapid IFN-γ response, confirming several studies (Heufler C *et al.* 1996; Gorak PM *et al.*1998). DCs can produce IL-12 in its bioactive form: the assay used here was specific for the 70-kDa heterodimer, which favors the differentiation of precursor TH0 cells into TH1 effectors (Trinchieri G, 1998). Furthermore Coley's Toxin was a more powerful inducer of IL-12 than the MBV (no IL-12 was detected after incubation with the MBV, even after 12 hours of culture). IL-12 production is critical for the promotion of an effective cellular immune response by activating and differentiating T lymphocyte to the TH1 pathway.

The data presented here suggest that both Coley's Toxin and the MBV might be stimulators for maturing DCs for TH1 responses designed for use in cancer therapy or for intracellular infections. The results of other studies showed that myeloid DCs seem to control the type of inflammatory response by secreting two functionally conflicting factors, and the ratio of IL-12 to IL-10 is thought to determine the balance of a TH1 versus TH2 response. The absolute amount of IL-10 was larger than that of IL-12 for both Coley's and the MBV, but IL-4 was not secreted in the case of MBV. The interaction of DCs with Coley's Toxin, leading to IL-12 production followed by the rapid production of IFN-γ, is an important response at the innate level. These cytokines are capable of promoting TH1 development and thus influencing the development of the adaptive immune response (Kikuchi T *et al.*2004).

PBMC's incubated with Coley's and MBV both produced high levels of the TH2-polarizing cytokine IL-10. It is well established that monocytes and macrophages

synthesize IL-10. It is also well known that IL-10 has an important regulatory role on monocyte function and on DC maturation. The MBV and Coley's Toxin stimulation induced secretion of TNF-α and IFN-γ at a high level that was markedly and significantly higher than the unstimulated value. Furthermore the MBV and Coley's Toxin stimulation also induced IL-6 and IL-10 secretion at a high level that was markedly and significantly higher than the unstimulated value. These results suggest that cells stimulated with MBV and Coley's Toxin secrete TH1 and TH2-type cytokines at a high level.

IFN-γ, TNF-α, IL-1β, IL-6 and IL -8 are typical inflammatory cytokines that are secreted by the PBMCs in the presence of Coley's Toxin and the MBV. Sallusto *et al*. (2000) showed in his study that immature DCs migrate toward increasing concentrations of inflammatory chemokines. The DCs are also exposed to increasing concentrations of proinflammatory cytokines like TNF-α, IL- 1 and the pathogen products that caused the inflammatory response. In response to these DCs mature and switch the usage and expression of chemokine receptors from inflammatory to lymphoid homing receptors (MacPherson GG, 1993).

Chapter 7

General Discussion and Conclusions

The aim of this study was to determine whether a suspension of MBV and a preparation of Coley's Toxin would have any significant effects on the activation and maturation status of DCs. This was of importance because only mature DCs can present the antigens of pathogens to T cells in order to activate the immune cells which carry out the effector functions. Measuring the influence of these two vaccines on DCs could help us to understand the requirements for the functions of DCs and thereby, develop DC based therapies. DC based therapies have potential application across a wide variety of diseases, ranging from infectious diseases such as HIV to cancer, inflammatory disease and autoimmune diseases. In recent years, we have seen several published clinical studies whereby the authors have generated activated DCs *in vitro* and used these for the treatment of the patient from whom it was generated (autologous immune therapies) (Lu W, Arreaes LC, Ferreira WT *et al.* 2004; Andrieu JM and Lu W :2007; Egan MA: 2007).

We presented this study with a detailed literature review on the immune system outlying the roles of the different cells involved in the immune system and the effect of different cytokines produced by these different cells. In Chapter 2, we discussed DCs in general. During this literature review it became clear the DC system of APCs is the initiator and modulator of the immune response and they determine the outcome of a successful antigen clearance. Mature DCs arise from immature precursors which are the cells that exhibit endocytic activity (Trombetta *et al*: 2003). Maturation is characterized by reduced phagocytic uptake, the development of cytoplasmic extensions or "veils", migration to lymphoid tissues, and enhanced T-cell activation potential (Romani *et al.*1996b; Rovere *et al*.1998).

In chapter 3 we described a flow cytometric method using a combination of monoclonal antibodes describing various surface markers in order to identify different types of DCs: we could distinguish between the major DC subsets by the absence of expression of several lineage markers for lymphocytes, monocytes and NK cells and the expression of CD11c in the case of mDCs and CD123 in the case of pDCs. The inclusion of the HLA-DR marker in addition to the previous described markers allows the discrimination of $CD123^{+}$ DCs from basophils.

Once we had shown our ability to identify the two subsets of DCs, we investigated the relative frequency of these cells in blood samples obtained from patients diagnosed with various infectious diseases. These included patients with cancer, HIV-infected individuals and other patients co-infected with HIV and TB. Our reasoning for the selection of these diseases is as follows:

- The cancer microenvironment consists of a variable combination of tumor cells, stromal fibroblasts, endothelial cells and infiltrating leukocytes, such as macrophages, T lymphocytes, and DCs. DCs are highly potent antigen presenting cells (APCs) which play a critical role in the regulation of the adaptive immune response. Because of their instrumental role in the immune system and their natural adjuvant properties, there is a great interest in exploiting DCs to develop immunotherapies for cancer, chronic infections and autoimmune disease.
- Several studies have shown that during HIV infection, both DC subsets are reduced in the blood (Pacanowski J *et al.* 2001; Schmidt B *et al.* 2005). Our results (although small in sample size, n = 10) showed a decrease in the %mDCs and %pDC in bloods from HIV infected patients when compared to healthy controls, thereby confirming the findings of (Pacanowski J *et al.* 2001 and Schmidt B *et al.*

2005a). Further studies are needed in order to reach final conclusions since we had no details of the medical status of these patients, especially as to their treatments (or lack thereof). It is however interesting that new studies are being published whereby the manipulation of peripheral blood DCs of HIV-infected patients have shown tremendous promise as to the control of viraemia and the ability to induce a CD4 cell recovery (Lu W, Arreaes LC, Ferreira WT *et al.* 2004; Andrieu JM and Lu W: 2007; Egan MA: 2007).

- A third group of patients were only discovered when we recruited blood samples to include in our study. These patients turned out to be a subset of HIV-infected patients (n = 5): the results of these patients looked different and it is only when we requested details from the clinician that it was discovered that these patient had TB as well as HIV, in other words, they were co-infected. The subsets of DCs measured appeared different: the %mDC and %pDC showed an increase in the peripheral blood and were in fact, significantly higher than the levels observed in blood samples from healthy controls. Furthermore, the maturation markers CD80 and CD83 in mDCs were up regulated. In the case of pDCs, the maturation marker CD83 was upregulated but this did not apply to the expression of CD80 (this was almost the same as for the control group). In this small group of patients, the %CD80 expression was higher than in the HIV infection only group, which implies that TB plays a role in the maturation of DCs and that the mycobacterial infection may over-ride an underlying immune suppressed state. This deserves further investigation since it may also represent a new biomarker of tuberculosis infection and the levels of the maturation markers on DCs may give an indication of the clinical stage of tuberculosis disease.

- In the case of cancer we only recruited 5 patients with newly-diagnosed lymphoma: the results of this study showed that the %mDCs were slightly increased as well as the maturation markers for mDCs (not significant). The % pDCs and the maturation markers for pDCs were slightly decreased (not significant). Studies done by Sevko AL *et al.* (2007) showed a decrease in the maturation markers CD80 and CD83.

Interactions between DCs and microbial pathogens are fundamental to the generation of innate and adaptive immune responses. Upon stimulation with bacteria or bacterial components such as lipopolysaccharide (LPS), immature DCs undergo a maturation process that involves expression of costimulatory molecules, HLA molecules, and cytokines and chemokines, thus providing critical signals for lymphocyte development and differentiation. In this study, we investigated the response of human DCs to MBV and LPS. Previous studies showed that DCs could be activated with killed *Streptococcus pyogenes.* With this study in mind it was hypothesized that the MBV used in this study might modulate DC maturation. The results of this study showed that the MBV and LPS all significantly induced DC maturation as measured by increased surface expression of costimulatory molecules such as CD80 and CD83.

Similarly, we investigated the effects of Coley's Toxin on the maturation status of DCs. We hypothesized that the Coley's Toxins used in this study might modulate DC maturation. Coley's is a mixture consisting of peptides of killed bacteria of species *Streptococcus pyogenes* as well as *Serratia liquefaciens.* The results of this study showed that Coley's Toxin could be used as effective agent to induce maturation of DCs, which appears to be a powerful tool for various types of immunotherapy. At present, it is one of the agents used by complimentary oncologists in Germany for the treatment of patients

post-surgical removal of the tumour burden (Dr. Brigitte Riedelsheimer, personal communication). Our findings therefore represent some *in vitro* data which supports the use of this biological agent.

The data shown in chapter 6, namely the measurement of different cytokines in the cell supernatants of the activated cells, showed that both the MBV and Coley's Toxin induced the secretion of TNF-α and IFN-γ at very high levels that were markedly and significantly higher than the unstimulated value. Furthermore the MBV and Coley's Toxin stimulation also induced IL-6 and IL-10 secretion under the same culture conditions, suggesting that these two experimental agents are able to induce the secretion of both TH1 and TH2-type cytokines from the T cells in the cultures. Previous studies have shown clearly that bacterial products do indeed induce the secretion of a TH1 response (Nakahara S *et al.* 2003).

Suggestions for future studies:

DC based therapies have potential application across a wide variety of diseases, ranging from infectious diseases such as HIV to cancer, inflammatory disease and autoimmune diseases. The market potential for a successful treatment is large (Cerundolo V *et al.* 2004). Mohamdzadeh M *et al.* (2004) states that market opportunities include the manipulation of DC acivity for the development of effective therapeutic vaccines, the modulation of DC activity to manage autoimmune diseases and targeting the underserved markets through the development of novel therapies that make use of the immune system to prevent and treat disease. These cells have the potential to be used as enhancers of responses to infectious diseases and due to their capacity to induce a primary immune

response, DCs can be used as vectors (vaccine carriers) for immunotherapy (Paczesny S *et al.* 2003).

One of the shortcomings of the present study is that we did not determine the longevity of the activated DCs cells once they had been induced to mature *in vitro* and to secrete the immune regulatory cytokines. This needs to be addressed in future studies since it would determine the need for the frequency for re-stimulation or the need for "booster" administrations of the cells when being used for immunotherapy. The need for the addition of the "survival" growth factors such as GM-CSF post activation has not been investigated and certainly deserves attention.

The observation of the increased mDCs and pDCs in the circulation of HIV and TB co-infected patients is certainly an interesting observation and this requires thorough investigation in larger groups of patients with better defined clinical staging and treatment status.

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Glossary

Adaptive immunity: response of antigen specific lymphocytes to antigen, including the development of immunological memory.

Agglutination: The aggregation of particulate antigen by antibodies. Agglutination applies to red blood cells as well as to bacteria and inert particles coated with antigen.

Allergen: An antigen responsible for producing allergic reactions by inducing IgE formation.

Allergy: A term covering immune reactions to non-pathogenic antigens, which lead to inflammation and deleterious effects in the host.

Anergy: The condition of exhibiting no response to an antigen or antibody.

Antibody: Plasma proteins that bind specifically to antigens. Serum protein formed in response to immunization; antibodies are generally defined in terms of their specific binding to the immunizing antigen, serum protein made in response to antigen; also called immunoglobulin (Ig)Specific molecules of the humoral immune response that bind to and neutralize pathogens or prepare them for uptake and destruction by paghocytes.

Antigen binding: physical association of antigen with antibody, TCR, or MHC.

Antigen processing: Large molecules are broken down (processed) within macrophages into peptides and presented within the groove of MHC molecules, enzymatic digestion of antigen and association with MHC for presentation to T cells.

Antigen receptor: The specific antigen-binding receptor on T or B lymphocytes; these receptors are transcribed and translated from rearrangements of V genes.

Antigen: molecule that react with antibodies. Any foreign material that is specifically bound by specific antibody or specific lymphocytes; also used loosely to describe materials used for immunization. Antigens may also be immunogens if they are able to trigger an immune response, or haptens if not.

Antigen-binding site: The part of an immunoglobulin molecule that binds antigen specifically.

Antigen-presenting cell (APC): A specialized type of cell, bearing cell surface class II MHC (major histocompatibility complex) molecules, involved in processing and presentation of antigen to inducer, or helper, T cells. Examples: macrophage, dendritic cells.

Autoimmunity (autoallergy): An immune response to "self" tissues or components. Such an immune response may have pathological consequences leading to autoimmune diseases.

A**ctive immunity: immunity** acquired through exposure to antigen and response of one's own immune system.

B lymphocyte (B cell): The precursors of antibody-forming plasma cells; these cells carry immunoglobulin and class II MHC (major histocompatibility complex) antigens on their surfaces, lymphocyte which develops in the bone marrow and binds antigen with membrane antibody (immunoglobulin).

Basophil: A polymorphonuclear leukocyte, whose basophils granules contain heparin, histamine and other vasoactive amines. Within tissues, these cells are known as mast cells q.v.

Bone marrow: The site of hematopoiesis, the generation of the cellular elements of blood, including red blood cells, polymorphonuclear leukocytes, platelets and monocytes.Also the site of B cell development and the source of stem cells that give rise to T cells upon migration to the thymus

CD: Cluster of Differentiation; membrane proteins on immune system cells that allow for their identification and isolation. Groups of monoclonal antibodies that identify the same cell surface molecule. The cell surface molecule is designated CD followed by a number The designation CD stands for "cluster of differentiation", a historical term that was coined to define cell-surface molecules that are recognized by a given set of monoclonal antibodies. This cluster of differentiation then received a number, for example CD1, CD2 etc., which stands only for the order of discovery. In general, each CD is associated with one or more functions, which were discovered through the effects on cell or tissue function of the antibodies that define it.

CD3: part of the T cell (antigen) receptor complex. CD3 transduces the antigen binding signal outside the plasma membrane into chemical signals (phosphorylation) in the cytoplasm.

CD4:co-receptor on helper T cells that binds Class II MHC and participates in T cell activation by antigen.

CD8: co-receptor on cytotoxic T cells that binds Class I MHC and participates in T cell activation by antigen.

Cell-mediated cytotoxicity (CMC): Killing (lysis) of a target cell by an effector lymphocyte.

Cell-mediated immunity (CMI): Immune reaction mediated by T cells; in contrast to humoral immunity, which is antibody mediated. Also referred to as delayed-type hypersensitivity. Describes any adaptive immune response in which antigen specific T cells play the main role.

Cellular immunity : immunity which can be transferred between individuals with the transfer of T cells.

Chemokine: small molecule secreted by white blood cells that attracts leukocytes. Small cytokines that are involved in the migration and activation of cells, especially phagocytic cells and lymphocytes. They play a centre part in inflammatory responses.

Chemotaxis: The characteristic movement or orientation of an organism or cell along a chemical concentration gradient either toward or away from the chemical stimulus.

Class I, II and III MHC molecules: Proteins encoded by genes in the major histocompatibility complex (q.v.). Class I molecules are designated HLA-A, B, or C. Class II molecules are designated DP, DQ or DR

Cluster determinant (CD): Cluster of antigens with which antibodies react that characterize a cell surface marker.

Complement components: An enzymatic system of serum proteins triggered by the classical and alternative pathways, and resulting in target cell lysis, phagocytosis, opsonization and chemotaxis.

Complement receptor: A structure found on erythrocytes, lymphocytes, neutrophils, monocytes and macrophages that binds C3 fragments.

Complement: A series of serum proteins involved in the mediation of immune reactions. The complement cascade is triggered classically by the interaction of antibody with specific antigen. Collection of plasma proteins that can be activated to promote inflammation and antigen elimination. They complement (work with) antibody.

Co-receptor: A cell surface protein that increases the sensitivity of the antigen receptor to antigen by binding to associated ligands and participating in signaling for activation

CTLA-4.: expressed on the surface of T cells after T cell activation - it competes with CD28 for binding to CD80 and CD86 on the surface of antigen-presenting cells (APCs). The CTLA-4-Ig attempt to suppress T cell activation uses a manufactured free-floating version of CTLA-4 that binds to CD80/86 and so prevents their signalling to CD28 and the ensuing costimulation of T cells, thus blocking the development of an effective T cell response.

Cytokine: small molecule secreted from one cell that signals another cell by binding to its specific receptor. Cytokines are proteins made by cells that affect the behavior of other cells. Cytokines produced by lymphocytes are called lymphokines or interleukins. Cytokines act on specific cytokine receptors on the cells they affect.

Cytokine receptor: Cellular receptor for cytokines. Binding of the cytokine to the cytokine receptor induces new activities in the cell, such as growth, differentiation or death.

Cytotoxic (Cytolytic) T cell: Cell that kills target cells bearing appropriate antigen within the groove of an MHC class I molecule that is identical to that of the T cell. T cell which kills virus-infected cells and tumor cells.

DC-SIGN or CD209 is a C-type lectin receptor present mainly on myeloid and pre-plasmacytoid dendritic cells that mediates dendritic cell rolling interactions with blood endothelium and activation of CD4+ T cells, as well as recognition of pathogen haptens. This molecule is involved in the initial stages of the Human Immunodeficiency Virus infection, as the HIV gp120 molecule causes co-internalization of the DC-SIGN molecule and HIV virion. The dendritic cell then migrates to the cognate lymphoid organ, whereupon recycling of the DC-SIGN/HIV virion complex to the cell periphery facilitates HIV infection of T cells by interaction between DC-SIGN and ICAM-3.

Dendritic cell: cell with long processes (dendrites) that presents antigen. These cells are found in T-cell areas of lymphoid tissues. They are the most potent stimulators of T cell responses. The dendritic cell derives from bone marrow precursors.

Determinant: Part of the antigen molecule which binds to an antibody-combining site or to a receptor on T cells (see hapten and epitope).

Diapedesis: The blood vessels are lined with the endothelium, a layer of cells that tends to protect blood cell migration outside of the cells. However, injury or trauma can cause white blood cells to migrate across the endothelium. This process is called diapedesis.

Domain: A compact segment of an immunoglobulin molecule, made up of about 110 amino acids around an S-S bond, and encoded by a unique segment of DNA, surrounded by nontranslated sequences.

DR antigens: MHC class II molecules found on B cells and antigen-presenting cells of humans.

Enzyme: organic reaction catalyst, which facilitates a chemical reaction without being used up. Most enzymes are proteins, but some RNA has catalytic activity for processing mRNA.

Enzyme-linked immunosorbent assay (ELISA): An assay in which an enzyme is linked to an antibody and a coloured substrate is used to measure the activity of bound enzyme and, hence, the amount of bound antibody.

Eosinophil: A polymorphonuclear leukocyte with large eosinophilic (i.e. red) cytoplasmic granules.

Epitope: An alternative term for antigenic determinant.

Fab: Fragment of antibody containing the antigen-binding site, generated by cleavage of the antibody with the enzyme papain, which cuts at the hinge region N-terminally to the inter-H-chain disulphide bond and generates two Fab fragments from one antibody molecule.

FACS : Fluorescence-Activated Cell Sorter; equipment used to count and separate leukocytes labeled with fluorescent-tagged antibodies to cell surface molecules.

Fc receptor (FcR): A receptor on a cell surface with specific binding affinity for the Fc portion of an antibody molecule. Fc receptors are found on many types of cells.

Fc: Fragment of antibody without antigen-binding sites, generated by cleavage with papain; the Fc fragment contains the C-terminal domains of the heavy immunoglobulin chains.

FDC: Follicular Dendritic Cell. Found in the B cell areas of secondary lymphoid

Ficoll Histopaque: material used to separate leukocytes by their density, especially lymphocytes from other white blood cells and erythrocytes.

Flow cytometry :process used to detect and quantify fluorescent antibody bound to leukocytes.

Fluorescent antibody: An antibody coupled with a fluorescent dye, used with a fluorescence microscope to detect antigen on cells, tissues, or microorganisms.

GM-CSF : A cytokine involved in the growth and differentiation of myeloid and monocytic lineage cells, including dendritic cells , monocytes and tissue macrophages.

Helper T cells: A class of T cells which help trigger B cells to make antibody against thymus-dependent antigens. Helper T cells also help generate cytotoxic T cells. Regulatory T cell which used cytokines to stimulate B cells to produce antibodies, macrophages to become activated, or cytotoxic T cells to become effector CTL.

Histocompatibility: Literally, the ability of tissues to get along; in immunology, it means identity in all transplantation antigens. These antigens, in turn, are collectively referred to as histocompatibility antigens.

HIV : Human Immunodeficiency Virus, the virus that causes AIDS.

HLA: The acronym for **Human** Leukocyte Antigen, human MHC contains an MHC Class II that encode HLA –DM that is involved in the loading of peptides onto MHC Class II molecules.

Humoral immunity: Any immune reaction that can be transferred with immune serum is termed humoral immunity (as opposed to cell-mediated immunity). In general, this term refers to a resistance that results from the presence of specific antibody.

Hypersensitivity: State of reactivity to antigen that is greater than normal for the antigenic challenge; hypersensitivity is the same as allergy and denotes a deleterious outcome rather than a protective one.

ICOS(CD278): is a CD28-superfamily costimulatory molecule that is expressed on activated T cells. It is thought to be important for Th2 cells in particular.

IFN-α : alpha interferon; cytokine produced in response to virus infection that interferes with virus replication.

IFN-β : beta interferon; cytokine produced in response to virus infection that interferes with virus replication.

IFN-γ: gamma interferon; cytokine produced by T cells that signals APC to express more membrane MHC.

Immune complex: Antigen bound to antibody.

Immunofluorescence: A technique used for detecting molecules using antibodies labeled with fluorescent dye. The bound fluorescent antibody can be detected by microscopy, flow cytometry or by fluorimetry, depending on the application being used.

Immunoglobulin (Ig): A general term for all antibody molecules. Each Ig unit is made up of two heavy chains and two light chains and has two antigen- binding sites.

Innate immunity: immunity present from birth and not dependent on prior antigen exposure. Innate immunity includes physical and chemical barriers to infection, phagocytes, complement, and Natural Killer cells.

Interdigitating dendritic cells: leukocytes with long processes (dendrites) that present antigen to T cells.

Interferon: A group of proteins having antiviral activity and capable of enhancing and modifying the immune response.

Interleukins: Glycoproteins secreted by a variety of leukocytes which have effects on other leukocytes.

Killer T cell: A T cell with a particular immune specificity and an endogenously produced receptor for antigen, capable of specifically killing its target cell after attachment to the target cell by this receptor. Also called cytotoxic T cell.

Leukocyte: white blood cell: neutrophil, basophil, eosinophil, or lymphocyte.

Ligand: molecule which specifically binds a receptor and triggers a response from the cell. Antigen is the ligand for the memory Ig on B lymphocytes and triggers B cell immune responses; insulin is the ligand for insulin receptor and triggers glucose uptake.

LPS: LipoPolySaccharide; endotoxin. Present in the outer membrane of Gram negative bacteria.

Lymphatic: vessel which transports lymph and leukocytes into and out of the lymph nodes and back into the circulatory system at the vena cava.

Light chain (L chain): The light chain of immunoglobulin is a structural feature that occurs in two forms: kappa and lambda.

Lymphocyte: Small cell with virtually no cytoplasm, found in blood, in all tissue, and in lymphoid organs, such as lymph nodes, spleen, and Peyer's patches, and bears antigen-specific receptors.

Lymphokines: Soluble substances secreted by lymphocytes, which have a variety of effects on lymphocytes and other cell types. **MIIC vesicle**:cytoplasmic vesicle in antigen-presenting cells where class II MHC binds processed exogenous antigen.

Macrophage: phagocytic white blood cell found in the tissues; the circulating form of the cell is called a monocyte. Acts as an antigen-presenting cell.

Macrophage-activating factor (MAF): Actually several lymphokines, including interferon, released by activated T cells, which together induce activation of macrophages, making them more efficient in phagocytosis and cytotoxicity.

Mannose receptor: The mannose receptor (MR) is an endocytic receptor and is expressed by immature cultured dendritic cells (DC), where it mediates high efficiency uptake of glycosylated

Major histocompatibility complex (MHC): A cluster of genes on chromosome 6 in humans, encoding cell surface molecules that are polymorphic and that code for antigens which lead to rapid graft rejection between members of a single species which differ at these loci. Several classes of protein such as MHC class I and II proteins are encoded in this region. These in humans, are known as 'Human leukocyte antigens' (HLA).

Memory: In the immune system, memory denotes an active state of immunity to a specific antigen, such that a second encounter with that antigen leads to a larger and more rapid response.

MHC class I molecule: A molecule encoded to genes of the MHC which participates in antigen presentation to cytotoxic T (CD8+) cells.

MHC class II molecule: A molecule encoded by genes of the MHC which participates in antigen presentation to helper T (CD4+) cells.

MHC: Major Histocompatibility Complex; refers to the proteins used to present antigen to T cells or the genes which encode them. MHC proteins on body tissues must be matched between organ donor and recipient for an organ transplant to avoid rejection.

monoclonal antibody: antibody that is homogenous, every molecule identical to the next in physical structure and antigen binding; produced by a hybridoma.

Monoclonal: Literally, coming from a single clone. A clone is the progeny of a single cell. In immunology, monoclonal generally describes a preparation of antibody that is monogenous, or cells of a single specificity.

Monocyte: Large circulating white cell, 2-10% of total white cells, phagocytic, indented nucleus. Migrates to tissues, where it is known as a macrophage.

Myeloma: A tumor of plasma cells, generally secreting a single species of immunoglobulin.

NK cell: Naturally occurring, large, granular, lymphocyte-like killer cells that kill various tumor cells; they may play a role in resistance to tumors. Also, they participate in ADCC. They do not exhibit antigenic specificity, and their number does not increase by immunization.

Opsonin: A substance, usually antibody or complement component, which coats a particle such as a bacterium and enhances phagocytosis by phagocytic cells.

Opsonization: Literally means "preparation for eating". The coating of a bacterium with antibody and/or complement that leads to enhanced phagocytosis of the bacterium by phagocytic cells.

OX 40(CD134): is a member of the TNFR-superfamily of receptors which is not constitutively expressed on resting naïve T cells, unlike CD28. OX40 is a secondary costimulatory molecule, expressed after 24 to 72 hours following activation; its ligand,

OX40L, is also not expressed on resting antigen presenting cells, but is following their activation. Expression of OX40 is dependent on full activation of the T cell

Passive immunization: Immunization by the administration of preformed antibody into a non-immune individual.

Pathogen: disease-causing organism.

PBMC: Peripheral Blood Mononuclear Cell .Lymphocytes and monocytes isolated fro peripheral blood, usually by Ficoll Histopaque density centrifugation.

Phagocytosis: The engulfment of a particle or a microorganism by leukocytes. The ingested material is contained in a vesicle called a phagosome, which then fuses with one or more lysosomes to form a phagolysosome. The lysosomal enzymes play an important role in pathogen destruction and degradation

Phagosome: cytoplasmic vesicle containing the engulfed particle.

Phenotype: The physical expression of an individual's genotype.

Pinocytosis: Ingestion of liquid or very small particles by vesicle formation in a cell.

Plasma cell: End-stage differentiation of a B cell to an antibody-producing cell. Plasma cells are the main antibody secreting cells of the body. They are found in the medulla of the lymph nodes and in the bone marrow.

Polyclonal activator: A substance that induces activation of many individual clones of either T or B cells. See Mitogen.

Polymorphism: Literally, "having many shapes"; in genetics polymorphism means occurring in more than one form within a species; the existence of multiple alleles at a particular genetic locus.

Polymorphonuclear leukocyte: White blood cells with multi lobed nuclei and cytoplasmic granules. There are three types: neutrophils with granules that stain with neutral dyes - most frequent, Eosinophils with granules that stain with eosin and basophils with granules that stain with basic dyes

Primary lymphoid organs: Organs in which the maturation of T and B lymphocytes take place and antigen-specific receptors are first acquired.

Primary responses: The immune response to a first encounter with antigen. The primary response is generally small, has a long induction phase or lag period, consists primarily of IgM antibodies, and generates immunologic memory.

Receptor: molecule which specifically binds a ligand; ligand binding triggers a specific response from the cell. Most receptors are on the cell's plasma membrane, but some can be cytoplasmic. Antigen is the ligand for the mIg on B lymphocytes and triggers B cell immune responses; insulin is the ligand for insulin receptor and triggers glucose uptake.

Secondary lymphoid organ: lymphoid organ in which lymphocytes are activated by antigen and accessory cells and become effector cells and memory cells. Includes the lymph nodes, spleen, mucosal lymphoid tissue and bone marrow.

Secondary response: repeat immune response to an antigen; memory response. This response starts sooner and is of higher affinity than the primary response. This response is dominated by IgG antibodies.

Stem cell: cell which can develop into more mature functional cells.

Suppressor T cell: T cell which inhibits the function of B cells or other T cells.

T cell: lymphocyte which has developed in the thymus and has membrane TCR that binds antigen plus self MHC. T cells are either helper (CD4) or cytotoxic (CD8) phenotype.

T cell activation: biochemical process of stimulating a resting T cell to become an effector cell.

T cell education: selection of T cells which can bind self MHC with appropriate affinity to be MHC-restricted.

TCR: T cell (antigen) receptor, membrane molecule that binds antigen peptide.

TH1 cell: helper T cell that activates macrophages and cytotoxic T cells.

TH2 cell: helper T cell that activates B cells

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TNF-α: Tumor-Necrosis Factor alpha, a cytokine produced by macrophages, mast cells and NK cells.

Vaccination: Originally referred to immunization against smallpox with the less virulent cowpox (vaccinia) virus; more loosely used for any immunization against a pathogen.