DEVELOPMENT AND VALIDATION OF STABILIZED WHOLE BLOOD SAMPLES EXPRESSING T-CELL ACTIVATION MARKERS AS QUALITY CONTROL REFERENCE MATERIAL

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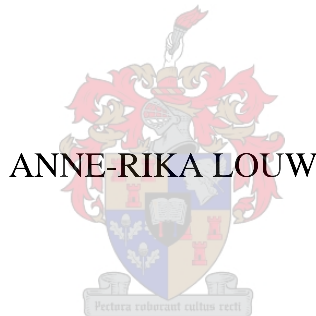

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis 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

Introduction: Flow cytometry has progressively replaced many traditional laboratory tests due to its greater accuracy, sensitivity and rapidity in the routine clinical settings especially clinical trails. It is a powerful tool for the measuring of chemical (the fluorochrome we add) and physical (size and complexity) characteristics of individual cells. As these instruments became major diagnostic and prognostic tools, the need for more advanced quality control, standardized procedures and proficiency testing programs increased as these instrumentations and their methodology evolve. Minor instrument settings can affect the reliability, reproducibility and sensitivity of the cytometer and should be monitored and documented in order to ensure identical conditions of measurement on a daily basis. This can be accomplished by following an Internal Quality Assurance (IQA) and/ or External Quality Assurance (EQA) program. Currently there are no such programs available in South Africa and poorer Africa countries. HIV is a global concern and the laboratories and clinics in these places are in need of such IQA programs to ensure quality of their instrumentation and accurate patient results. Quality assurance programs such as CD Chex® and UK Nequas are available but due to bad sample transport, leave the receiving laboratories with nightmares. It would be best if there was a laboratory in South Africa that could provide the surrounding laboratories with stabilized whole blood samples that can be utilized as IQA. The transport of these samples can be more efficient due to shorter distance and thus the temperature variations limited.

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Aims and Objectives: The aim of Chapter one is to familiarize the reader with general terminology and concepts of immunology. Chapter two describes in detail the impact stabilized whole blood had on clinical immunology concerning Quality Control and Quality Assurance. The objective of this study is to stabilize whole blood with a shelf life of greater than 30 days to serve as reference control material for South African Immunophenotyping. It is further an objective to use these in-house stabilized control samples for poorer African countries as Internal Quality Assurance reference material. It is a still further objective to stimulate various lymphocyte subsets to express activation antigens and then stabilize these cells for more specialized immunological test and can serve as a QC for those required samples.

Study design: In Chapter three, the method currently used to stabilize whole blood was modified. The stability of different concentrations of a first stabilizing agent (Chromium Chloride hexahydrate) was investigated. Incubation periods and concentrations of paraformaldehyde as second stabilizing agent were investigated. Blood samples from healthy individuals (n=10) were stabilized and monitored for the routine HIV phenotypic surface antigens over a period of 40 days. These samples (n=10) were compared on the Becton Dickinson Biosciences (BD) FACSCalibur™ versus BD FACSCount™ instrumentation. Blood samples (n=3) were stabilized and monitored to identify phenotypic cell surface molecules for as long as possible. They were quantified on both flow cytometric instruments. In addition, these stabilized samples (n=3) were investigated as control blood for calibration purposes on the BD FACSCount™ instrument.

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In Chapter four, lymphocytes were isolated and activated with various stimuli to express sufficient activation antigens such as CD25, CD69, HLA-DR and CD40 Ligand on the T helper cell surfaces. These activated antigens were analyzed on the BD FACSCalibur™ and further stabilized to serve as possible IQA samples in future.

Results: In Chapter three, the ten individual stabilized samples had non-significant P values ($P > 0.05$) for CD3, CD4 and CD8 percentages and absolute values comparing day 3 until day 40. Comparing the BD FACSCalibur™ versus BD FACSCount™, resulted in a $R^2 = 0.9848$ for CD4 absolute values and a $R^2 = 0.9636$ for CD8 absolute values. Stabilized blood samples (n=3) were monitored for routine HIV phenotypic markers until day 84. The cells populations were easily identifiable and could be quantified on both BD FACSCalibur™ and BD FACSCount™ instruments.

In Chapter four; for the activation study purposes, activated T helper lymphocytes expressed approximately 25 to 35% CD40 Ligand cell surface molecules. The stimulant of choice was Ionomycin at a 4µM concentration. Cells were incubated for four hours at 37 degree Celsius in a 5% CO_2 environment. For CD69 surface expression, 6 hour incubation was optimum. The stimulus of choice in this case was 4µM Ionomycin which induced 84.21% CD69 expression in the test samples. For CD25 expression; 6 hour incubation with PHA resulted in approximately 43% of CD25 expression. For HLA-DR surface expression; 6 hour incubation with PHA resulted in approximately 43.32% of HLA-DR expression. Activated lymphocytes expressing CD40 Ligand showed stability until day 23. Activated Lymphocytes expressing CD69, CD25 and HLA-DR were stabilized in the same manner and stability could be achieved until day 16.

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Conclusion: This thesis was related to the preparation of control samples (IQA) designed to simulate whole blood having defined properties in clinical laboratory situations. In future kits can be developed with a low, medium and high control sample for the various immunological phenotypic determinants. Another kit can be compiled where various activation markers can be identified, quantified with a “zero”, low and high control. These whole blood IQA kits and “activation IQA kits” can be implemented for training of newly qualified staff, competency testing of staff, method development, software testing, panel settings and instrument setting testing. Control samples ideally must have a number of properties in order to be effective. For instance stability during storage times, preferably lasting more than a few weeks, reproducibility and ease of handling. These will provide the information on day-to-day variation of the technique or equipment which will enhance accuracy and improve patient care.

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OPSOMMING

Inleiding: Vloeisitometrie tegnologie het verskeie tradisionele laboratorium toetse vervang as gevolg van beter akuraadheid, sensitiwiteit en vinniger beskikbaarheid van resultate in ‘n kliniese omgewing, veral kliniese proewe. Vloeisitometrie is ‘n kragtige tegniek om chemiese (fluorokroom byvoeging) en fisiese (sel grote en kompleksiteit) karakter eienskappe van individuele selle te meet. Met die toename in gebruik en gewildheid van hiedie instrumente, neem die behoefde toe vir gevorderde kwaliteit kontroles, gestandardiseerde prosedures, met profesionele toets programme tesame met metode ontwikkeling.

Klein verstellings aan instrument parameters beinvloed die betroubaarheid, herhaalbaarheid en sensitiwiteit van ‘n sitometer en moet gemonitor (en dokumenteer) word om identiese kondisies van leesings op ‘n daaglikse basis te verseker. Dit kan bereik word deur in te skakel met ‘n interne kwaliteits versekerings program [IQA: “Internal Quality Control”] en/of ‘n eksterne kwaliteits versekerings program [EQA: “External Quality Control”] te volg. Op die oomblik is daar geen sulke kwaliteits versekerings programme in Suid Afrika en/of in die verarmende Afrika lande beskikbaar nie. MIV is ‘n wêreldwye bekommernis en laboratoriums en klinieke in hierdie gedeeltes van die land verlang ‘n dringende behoefdte vir sulke “IQA” programme om kwaliteit van instrumentasie en akkurate pasiënt resultate te verseker wat tot beter behandeling van pasiënte lei. Kwaliteit versekerings programme soos “CD Chex®” en “UK Nequas” is beskikbaar, maar baie probleme met verwysing na monster integriteit as gevolg van tydsame vervoer en aflewering kondisies word hiermee geassosieër.

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Die behoefte het ontstaan vir ‘n laboratorium in Suid Afrika wat direk die omliggende laboratoriums, hospitale en klinieke kan voorsien met gestabiliseerde blood monsters wat gebruik kan word as “IQA”. Die vervoer en aflewerings kondisies van hierdie monsters sal aansienlik verbeter as gevolg van die korter aflewerings afstand wat direk die beperkte temperatuur wisseling beinvloed.

Doel van studie: Die doelwit van hoofstuk een is om vir die leser ‘n inleiding te gee tot terminologie en konsepte van immunologie en die immune sisteem. Hoofstuk twee beskyf die impak wat gestabiliseerde heelbloed het op die kliniese immunologie met betrekking tot kwaliteit beheer en kwaliteit versekering. Die doelwit van hierdie studie is om heelbloed te stabiliseer sodat die rakleeftyd meer as 30 dae is en sodoende as verwysings-materiaal kontroles vir Suid Afrikaanse immunofenotipering kan dien. Dit is ‘n verdere doelwit om hierdie tuis-gestabiliseerde kontrole monsters te gebruik as “IQA” verwysings materiaal in verarmende Afrika lande. Die doelwit van hoofstuk vier is om limfosiete te stimuleer om verskeie aktiverings merkers uit te druk op hul selmembrane en dan te stabiliseer en dié te gebruik as Kwaliteits Kontroles vir die meer gespesialiseerde immunologiese toetse.

Studie ontwerp: Hoofstuk drie beskryf ‘n aangepaste en verbeterde metode van heel bloed stabiliseering. Stabiliteit word ondersoek in ‘n verskyndenheid konsentrasies van ‘n primêre stabiliseerings agent (chromium chloried heksahidraat) en inkubasie periodes met paraformaldehied as tweede stabiliseerings agent word deeglik gedokumenteer. Bloedmonsters van gesonde indiwidië (n=10) was gestabiliseer en gemonitor vir roetine MIV membraanoppervlak antigene oor ‘n periode van 40 dae.

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Hierdie monsters (n=10) was gelees en geanaliseer op ‘n BD FACSCalibur™ en vergelyk met ‘n BD FACSCount™ vloeisitometer instrument. Drie gestabiliseerde heelbloed monsters (n=3) was gemonitor vir ‘n periode vir so lank moontlik die fenotipiese selmembraan molekules identifiseerbaar was en die kwantiteit bepaalbaar was. Hierdie drie monsters was gemeet op beide instrumente. As ‘n addisionele doelwit, was hierdie drie gestabiliseerde monsters ondersoek om as moontlike kalibrasie materiaal (verteenwoordig ‘n normale bloedmonster) te dien vir die BD FACSCount™ instrument in die oggende voor pasiënt monsters gelees kan word.

In hoofstuk vier was limfosiete geïsoleer en geaktiveer met ‘n verskyndenheid stimulante om optimale aktiveerings-antigene uit te druk op T helper selmembrane (byvoorbeeld CD25, CD69, HLA-DR en CD40 Ligand). Hierdie geaktiveerde monsters was geanaliseer op die BD FACSCalibur™ en daarna gestabiliseer. Na stabilisasie van die geaktiveerde limfosiet monsters was dit gemonitor oor ‘n tydperk so lank moontlik data plotte leesbaar en selpopulasies identifiseerbaar was. Hierdie monsters kan dien as ‘n moontlike “IQA” toets stel vir ‘n meer gespesialiseerde immunologiese aktiveerings kontrole doeleindes.

Resultate: In hoofstuk drie; tien individiële gestabiliseerde heelbloed monsters het gedui op geen-beduidende P waardes ($P > 0.05$) vir CD3, CD4 en CD8 persentasies en absolute waardes; gemeet vanaf DAG 3 vergelykbaar tot-en-met DAG 40.

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Met korrelasie statistiek en vergelyking van die BD FACSCalibur™ met die FACSCount™ instrumente, is die volgende opgemerk; R^2 = 0.9848 vir die CD4 absolute waardes en 'n R^2 = 0.9636 vir die CD8 absolute waardes. Drie gestabiliseerde monsters (n=3) was gemonitor vir MIV roetine fenotipeering tot en met DAG 84. Die selpopulasies was duidelik identifiseerbaar en die kwantitatief meetbaar op albei instrumente (BD FACSCalibur™ en BD FACSCount™).

Hoofstuk vier: geaktiveerde T helper lymphosiete het 25 – 35% membraan CD40 Ligand uitgedruk op hul selmembrane. Die stimulant van keuse was ionomysien teen 'n optimale konsentrasie van 4µM. Die optimale inkubasie tydperk was vier ure by 37˚C in 5% CO_2 kondisie. Ses uur inkubasie in 4µM ionomysien by 37˚C in 'n 5% CO_2 omgewing was optimal vir die CD69 selmembraan uitdrukking en het 84.21% opgelewer. Vir CD25 selmembraan uitdrukking was die selle vir ses ure met phietoheamagglutinin (PHA) gestimuleer by 37˚C in 5% CO_2 kondisie en het 43% CD25 selmembraan uitdrukking opgelewer. HLA-DR selmembraan uitdrukking: selle was vir ses ure saam met PHA by 37˚C in 5% CO_2 kondisie inkubeer en het 43.32% opgelewer. CD40 Ligand aktivering/gestabiliseerde limfosiete het tot en met dag 23 stabiliteit getoon. Die ligand was duidelik identifiseerbaar en kwantifiseerbaar. Geaktiveerde lymphosiete wat CD69, CD25 en HLA-DR selmembraan merkers uitdruk het na die stabiliseerings proses stabiliteit getoon tot-en-met dag 16.

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Gevolgtrekking: Die doel van hierdie studie was om verwysingskontroles voor te berei sodat dit vars heelbloed naboots met uitkenbare eienskappe vir kliniese situasies. ‘n Toets kontrolestel met verwysings materiaal vir drie vlakke (byvoorbeeld ‘n lae, medium en hoë kontrole) absolute selwaardes en persentasies kan voorberei word vir roetine immunologiese fenotiperings merkers (CD3/CD4/CD8/CD45). Meer gespesialiseerde kontrolestelle vir meer spesifieke doeleindes kan opgemaak word wat ‘n verskydenheid van limfosiet aktiveringsmerkers bevat met byvoorbeeld ‘n “nul”, lae en hoë verwysings kontrole daarin. Hierdie heelbloed kan dien as “aktiveerde interne kwaliteits verwysings materiaal” en kan gebruik word om nuut aangestelde laboratorium werkers en nuut gekwalifiseerde studente op te lei. Hierdie verwysings materiaal / kontroles kan aangewend word vir bevoegdheids doeleindes (byvoorbeeld vir SANAS akkreditasie doeleindes), vir metode ontwikkeling, vir sagteware toetsing, vir paneel opstelling en instrument verstellings doeleindes. Die kontroles moet ‘n verskydenheid eienskappe bevat om effektief te wees. Byvoorbeeld, stabiliteit tydens storing, gewenslik meer as ‘n paar weke, herhaalbaar en maklik handteerbaar. Hierdie kontroles sal inligting voorsien op ‘n daaglikse basis tydens wisseling van tegnieke of instrumentasie wat akuraatheid beinvloed en op die ou-end direk pasiënt versorging bevoordeel.

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ABBREVIATIONS

- **ABS** - Absolute values or absolute counts
- **AIDS** - acquired immunodeficiency syndrome
- **APC(s)** - antigen presenting cell(s)
- **APC** - allophycacyanin
- **B cells** - B lymphocytes
- **BCR** - B cell receptor
- **CAP** - College of American Pathologists
- **CD** - Cluster of Differentiation
- **CD40L** - CD40Ligand
- **CDC** - Centers for Disease Control and Prevention
- **CPD-A** - Citrate phosphate dextrose-adenosine
- **CTN** - Canadian HIV trials Network
- **CV** - Coefficient of Variation
- **CVI** - common variable immunodeficiency
- **DCs** - Dendritic cells
- **DNA** - Deoxyribonucleic acid
- **EQA** - External quality assurance
- **FDC** - Follicular dendritic cells
- **FITC** - fluorescein isothiocyanate
- **FOXP3** - forkhead-winged-helix transcription factor
- **FSC** - Forward scatter

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- **GLP** - Good Laboratory Practice
- **GM-CSF** - granulocyte-macrophage colony-stimulating factor (GM-CSF)
- **HAART** - Highly Active Anti Retroviral Therapy
- **HIV** - Human immunodeficiency virus
- **ICAM-1** - intracellular adhesion molecules
- **ICAM3** - intercellular adhesion molecule 3
- **ICOS** - inducible T cell co-receptor molecule
- **Ig** - Immunoglobulin
- **IL** - Interleukin (e.g. IL-2)
- **IQA** - Internal quality assurance
- **K_2 EDTA** - Disodium Ethylenediaminetetraacetic acid
- **kDa** - kilodalton
- **LFA-1** - leukocyte function antigen -1
- **LPS** - lipopolysaccharides
- **MALT** - Mucosal Associated Lymphoid Tissues
- **mDCs** - myeloid dendritic cells
- **MDDCs** - Monocyte-derived DCs
- **MF** - maturation factors
- **MHC** - Major Histocompatibility Complex
- **NH_4Cl** - ammonium chloride
- **NK** - Natural Killer cells
- **NOD** - nonobese diabetic
- **NQAAPI** - National Quality Advisory Panel for Immunology

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- **PBS** - Phosphate buffered saline
- **pDCs** - plasmacytoid dendritic cells
- **PE** - phycoerythrin
- **PEG -** Polyethylene glycol
- **PerCP** - peridinin chlorophyll protein
- **PHA** - Phytohemagglutinin (a lectin from *Phaseolus vulgaris* – derived from the red kidney bean)
- **PMA** - phorbol-12-myristate-13 (also known as TPA)
- **PMN** - polymorphonuclear
- **PMTs** - Photo multiplier tubes
- **QA** - Quality Assurance
- **QASI** - Quality Assessment and Standardization for Immunological Measures
- **QC** - Quality Control
- **QMP-LS** - Canadian Quality Management Program – Lab Services
- **RNA** – ribonucleic acid
- **RPMI** - Roswell Park Memorial Institute medium
- **sCD40L** - soluble CD40L
- **SD** - standard deviation
- **SLE** - Systemic Lupus Erythematosus
- **SOP's** - Standard Operating Procedures
- **SSC** - Side scatter
- **T cells** - T lymphocytes
- **T_c cells** - Cytotoxic T cells/ $CD8^+$ T cells
- **TCR** - T cell receptor

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- **T_h cells** - Helper T cells/ $CD4^+$ T cells
- **TLR** - Toll-like-receptors
- **TNF** - tumor necrosis factor
- **T_{reg} cells** - Regulatory T cells
- **UK NEQAS** - United Kingdom National External Quality Assessment Scheme
- **v/v** - volume per volume
- **w/v** - weight per volume
- **WBC** - White blood cell
- **WBL -** whole blood

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CHAPTER 1

GENERAL INTRODUCTION

1.1 UNDERSTANDING THE IMMUNE RESPONSE

The early heroes of immunology were Edward Jenner (1749 – 1823) who introduced cowpox as the first reliable vaccine and Louis Pasteur (1822 – 1895) who invented the generic term ‘vaccine’ in honor of Jenner’s achievement. At the end of the nineteenth century, immunology birthed as an offspring of infectious biology and vaccinology when Paul Ehrlich (1845 – 1915) and Emil Behring (1854 – 1917) joined forces to develop passive vaccination to elucidate the principles of acquired immunity. Robert Koch (1843 – 1910) was the founder of medical microbiology. At the institute of Louis Pasteur, Elie Metchnikoff (1845 – 1916) developed the principle of innate immunity. Hence, vaccinology and infection microbiology were instrumental in establishing immunology. Immunity is the human’s way to protect its body against “foreign” invaders which might cause infectious diseases. The cells, organs and molecules involved in these protective processes make up the immune system. A response induced by introduction of a foreign agent, for example, infection-causing organisms such as bacteria, viruses, parasites, and fungi is known as the Immune Response.

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Not all immune responses protect the body from disease; some individuals mount immune responses to their own tissues as if they were foreign agents, this is known as autoimmunity. For these individuals, an immune response can be induced by means of allergens found in house dust mite, cat dander or rye grass pollen and these allergens causes disease (i.e. hypersensitivity or allergy).

1.2 THE STRUCTURES OF THE IMMUNE SYSTEM

The organs of the immune system are called lymphoid organs because they are the home to lymphocytes. Lymphocytes are small white blood cells that are key players in the immune system. Lymphoid tissues are divided into the central (primary) and peripheral (secondary) organs. **Figure 1.2.1** demonstrates the various lymphoid organs and where they can be found in the human body.

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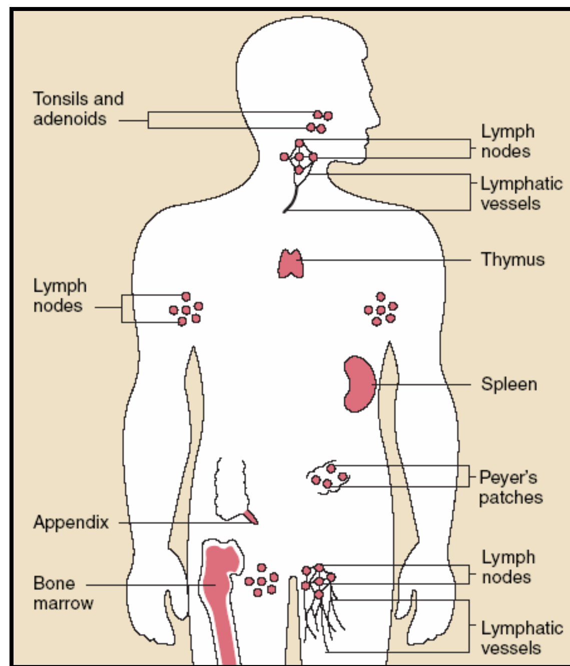

Figure 1.2.1: The organs of the immune system are positioned throughout the human body. (Adapted from National Institutes of Health, Sept, 2003, p.8)

1.2.1 Primary lymphoid organs

The primary lymphoid organs include the bone marrow and thymus. These are the sites where lymphocytes mature. The Thymus is situated behind the breastbone. The function of the thymus was discovered in 1961 by Miller. Bone marrow is the soft tissue in the hollow center of bones and the source of different blood cells. There the lymphocytes, monocytes and granulocytes originate from precursor stem cells. These white blood cells (leucocytes) are destined to become immune cells. The bone marrow and thymus are more involved in generating precursor lymphocytes rather than immune responses.

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B lymphocytes also called B cells migrate from the marrow to the peripheral lymphoid tissue. T lymphocytes also called T cells undergo further maturation in the thymus before they enter the immune system. Once lymphocytes are released from the bone marrow and thymus their life of patrol and response begins [Janeway *et al*. (2002)].

1.2.2 The secondary lymphoid organs

The secondary lymphoid organs comprise lymph nodes and spleen. The lymph node is where antigens from the tissues are collected and the spleen is where blood-borne antigens (especially bacteria) encounter the immune system. Clusters of specialized antigen-collecting epithelial cells and clusters of lymphocytes line the mucous membranes of the respiratory, digestive, and urogenital systems where contact with pathogens is the highest. There is also the tonsils, appendix, and the Peyer's patches also called the Mucosal Associated Lymphoid Tissues (MALT) – please follow **Figure 1.2.1** for the secondary lymphoid organ demographics.

Lymphocytes travel throughout the body using blood vessels and they also travel through a system of lymphatic vessels that closely parallels the body's veins and arteries. The lymphatic vessels carry lymph, a clear fluid that batches the body's tissues. The word "lymph" in Greek means a pure, clear stream. Cells and fluids are exchanged between blood and lymphatic vessels, enabling the lymphatic system to monitor the body for invading microbes. Secondary (peripheral) lymphoid organs are designed to bring together leucocytes and antigens. If the tissues are infected, antigen is carried to the nearby (draining) lymph nodes where it comes into contact with phagocytes and lymphocytes to initiate an adaptive immune response.

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The lymph nodes are small bean-shaped structure found along the lymphatic vessels, with clusters in the neck, armpits, abdomen, and groin. **Figure 1.2.2** illustrates within each lymph node the specialized compartments where immune cells congregate, and where they can encounter the foreign antigens. Immune cells and foreign particles enter the lymph node via incoming (afferent) lymphatic vessels or the lymph node's tiny blood vessels. The lymphocytes pass sinuses lined with macrophages and exit the lymph nodes through outgoing (efferent) lymphatic vessels. Finally all drain into the portal vein, and once in the blood stream, they are transported to tissues throughout the body [Janeway *et al*. (2002)]. The immune cells patrol the body for foreign invaders, and then gradually drift back into the lymphatic system, to begin the cycle all over again.

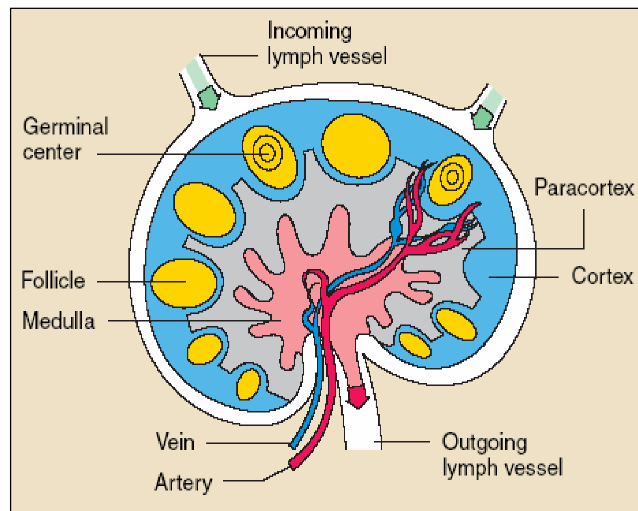

Figure 1.2.2: The lymph node contains numerous specialized structures. T cells concentrate in the Paracortex, B cells in and around the germinal centers, and plasma cells in the medulla. (Figure adapted from National Institutes of Health, 2003, p.9)

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1.3 INNATE AND ADAPTIVE IMMUNITY

Immunity can either be strong or weak, short-lived or long-lasting, depending on the type of antigen, the amount of antigen, and the route by which it enters the body. There are two levels of defense against foreign antigens. The first type of defense is present in neonatal animals and in invertebrates and is called natural or innate immunity. The second type of immunity is the adaptive or acquired immunity and is confined to vertebrates. The innate immunity is sometimes referred to as non-specific or broadly specific because the receptors recognize a limited number of molecules; some are shared by many infectious agents such as lipopolysaccharides (LPS), peptidoglycans and double stranded RNA [Janeway *et al*. (2002)]. For 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 second level of defense increases in strength and effectiveness with each encounter. The foreign agent is recognized in a specific manner and the immune system acquires memory towards it [Sprent and Surh (2001)].

1.3.1 Innate immunity

Until a decade ago, the innate immune system was considered solely as a first line of defense that rapidly attacked invading pathogens via non-specific stimulation of host effector cells. This mindset was changed with the identification of the Toll-like-receptors (TLRs) as sensors that specifically recognized microbial components or patterns [Akira *et al.* (2006)]. The innate immunity is made up of the following components: phagocytic cells, physical barriers, physiological factors and protein secretions.

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The physical barriers are the first line of defense against infections and comprises of the skin, and mucous membranes with mechanical protection through cilia and mucous. The physiological factors are such as pH, temperature and oxygen tension that might limit microbial growth. The acid environment in the stomach in combination with microbial competition (from the commensal flora) inhibits gut infection. Protein secretions help resist invasion and one example is lysozyme that is secreted into external body fluids. Complement, interferons, collectins and other “broadly specific” molecules such as C-reactive protein are soluble factors that are considered important against infection. Another significant component of innate immunity is the phagocytes which are cells that engulf large particles or other cells. They are critical in the defense against bacterial and simple eukaryotic pathogens. Phagocytes such as macrophages and polymorphonuclear leucocytes can recognize bacterial and yeast cell walls (through broadly specific receptors) and this recognition is greatly enhanced by activated complement (opsonin). In other words, as mentioned by Pulendran and Ahmed (2006), the innate immune system senses the type of infectious agent that has invaded the host and instructs the acquired immune system how to generate the appropriate response in defense against the invader.

1.3.2 Adaptive Immunity

Acquired immunity improves the effectiveness of the innate immune response by focusing the response to the site of invasion or infection. It provides an additional effector mechanism that is unique to lymphocytes in that it responds more quickly due to immune memory.

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The major difference between innate immunity and acquired immunity lies in the antigen specificity of lymphocytes: acquired immunity discriminates between self and non-self molecules. Adaptive immunity is usually acquired actively by natural infection or by vaccination with attenuated pathogen or inactive toxin (killed or weakened). Active immunity requires 2-3 weeks to become established and may be long-lasting (even a lifetime). Adaptive immunity may also be acquired passively from an immune person by the transfer of antibodies or even immune cells (rarely). Although the antibodies protect the person as soon as they are transferred, this protection only lasts weeks because the antibodies are removed from the circulation in a natural process called "turnover". Humoral immunity can also be transferred in serum. This includes antibodies transferred across the placenta and in breast milk from the mother to her baby. Another example is horse antibodies that are used to treat the venom from a snake bite. Both the humoral immune response mediated by antibodies and the cellular immune response mediated by T cells are controlled by T helper cells [Abbas *et al*. (1996)]. **Figure 1.3.2a** illustrates the concept of humoral immunity. Cellular immunity on the other hand, is conferred via T cells: foreign transplanted cells are limited by passive cellular immunity for example human bone marrow transplants, tissue transplantation or even organ transplantations. **Figure 1.3.2b** illustrates the concepts of cellular immunity.

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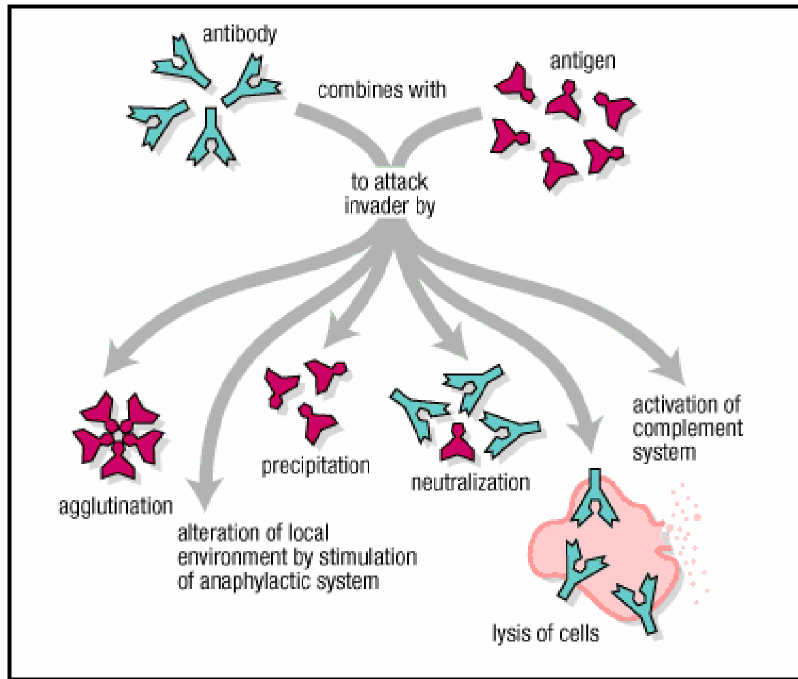

Figure 1.3.2a illustrates humoral immunity that is associated with circulating antibodies, in contrast to cellular immunity.

(Figure adapted from phoenix-cfs.org/The%20SITE/glossaryCFS.htm.)

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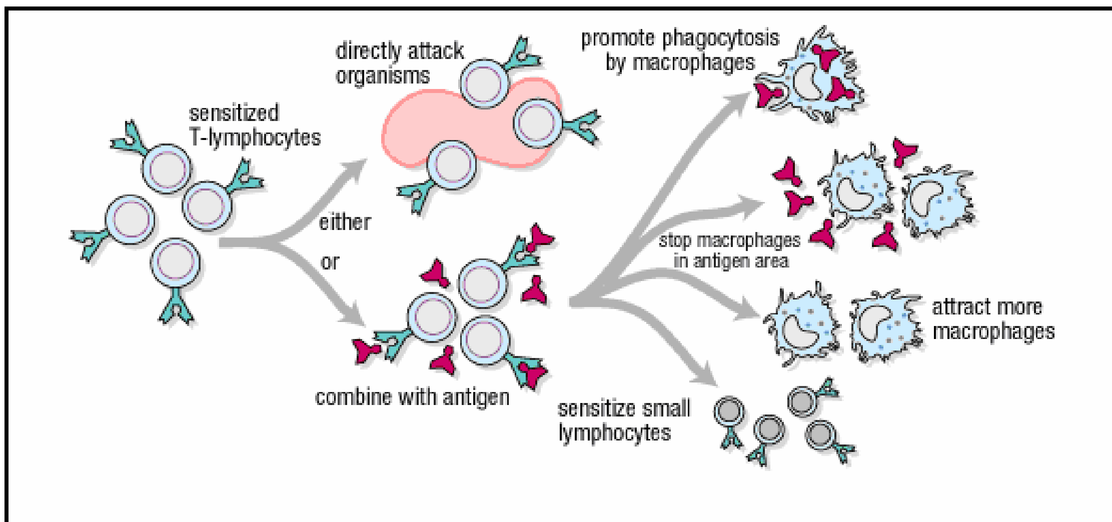

Figure 1.3.2b illustrates cellular immunity, where the immune response is initiated by an antigen-presenting cell interaction with and mediated by T lymphocytes (e.g. graft rejection, delayed-type hypersensitivity).

(Figure adapted from phoenix-cfs.org/The%20SITE/glossaryCFS.htm)

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1.4 CELLS OF THE IMMUNE SYSTEM

1.4.1 Phagocytes: Granulocytes and Monocytes / Macrophages

Phagocytes are divided into two types of leukocytes: blood monocytes and polymorphonuclear leukocytes (PMNs or granulocytes). Blood monocytes are called macrophages when they leave the circulation and enter the tissues where they will reside and carry out specialized functions. **Figure 1.4.1a** illustrates the various leukocytes derived from the same pluripotent stem cell: a pluripotent stem cell gives rise either to the lymphoid stem cells or to the myeloid stem cells. Granulocytes and monocytes derive from the common myeloid stem cell.

There are three types of granulocytes, namely the neutrophils, the eosinophils and the basophils: these cells are distinguished microscopically according to their cytological staining patterns. Polymorphonuclear leukocytes have lobed nuclei and many granules in their cytoplasm. Neutrophils express receptors for immunoglobulins and complement factors. They are involved in the acute inflammatory response. Eosinophils carry receptors for Immunoglobulin E (IgE) and they are involved in the destruction of IgE-coated parasites, such as helminthes (worm parasites). These cells also contribute to the response to allergens. Basophils are the circulating counterparts of tissue mast cells: they express high affinity receptors for IgE and when they are stimulated / activated, they secrete the chemicals which are responsible for immediate hypersensitivity. The mast cell is a twin of the basophil: it is found in the tissues, for example the lungs, skin, tongue and linings of the nose and intestinal tract. Mast cells are responsible for the symptoms of allergy [Maurer *et al.* (2003)].

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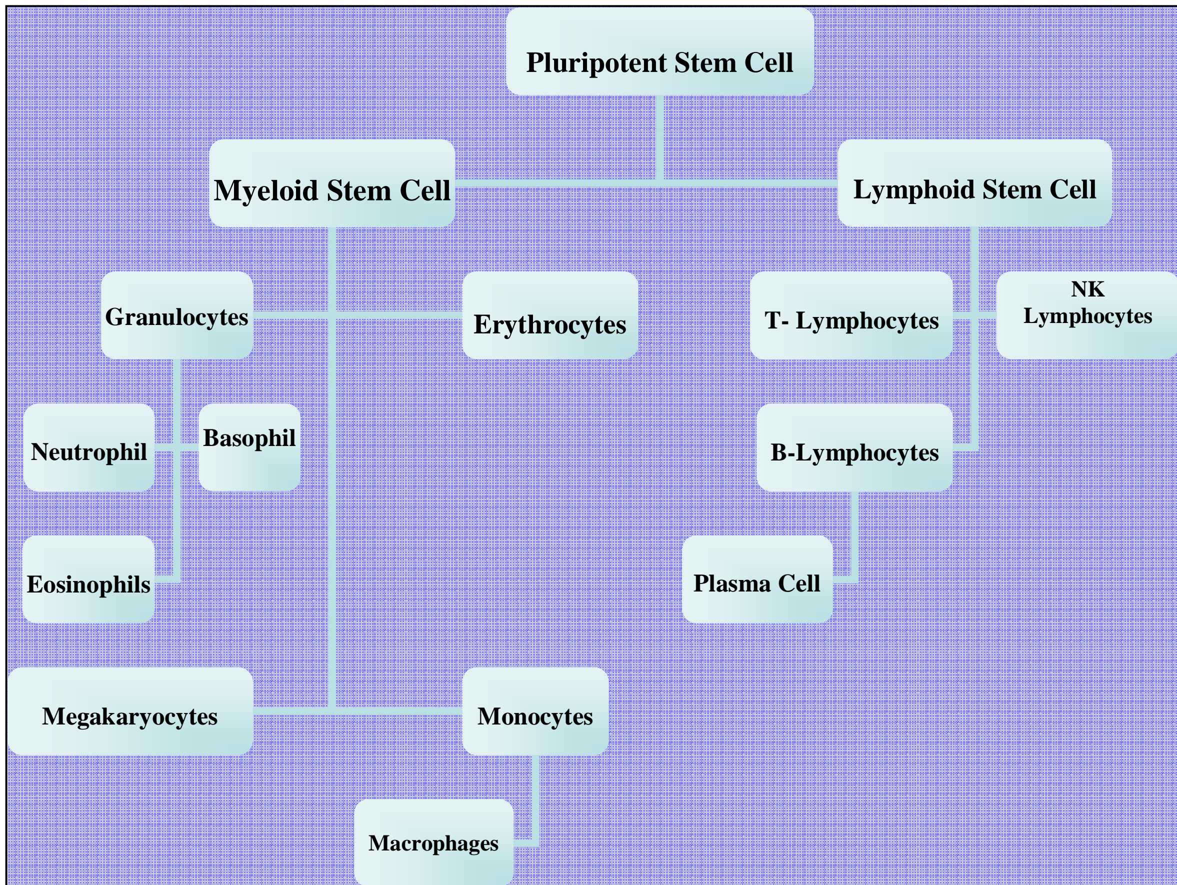

Figure 1.4.1a: Cells of the immune system: all deriving from the pluripotent stem cell (Diagram compiled by Anne-Rika Louw July 2007).

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Monocytes are large cells with round, horse-shoe shaped nuclei. They circulate in the blood and as they leave the blood to enter tissue, they become macrophages. These cells can put out pseudopodia to surround an antigen, engulf and kill and act as scavengers for cell debris and senescent cells. Macrophages and other polymorphonuclear cells comprise the innate immunity because they bind common surface molecules on pathogens or antibody-coated pathogens. Another key function of macrophages leading to inflammation is their ability to produce cytokines that attract other leucocytes (recruitment during an acute response) and other cytokines which make the blood vessels leaky (cause vaso-dilation and slowing down of blood flow in the region affected). Cells of the monocyte-macrophage lineage (also dendritic cells) take up large particulate antigens, pieces of tissues, senescent cells, bacteria, etc. by phagocytosis. **Figure 1.4.1b** illustrates a macrophage that is phagocytosing a bacterium in the close proximity. They express a myeloid receptor (CD14) which recognizes molecules from a wide variety of bacterial envelopes. Ligation (binding to the antigen) of this receptor leads to macrophage activation and finally antigen presentation to the T cells. In turn the T cells secrete cytokines that increase phagocytosis and microbicidal activity, a positive feedback loop until the antigen is eliminated. The microbicidal activity is associated with degradative enzymes, nitrogen and oxygen free radical production and prostaglandin etc. These cells express receptors for antibody and complement. They bind to the immune complexes, especially if the antibody involved has complement components bound to it (if the antibody has fixed complement), and phagocytose these rapidly.

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To explain cytokines in little more detail, cells of the immune system secrete chemical messengers (called cytokines) to communicate with each other. Cytokines are proteins secreted by cells to act on other cells to coordinate an appropriate immune response by switching certain cell types on and off. They include interleukins, interferons and growth factors. For example, one cytokine, interleukin 2 (IL-2), is secreted by the antigen activated T cells and in turn triggers the immune system to produce more T cells. Other cytokines chemically attract specific immune cells to the site of infection. They are called chemokines. Chemokines are released by cells at the site of injury/ infection and "call" other immune cells to that region to help repair the damage and fight off the villain.

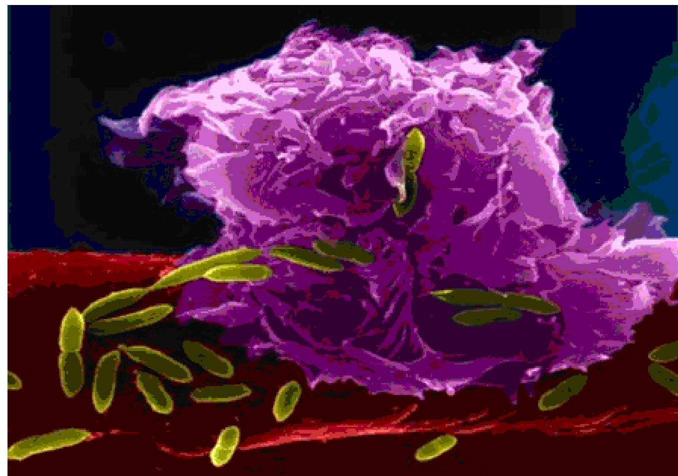

Figure 1.4.1b illustrates an activated machrophage phagocytosing bacteria upon contact. (Figure adapted from www.itb.cnr.it/.../L/UK/IDPagina/86, 29 Mar 2007.)

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1.4.2 Dendritic cells

Dendritic cells (DCs) are present in small numbers in tissues that are in contact with the external environment, the skin (called Langerhans cells) and in the inner lining of the nose, lungs, stomach and intestines. They are also found in an immature state in the blood circulation: once activated, they migrate to the lymphoid tissues where they interact with T cells and B cells. DCs are antigen-presenting cells that initiate a primary immune response by activating lymphocytes and secreting cytokines. **Figure 1.4.2a** illustrates the role of DCs interacting with cells both in the innate and the adaptive immune responses.

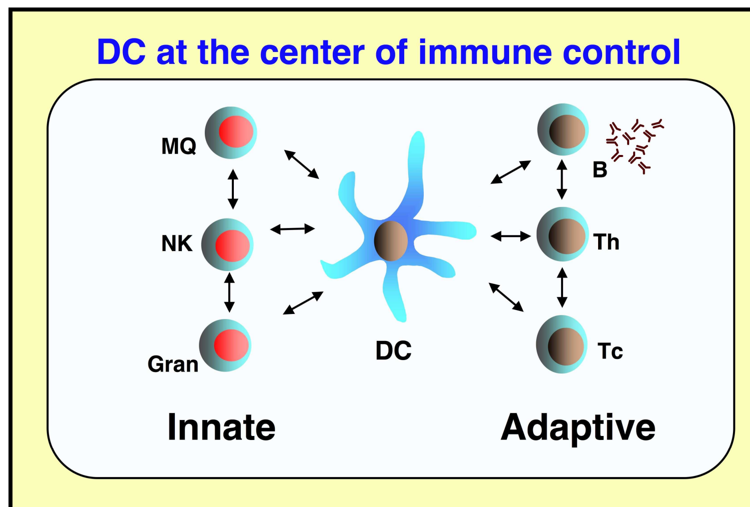

Figure 1.4.2a focus on the immune cells that play a key role in maintaining the immune balance: the professional antigen presenting dendritic cells (DC) and the adaptive and innate immune cells. (Figure adapted from www.ncmls.eu/til/ research/research.html.)

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At certain development stages, DCs grow branched projections, dendrites, which give the cell its name. Immature dendritic cells are also called veiled cells and they possess large cytoplasmic 'veils' rather than dendrites. DCs were discovered in 1973 by Steinman and Cohn. There are three categories of DCs: myeloid DCs (mDCs), plasmacytoid DCs (pDCs) and follicular dendritic cell. The first category of DCs function is antigen presentation and activation of T cells (Follow **Figure 1.4.2a**). The second category of DC function is not as well established, but it has been suggested that a different class of DCs exist with the function of inducing and maintaining immune tolerance [Steinman *et al*. (2003)]. **Figure1.4.2b** illustrates the deregulation of a complicated balance that is directly associated with human diseases, ranging from inflammatory and autoimmune disorders to infection and cancer.

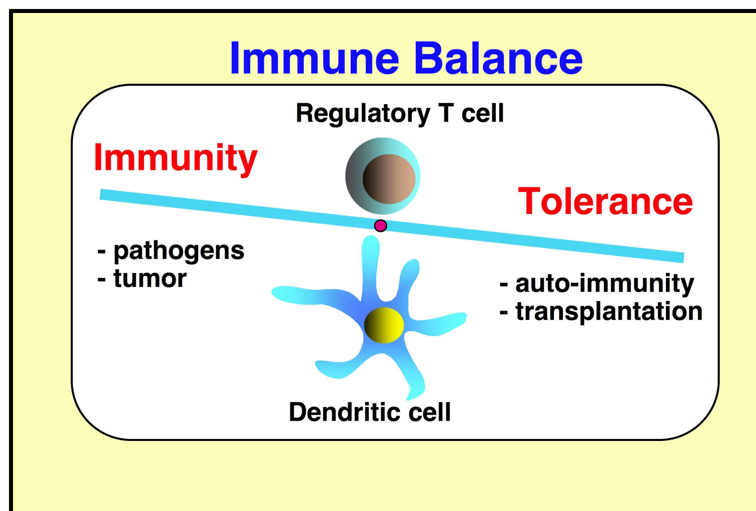

Figure 1.4.2b illustrates the immune system has the tremendous task to eliminate pathogens and eradicate arising tumors, while preventing auto-reactive responses that are harmful to the host. (Figure adapted from www.ncmls.eu/til/research/research.html.)

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The third category of DCs is known as follicular DCs they appear to work to maintain immune memory in tandem with B cells as seen in **Figure 1.4.2a**. Myeloid DCs are similar to monocytes and are made up of two subsets; mDC-1 which is a major stimulator of T cells and mDC-2, which may have the function in fighting wound infection. In general mDC cells are characterized by their ability to produce high level of IL-12 involving the Th1 response [Rissoan *et al*. (1999)]. Plasmacytoid DCs prime antiviral adaptive immune responses by producing high levels of type 1 Interferons [Banchereau *et al*. (1998); Cella *et al*. (1999); Siegal *et al*. (1999) and Liu (2005)] involving the Th1 responses. Langerhans dendritic cells are primarily found in the skin and express langerin (CD207) a Langerhans-cell-specific C-type lectin [Valladeau *et al*. (1999)]. Follicular dendritic Cells (FDCs) might not be considered a typical DC subset; they are not derived from the bone marrow and are not known to be processed and present antigens through MHC-restricted pathways [Banchereau *et al*. (1998)]. FDCs can be found in the B-cell follicles and germinal centres of peripheral lymphoid tissues; they trap and maintain infectious viruses for long periods of time.

Monocyte-derived DCs (MDDCs) are used in many experimental studies as an *in vitro* model due to low concentrations of DC populations *in vivo*. MDDCs share characteristics with myeloid DCs, immature dermal DCs and interstitial DCs, and they express high levels of the cell-surface markers MHC class II molecules, CD11c, CD25 and DC-specific intercellular adhesion molecule 3 (ICAM3)-grabbing non-integrin (DC-SIGN; also known as CD209). Immature MDDCs can be converted into mature MDDCs by various stimuli, including lipopolysaccharide, interferon-γ, tumour-necrosis factor and CD40 Ligand (CD40L) [Banchereau *et al*. (1998)]. This *in vitro* study is illustrated in **Figure 1.4.2c**.

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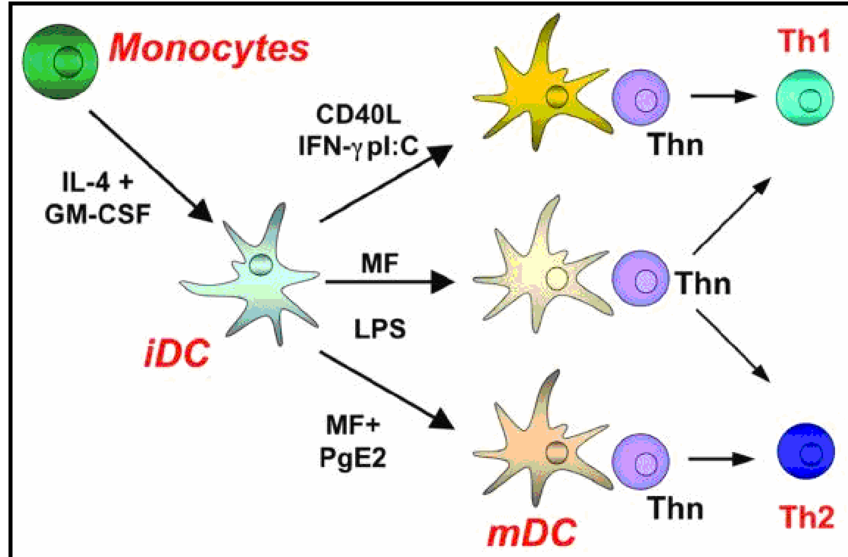

Figure 1.4.2c illustrates Dendritic-cell maturation. Monocyte-derived immature dendritic cells (DCs) may develop into T helper (Th1)-cell-promoting or Th2-cell-promoting effector subsets, depending on the activation signal they receive. Monocytes cultured in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) can develop into immature DCs, which can be further cultured with diverse stimuli to obtain different mature DC subtypes. (Figure adapted from Sanders *et al.* (2002))

CD40L: CD40 ligand; IFNγ: interferon-γ; pl:C: polyinosinic–polycytidylic acid; MF: maturation factors such as interleukin-1β (IL-1β) and tumour-necrosis factor (TNF); LPS: lipopolysaccharide; PgE_2: prostaglandin E_2; T_Hn: naïve T helper cells.

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1.4.3 Lymphocytes

Lymphocytes are small (approximately the size of a red blood cell), round cells with little cytoplasm and round nuclei. Lymphocytes have membrane receptors that bind to antigens. Each lymphocyte recognizes one specific antigen. Lymphocytes express receptors of varying affinity for the antigens and grant specificity to immunity. The cell with the highest affinity for the most abundant antigen will have growth advantage and will preferentially generate progeny of itself, or in other words, ‘offspring of parent cells’. This process is antigen driven and is called clonal expansion. From the pluripotent stem cell, lymphocytes develop from the lymphoid lineage. From **Figure 1.4.1a**, these lymphoid stem cells either become natural killer cells, T lymphocytes or B lymphocytes. B cells produce antibodies and some soluble mediators called cytokines. They arise in the bone marrow in adult mammals.

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T Lymphocytes

T cells originate in the bone marrow and mature in the thymus. Antigen receptors on T lymphocytes (T cells) are called T cell receptor (TCR). Their surface receptors are structurally related to immunoglobulins, but they do not produce antibody molecules. T cells recognize antigens in a different way to B cells. **Figure 1.4.3a** illustrates the way T cells recognize peptide fragments of antigen complexed with cell surface Major Histocompatibility Complex (MHC) glycoproteins on nearby antigen presenting cells. MHC class II molecules present antigenic peptides to T cells and are essential for the initiation of cellular and humoral immune responses [Pieters (2000); Hiltbold and Roche (2002)].

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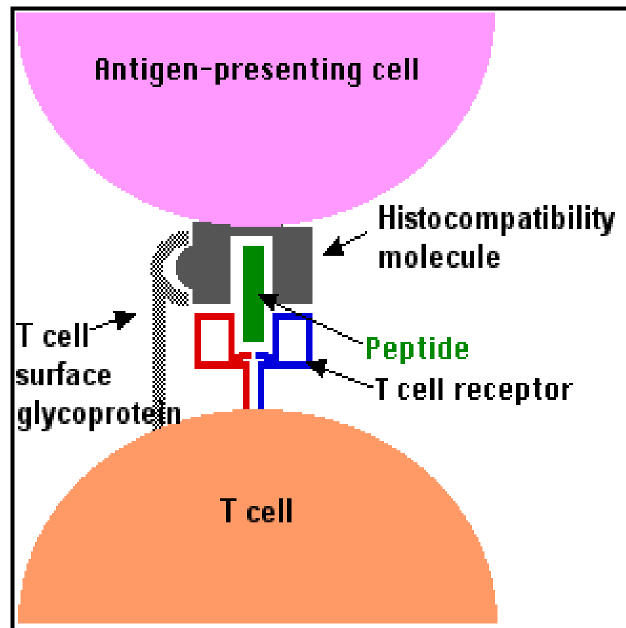

Figure 1.4.3a: T cell receptor binding to MHC-antigen complex. (Figure adapted from http://users.rcn.com/jkimball.ma.ultranet/BiologyPages.)

The cellular expression of MHC Class II molecules is limited to antigen-presenting cells such as B cells, macrophages, and dendritic cells. The class II molecules are highly polymorphic proteins. The presence of several class II isotypes (DP, DQ and DR) increases their diversity. There are at least two alleles of each of these three Class II subsets expressed in most humans. Lymphocytes specific for many diverse antigens are produced continually in the absence of antigen exposure. When a lymphocyte encounters its specific antigen and receives the proper co-stimulation signals, it proliferates and differentiates into a clone of effector cells, all with the same antigen specificity.

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NK Cells

Natural killer (NK) cells are the third subset of the lymphocytes; they arise from the same lymphoid stem cell. NK cells are large granular cells that lack specific antigen receptors. The NK cell is a special kind of lymphocyte that bridges the adaptive immune system and the innate immune system: they represent a first line of defense to infections, tumour growth and other pathologic changes. NK cells do not express antibodies or T cell receptors on their cells surfaces; they produce cytokines and express receptors for immunoglobulins which allow them to detect some infected host cells. These include tumour cells, virus or intracellular bacteria-infected cells, because they respond to altered or modified MHC proteins present on the virus-infected and cancer cells.

B – Lymphocytes

Antigen receptors on B lymphocytes (B cells) are called membrane immunoglobulin, antibody or B cell receptor (BCR). B cells work predominantly by secreting antibodies into the body's fluid. The antibodies ensnare antigens circulating in the bloodstream. Each B cell is programmed to make one very specific antibody: when a B cell encounters its triggering antigen, it gives rise to many large cells known as plasma cells. Each of the plasma cells descend from a given B cell; each plasma cell manufactures millions of identical antibody molecules and releases them into the bloodstream. An antigen matches an antibody much as a key matches a lock. Some match exactly, others fit more like a skeleton key. But whenever antigen and antibody interlock, the antibody marks the antigen for elimination [Janeway *et al*. (2002)].

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There are five different types of immunoglobulins. Immunoglobulin G (IgG) coats microbes which help in the rapid uptake by other cells of the immune system. IgD remains attached to B cells and plays a role in initiating early B cell response. IgA guards the entrances to the body: it concentrates in the body fluids for example tears, saliva, the secretions of the respiratory tract and the digestive tract. IgE protects the body against parasitic infections, but this antibody is responsible for the symptoms of allergy. When B cells are activated, they differentiate into plasma cells and during this process, a memory cell is generated. This ensures that immunological memory is safeguarded for future responses if required. A pathogen invading a vaccinated host is directly attacked by pre-existing antibodies that are produced by plasma cells [Manz *et al*. (2005]. Memory cells have a prolonged life span and can thereby "remember" specific intruders. T cells can also produce memory cells with an even longer life span than B memory cells. The second time an intruder tries to invade the body, B and T memory cells help the immune system to activate in a much faster and more robust protective response [Ahmed and Gray (1996) and Kalia *et al*. (2006)].

T - Lymphocyte subsets

Peripheral blood lymphocytes are similar in appearance; however, they consist of different subpopulations that may be defined phenotypically and functionally. Phenotypically, they are defined by the expression of lineage-specific cell surface proteins. The introduction of the Cluster of Differentiation (CD) nomenclature in early nineteen eighties, [Bernard and Boumsell, (1984)] made it much easier to describe the different cell phenotypes.

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CD numbers have been given in a systematic manner to leucocyte surface antigens identified by monoclonal antibodies submitted to leucocyte differentiation workshops. For example all leucocytes are CD45 positive, a marker expressed on the cell surface of all leucocytes. All T cells are CD3 positive in other words; all T lymphocytes express the CD3 glycoprotein at their surface. CD3 positive mature T cells can be either helper T cells (CD4 positive) or cytotoxic T cells (CD8 positive). Helper T cells are the "main officers" of the adaptive immune system. Once activated, they divide rapidly and secrete cytokines that "help" or regulate the immune response. These so called CD4 positive T cells are the target cell of HIV infection [Hsieh *et al*. (1993)]. The virus infects the cell using the CD4 proteins to enter the cell. After many debates it has been shown that loss of T helper cells as a result of HIV infection leads to the symptoms of AIDS [Mandy *et al*. (2002)]. Cytotoxic T cells on the other hand destroy virally infected and tumor cells. They are the cells that have a major influence in transplant rejection [Hayry and Defendi (1970)].

The third type of T helper cells is the regulatory T cells (Treg cells), formerly known as suppressor T cells. Treg cells are very important for the maintenance of immunological tolerance. Their role is to dampen down T cell mediated immunity toward the end of an immune reaction to minimize collateral damage [Belkaid and Rouse (2005); Jiang and Chess (2006)]. Please follow **Figure 1.4.2b** which illustrates this phenomenon in a summarized diagram. The Treg cells have an intracellular molecule called FOXP3 (forkhead-winged-helix transcription factor) which distinguishes this cell from other T cells. They are CD4+CD25+ T cells that comprise 5-10% of peripheral T cells in normal mice and exhibit potent immuno-regulatory functions *in vitro* and *in vivo.*

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More recently a forth type of T helper cell, Th17 cells, has been identified as a separate entity [Colgan & Rothman (2006), Tato *et al*. (2006) and Weaver *et al*. (2006)]. These cells produce IL-17 as a marker cytokine and are apparently highly pathogenic. It was described by Zheng (2007) that these cells are predominantly found in subjects suffering from autoimmune diseases.

The last of the T cell subset is the γδ T cells that possess a distinct TCR on their surface. They make up 5% of the total T cell population. However, γδ T cells do not seem to be MHC restricted and are unique in that they seem to be able to recognize whole proteins rather than requiring peptides to be presented by MHC molecules on antigen presenting cells [Janeway *et al*. (2002)]. These γδ T cells together with NK cells are so-called 'null' cells: whilst NK cells are CD3 – negative, the γδ T cells express CD3 antigen. NK cells are usually identified by CD16 and/ or CD56 expression and B cells are identified by CD19. If one would add up the percentage of T cells + %B cells + %NK cells, this should add up to 100% with +/- 10%, thereby accounting for all the lymphocytes in circulation. This is called the lymphosum. In a similar vein, CD4+ T cells + CD8+ T = CD3+ T cells with +/- 5%. **Figure 1.4.3b** illustrates the balancing this dual task, a complex interplay between Dendritic cells, NK cells, B cells, Th cells, Tc cells and Tregs exists of many stimulatory and inhibitory circuits that are in place to combat infection, inflammation, autoimmune disorders and cancer.

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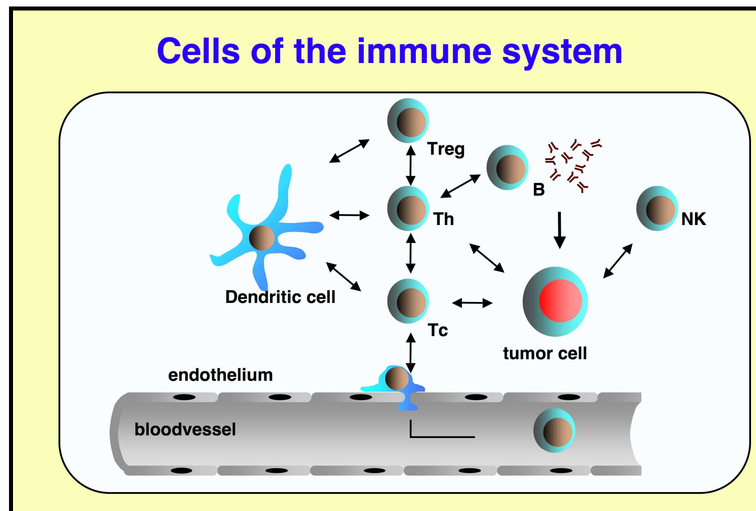

Figure 1.4.3b illustrates the complicated balance and cell interaction to regulate activation and inhibition of the immune system to combat human disease such as autoimmunity, inflammation and cancer.

(Figure adapted from www.ncmls.u/til/research /research.html.)

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Figure 1.4.3c demonstrates (naïve/precursor) helper T (Thp) cells that can be induced to differentiate towards T helper 1 (Th1), Th2, Th17 and regulatory (Treg) phenotypes according to the local cytokine environment. Stimulation of dendritic cells by microbial antigens causes production of interleukin (IL)-6, IL-23 and/or IL-12. Predominant production of IL-12 promotes commitment of Thp to a Th1 phenotype while IL-6 in combination with Treg-derived transforming growth factor (TGF)-β promotes skewing of Thp towards a Th17 phenotype. IL-23 produced by DCs causes proliferation and cytokine production by Th17 cells [Uhlig *et al.* (2006)].

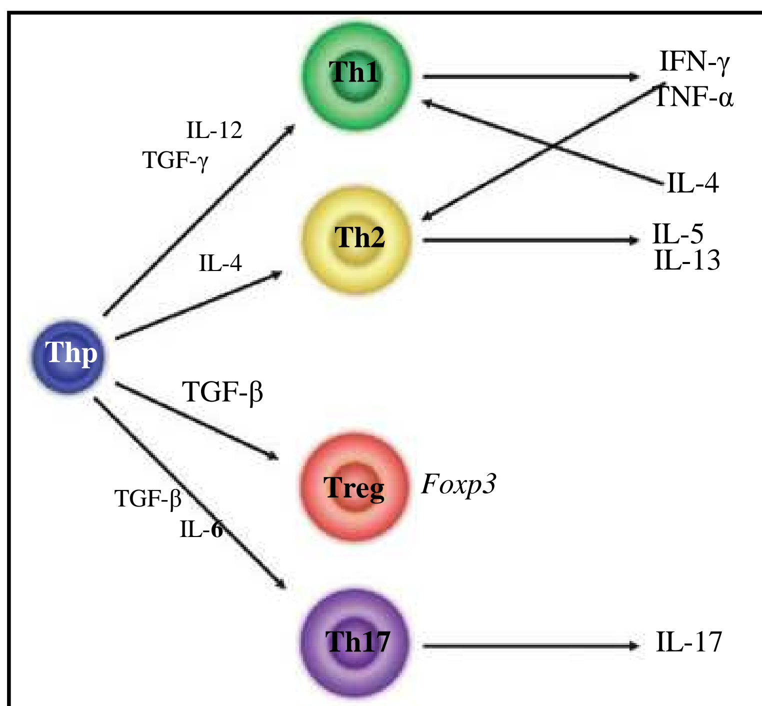

Figure 1.4.3c illustrates a schematic diagram of non committed precursor T helper cells (Thp) differentiating towards either Th1, Th2, Tregs or Th17 cells depending on the predominant cytokine environment. (Adapted from Fig 3 Clinical and Experimental Immunology 148: p39)

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1.5 ACTIVATION OF T LYMPHOCYTES

1.5.1 Activation of T helper cells

T helper cells orchestrate and regulate the immune response in that they activate cytotoxic T cells and provide help to the B cells. Activation of T helper cells requires two signals: the first one is when a helper T cell encounters an antigen presenting cell (APC), the TCR-CD3 complex binds to the peptide-MHC complex present on the surface of APCs (see **Figure 1.4.3a**). The second signal is the cytokine interleukin-1 (IL-1): the APC releases this molecule when the foreign particle is phagocytosed. The foreign particle must be displayed in combination with a Class II MHC molecule on the surface of the APC. The antigen-protein complex attracts a T helper cell and promotes its activation (see **Figure 1.5.1a**).

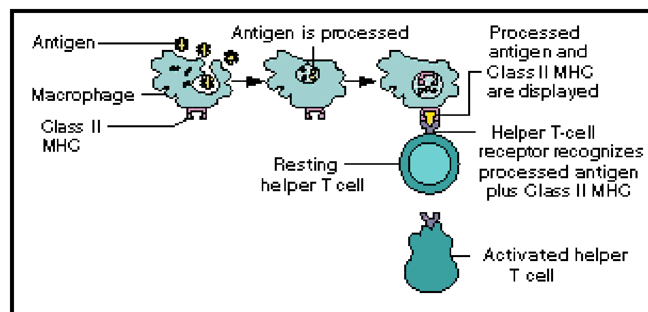

Figure 1.5.1a: Diagram explaining the activation of helper T lymphocytes.

(National Institute of Allergy and Infectious Diseases, Sept 2003) (Figure adapted from www.iba-go.com/streptamer/str_tcell.html.)

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Once activated, the cell undergoes clonal expansion and differentiation into effector cells. Some of the activated T helper cells will not proliferate but will become T-memory cells. The two signals mentioned above induce the expression of IL-2 receptors on the Th1 cells and also induce the production of IL-2 by the Th1 cell. The IL-2 receptor is also known as CD25 and was documented by Sakaguchi in 2000. Now the IL-2 induces growth of the Th1 cells. The CD4 positive T cells differentiate into Th1 and Th2 subsets depending on the cytokines present in the environment of the cells [Janeway *et al.* (2002)]. The idea is that each T helper subset has the ability to stimulate a set of anti-pathogen effector functions (for example for either intercellular of extracellular pathogens). This will promote the development of more of the same T helper subsets while inhibiting the development of the opposite subset "response". When IL-12 is produced by macrophages (or any other APC), the immune response tends towards the Th1 direction. IL-12 prevents gene transcription of IL-4, IL-5 and IL-10. Basophils for example produce IL-4 and this will drive the immune response towards a Th2 immune response. A population of naturally occurring CD4 positive T cells, distinct from Th1 or Th2 cells, which inhibited Th1-mediated intestinal inflammation, is described by Powrie *et al.* (1994 and 1996). This phenomenon gave way to the identification of suppressor T cells, now renamed 'regulatory T cells'. Co-stimulatory molecules act as quality control to ensure that the antigen recognized is foreign. These co-stimulatory molecules are the CD28 on CD4 positive T cells and the CD80 or CD86 proteins on the professional APCs. The CD80 (B7-1) and CD86 (B7-2) molecules on professional APCs are involved in stimulating naïve T cells that express the co-receptor CD28 [Greenwald *et al* (2005)]. This is necessary for the activation of naïve helper T cells, but its importance is best demonstrated during the similar activation mechanism of CD8 positive cytotoxic T cells. These co-stimulatory molecules are demonstrated in **Figure 1.5.1b.**

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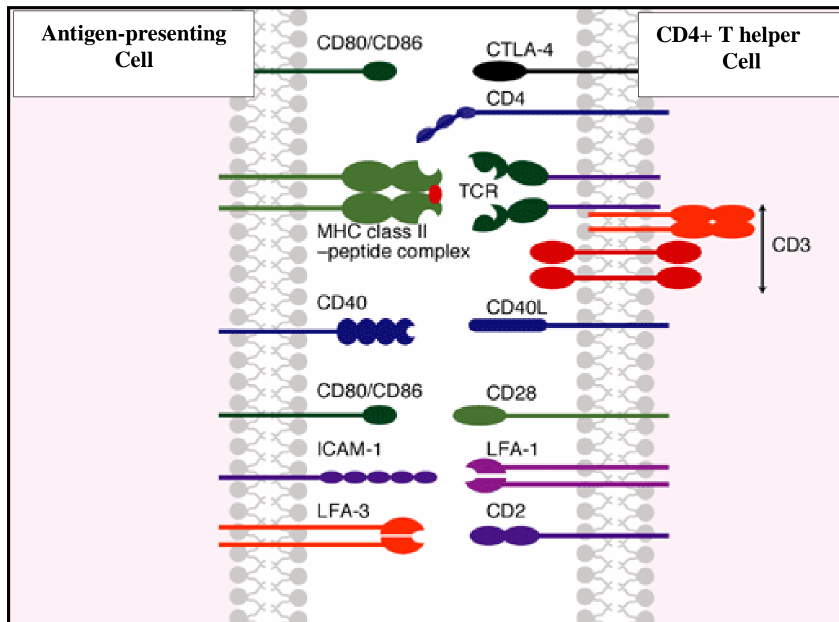

Figure 1.5.1b illustrates the cell surface molecules in T cell activation. Some of these are potential targets for immunotherapy. The interactions between the T cell receptor and the major histocompatibility complex (MHC) class II-peptide complexes are fundamental to T cell activation. (Figure adapted from www-ermm.cbcu.cam.ac.uk/9900126Xh.htm on 28 March 2007).

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1.5.2 Activation of cytotoxic T cells

T-helper cells are involved in cytotoxic T cell activation (see **Figure 1.5.2**). Again two signals are required for the activation of cytotoxic T cells: the first signal is IL-2 provided by the T helper cells, the cytotoxic T cells (CD8 positive) carries the IL-2 receptors for this molecule. The other signal is the cytotoxic T cell receptor in complex with a Class I MHC protein carrying the foreign epitope. A receptor on a circulating, resting cytotoxic T cell (CD8 positive) recognizes the antigen-protein complex and binds to it. This binding process and the help of CD4 positive T cells activate the cytotoxic T cell. Activated cytotoxic T cells can attack and destroy the virus infected cell, tumour cell or tissue graft by releasing cytokines (perforin and granzymes).

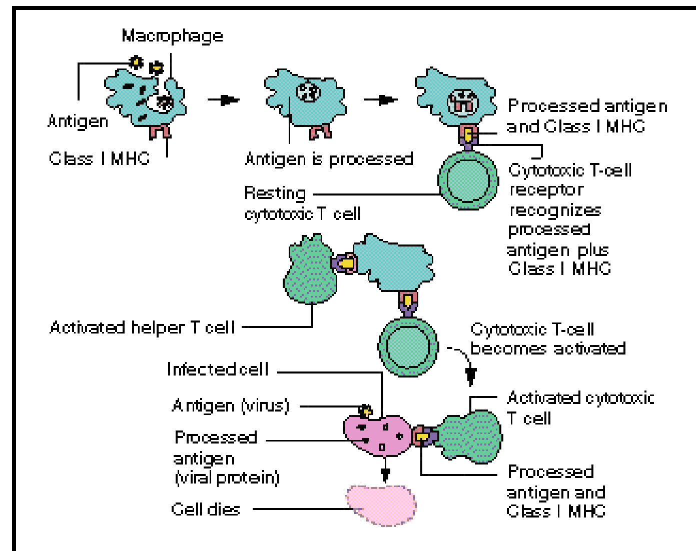

Figure 1.5.2: This diagram explains the activation of cytotoxic T lymphocytes. (National Institute of Allergy and Infectious Diseases, Sept 2003) (Figure adapted from www.iba-go.com/streptamer/str_tcell.html).

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1.5.3 T helper lymphocytes activate B cells

The B cells have surface immunoglobulins which bind to the specific foreign epitopes. A signal is transmitted to the nucleus and the antigens are delivered to intracellular sites where it is processed into peptides. The peptides bound to MHC class II return to the surface of the B cells. The effector T helper cell specific for that antigen recognizes and binds to the MHC/ peptide complex. The Th2 cells then produce surface molecules that bind complementary molecules on the B cell surface (also dendritic cells). One example of such surface molecules is the CD40 Ligand also known as CD154 activation markers. At the same time, the Th2 cells releases lymphokines such as IL-4, IL-5, IL-6 and IL-10. This leads to the activation of B cells, class switching can occur, affinity maturation can occur, and the B cells can develop into antibody producing plasma cells. Some of the activated B cells do not proliferate into plasma cells but become memory B cells. If no activation signal is received from the T cell, then the B cell becomes anergic (non-responsive to the antigen).

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1.6 HIV INFECTION AND IMMUNITY

The absolute CD4 positive T cell counts were the first (and remain) the most widely used laboratory measurement of immunodeficiency and the risk of clinical disease progression in HIV-positive persons. Together with changes in CD4 lymphocyte counts, viral loads (RNA copies) are widely used as indicators of HIV progression in infected patients. The first description of depletion of the helper T cell subset (CD4 T cells) was in a study of four previously healthy homosexual men who had contracted *Pheumocystis carinii* pneumonia and mucosal candidiasis. This was documented by Gottlieb *et al*. in 1981. In the very same article as the first clinical article published; a selective immunodeficiency of the T-helper cell subset and the use of flow cytometry in a variety of human immunodeficiency disorders were described. In 1984, Klatzmann *et al.* and Dalgleish *et al.* (1984) showed that the anti-CD4 specific monoclonal antibodies blocked the infection of CD4-bearing lymphocytes by a novel human retrovirus.

Both studies concluded that the CD4 molecule on T cells was an essential and specific component of the receptor, the causative viral agent of AIDS which was then renamed HIV-1. Mandy *et al.* (2002) demonstrated that the CD4 molecule on T cells is one of the receptors to which the HIV-1 attaches itself. HIV induces chronic activation of CD4 positive T cells and CD8 positive T cells but predominantly destroys the CD4 positive T cells. Therefore there is a decline of CD4 positive T cells in HIV infection and there is an association with increased levels of activation markers on the CD4 population [Mahalingam *et al.* (1993) and Savarino *et al*. (2000)].

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Considerable evidence suggests that the continuous activation of the immune system associated with HIV plays a key role in the progression to AIDS [Landay *et al.* (1990), Pantaleo *et al.* (1993), Pantaleo and Fauci (1995) and Fauci (1996)]. On the other hand, the basis of HIV-specific and T cell immune dysfunction has not been elucidated fully and it is likely that both HIV-specific and non-specific mechanisms are involved [Champagne *et al*. (2001), Lieberman *et al*. (2001), Lawn *et al.* (2001) and Douek *et al*. (2002)]. Powderly *et al.* (1998) mentioned that using HAART as treatment recovers not only CD4 positive T cell numbers but also their function. It was indicated by Holub *et al*. (2004) the usefulness for monitoring the efficacy of HAART therapy. This is done by measuring the CD4 positive T cell numbers with flow cytometry. Today as more effective antiretroviral combinations are discovered so are new immunophenotyping markers. These markers provide more direct information about how an individual living with HIV responds to the applied treatment. It also provides information on the overall immune status of these patients. For instance, it is important to determine the expression of CD38 antigen on CD8 positive T cells. It has been demonstrated that the CD38 molecule is a biological and clinical marker of HIV infection: infection with the virus induces lymphoid activation which results in increased T cell activation-associated antigens such as CD38 [Schlesinger *et al*. (1995), de Martino *et al*. (1998) and Navarro *et al*. (2001)]. To date, only CD38 has shown prognostic significance independent of the CD4 cell counts [Giorgi *et al.* (1989), Giorgi *et al.* (1993) and Mocroft *et al.* (1997)]. In other words the circulating levels of CD8+CD38+ cells increase as HIV infection progresses from seroconversion and asymptomatic stages to AIDS [Prince *et al.* (1991), Gruters *et al.* (1991), Bass *et al.* (1992), Kestens *et al.* (1992) and Levacher *et al.* (1992)].

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Similarly, decreased CD4 positive T cell counts have been reported to correlate with increasing percentages of CD8+CD38+ cells [Perfetto *et al.* (1998].

Although many clinical and laboratory markers, HIV-related symptoms [(Redfield *et al.* (1986)], depletion of $CD4^+$ T cells [Fahey *et al.* (1990)], cutaneous anergy [Redfield *et al.* (1986)], elevated serum $ß_2$-µglobulin and neopterin levels [Fahey *et al.* (1990)], HIV-1 p24 (core) and syncytium-inducing HIV-1 phenotype [(Koot *et al.* (1993)], have been used to estimate prognosis in patients with HIV-1 infection, none of these markers is ideal, because all have limitations in sensitivity, specificity, or predictive power. However it was shown by Liu *et al.* (1996 and 1997) that increasing levels of CD38 antigen expression on CD8+ T cells are a stronger prognostic indicator for risk of chronic HIV disease progression to AIDS and death than CD4 positive cell count, the soluble immune markers (elevated serum $ß_2$-µglobulin and neopterin levels [Fahey *et al.* (1990)]), or combination of HLA-DR and CD38 expression.

Today, for CD4 T cell enumeration, there are more than 20 methods requiring instruments with various levels of sophistication that can be implemented. Flow cytometry remains the undisputed choice for monitoring immune competence during antiretroviral therapy of HIV infection. Change in immunophenotyping is rapidly growing. This change is being facilitated by the arrival of powerful generic antiretroviral drugs to resource-poor areas of the world where they are needed most. Cost-effective implementation of T cell subset monitoring technology has escaped these regions. As a global effort, simpler, better and eventually more affordable tests for T-cell subsets are being developed [Janossy *et al.* (2001)].

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CHAPTER 2

QUALITY CONTROL

2.1 THE IMPORTANCE OF FLOW CYTOMETRY

In the early 1980s, flow cytometry was introduced to the clinical diagnostic laboratory and used for accurate quantification of peripheral blood cell populations. Followed shortly in 1984, the introduction of cluster of differentiation (CD) nomenclature [Bernard and Boumsell, (1984)] improved the "language" of flow cytometric typing results. Flow cytometry has gained wide popularity and now plays a crucial role in several aspects of medical immunology and hematology. Flow cytometry is still the method of choice for CD4 monitoring in human immunodeficiency virus (HIV) infected individuals. T-helper (CD4+) lymphocyte absolute values and percentages remains the most widely used laboratory measurement of the degree of immunodeficiency and risk of clinical disease progression in HIV-positive individuals [Mandy *et al*. (2002)]. Furthermore, a decrease in CD4 positive T lymphocytes is associated with the opportunistic infections of individuals infected with HIV. Subset of cells can be identified and characterized by staining intensity and patterns of maturation antigens and has a significant impact on the diagnosis, monitoring, and therapeutic outcome of diseases such as leukemia [Givan (1992) and Marcey (1994)]. Some of the major advantages of flow cytometry are speed of processing, number of events (cells) acquired in a short period, accuracy, reproducibility, automation and the wide choice of reagents available.

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Today flow cytometry is applied to monitor subsets of lymphocytes in routine immunology, typing of leukemia and lymphomas in hematology, S-phase determinations in oncology, chromosomal analysis and typing of bacteria in bacteriology.

And finally, thanks to commercial development and marketing, less sophisticated flow cytometric instruments can be used confidently with relative little human intervention. Such an example is the bench-top FACSCount™ by Beckton Dickinson. Flow cytometry has progressively replaced many traditional laboratory tests due to its greater accuracy, sensitivity and rapidity.

2.2 PRINCIPLES OF FLOW CYTOMETRY

Flow cytometry is a powerful tool for the measurement of chemical (the fluorochrome we add) and physical (size and complexity) characteristics of individual cells. In a nutshell, cells move in single file via the fluid stream (sheath fluid) and pass a laser sensing point. Large numbers of cells can be processed and measurements are made separately on each individual cell. As each cell passes in front of a laser, it scatters the laser light in different directions depending on the size and granularity. Please see **Figure 3.1.1** in **Chapter 3** illustrating a granulocyte passing through the laser beam. The degree of light scatter allows the flow cytometer to identify all major blood cell populations. At the same time fluorescent dye molecules conjugated to monoclonal antibodies specific for surface markers bound to the cell are excited. Once they are excited, they emit light at a specific wavelength. Photo multiplier tubes (PMTs) detect both emitted and scattered light from each fluorescent dye at specific emission frequencies.

Combination of filters allows measurement of 1, 2, 3, or 4 colors at the same time. Selected cells are shown to have different staining intensities: lymphocytes have the highest intensity of CD45 expression, and then monocytes and polymorphonuclear lymphocytes are the dimmest. Three- and four-color immunophenotyping are not unusual in the clinical laboratory, it allows the simultaneous measurement of five or six different parameters, respectively [Nicholson *et al.* (1996)]. Complex analyses are able to combine immunophenotyping of both surface and cytoplasmic antigens, DNA analysis and functional evaluations. With so many variables in these analyzers, standardization and validation of instrumentation and methodology is essential for quality assurance of the results [Carter *et a*l. (1992), National Committee for Clinical Laboratory Standards (1992), Hurley (1988, 1997a, b) and Brando & Sommaruga (1993)].

2.2.1 Dual Platform Technique for CD4+ cell counts

The absolute T cell counts are generally calculated from the absolute lymphocyte value and the percentage T cell subset value. The flow cytometer provides the T-cell percentage as a fraction of a "denominator" (reference populations of cells, for example white blood cells). The hematology analyzer provides the absolute WBC count together with a differential count, which must include the "denominator" [Glencross *et al*. (2002)]. Recent reports have highlighted the variability in absolute T cell counts as a result of using different hematology analyzers [Robinson *et al*. (1992)]. For example, the flow cytometer provides the following: CD3% = 75, CD4% = 34 and the CD8% = 36. The hematology analyzer provides the differential count; lymphocyte count of 2. 62 x 10^3. The CD4 absolute count = $^{34}/_{100}$ X 2620 = 890 / mm^3 blood.

2.2.2 Single Platform Technique for CD4+ cell count

Single platform flow cytometers capable of generating absolute T cell counts have been developed to overcome the problem of variability on an inter-laboratory basis. This technique depends on either precision fluidics [Connelly MC *et al*. (1995)] or microbead technology (counting beads) to assess absolute T cell counts directly on the flow cytometer [Nicholson *et al*. (1997) and Schlenke *et al*. (1998)]. Consistently lower Coefficients of Variations (CVs) were obtained using this technology and it is therefore the method of choice today [Barnett *et al*, (1999)]. The chief advantage is the elimination of the need of a second instrument. For example using the BD TruCount Bead Method where a known bead count (provided by the manufacturer) is used to calculate the absolute cell values (absolute cell values are measured as cells x 10^6 / L for this thesis).

.

Calculation:

$$\frac{\text{\# of Events in region containing cell populations}}{\text{\# of events in absolute count bead region}} \times \frac{\text{\# of beads per test}}{\text{test volume}} = \text{Abs count of cell population}$$

= number

Abs = absolute

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2.3 QUALITY CONTROL OF FLOW CYTOMETRY

The laboratory's responsibilities to generate accurate cell counts continue to increase since a patient's clinical management depends on such counts. This is further reinforced as new drugs enter the clinical trial phases and the primary outcome of such studies depends on the accurate reporting of the laboratory process. Unfortunately, among the very large number of applications, only a minority of flow cytometric protocols have been standardized.

There are many factors that are responsible for variation in analytical conditions and these may greatly affect results obtained. It was acknowledged that more advanced quality control, standardized procedures and proficiency testing programs were needed to be incorporated to improve the reliability of results generated. Hence the variables can be schematically divided into basically three all-purpose groups:

1. Factors related to biological samples,
2. Immunological and accessory reagent factors and
3. Last but not least, those factors associated with the use of the instruments.

Generally Good Laboratory Practice (GLP) is a good place to start and today this is implemented in more South African laboratories. GLP includes Quality Control (QC) and Quality Assurance (QA) and is most important in providing first-class patient care [Hurley and Zito (1998)].

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In 1992, the National Committee for Clinical Laboratory Standards, the Centers for Disease Control and Prevention (CDC), Association of State and Territorial Public Health Laboratory Directors, and the National Institute of Health's AIDS Clinical Trails Group built on recommendations developed previously and published guidelines for performing CD4 T lymphocyte determination on specimens of HIV infected individuals [Centers for Disease Control (1992)]. This guideline provided specific information about performing the test, with recommendations for quality control procedures, information about lymphocyte gating, a monoclonal antibody panel and reporting requirements. Today, flow cytometry has become such a major diagnostic and prognostic tool and the importance of Quality Control procedures including both participation in an external quality assurance (EQA) and an internal quality assurance (IQA) program cannot be emphasized enough. These quality control procedures that are implemented for the performance of the flow cytometer must be designed to asses all aspects of the procedure. The major instrument settings can affect the reliability, reproducibility and sensitivity of the cytometer and should be monitored and documented in order to ensure identical conditions of measurement on a daily basis.

More than one Major Concern

It has been difficult to provide testing facilities with internal quality control (IQA) material because fresh whole blood collected into EDTA becomes unsuitable for analysis 30 hours post-phlebotomy [Owens and Loken (1995)]: for instance, for CD4 monitoring, the antibody labeling is usually direct (by adding an aliquot of the conjugated antibody to a sample volume of the whole blood) and the test should preferably be performed within 48 hours but no later than 72 hours post-phlebotomy according to the published guidelines [CDC (1997) and CDC (2003)]. The rapid deterioration of the fresh blood samples places an additional burden upon the laboratory to find a suitable material representative of the patient sample. This usually comes down to blood being drawn from a member of the staff. Most clinical laboratories, therefore, employ either latex beads of frozen cells to monitor both sample processing and flow cytometer performance. These preparations, although of significance, fail to provide full-process quality control. We still do not have adequate control material which can be used to test the entire analytical procedure, factors related to the biological sample, immunological and accessory reagent factors and factors associated with the use of the instruments [Martini and D'hautcourt (1993)]. It has been mentioned by D'hautcourt (1996) that it is most unlikely that we will one day have at our disposal a control sample which will mimic the great diversity of properties specific to the leukemic blasts. In this situation, an internal quality control program must still be established, correct results produced by the flow cytometry laboratory ensured, before results can be compared to those of other laboratories via external quality control programs.

Once the internal quality control procedure has been correctly performed, it is necessary to establish an external quality control to perform a global evaluation of the laboratory performance on a regional, national and/or international basis. An additional upcoming requirement for Quality Control is the calibration of the equipment using whole blood samples or blood products to calibrate for example the BD FACSCount™ flow cytometer. For the hematology analyzers currently fixed bovine blood or blood from donkeys and turkeys is used because a suitable source of stabilized human blood is not available. For certain flow cytometers (for example BD FACSCount™) a fresh blood sample of a healthy individual is required to calibrate the instrument early every morning. It will contribute greatly to clinics (using this instrumentation) if stabilized whole blood can be used instead of a staff member donating fresh whole blood to calibrate the instrument every day.

2.3.1 Internal Quality Control Programs (IQA)

All laboratories worldwide are responsible for maintaining performance standards. Internal Quality Control program (IQA) monitors and evaluates all aspects of the procedures in a clinical setting. The IQA program should cover the following main aspects:

- Specimen collection, transportation and the maintenance of its integrity,
- Reagents used to perform test (Particularly monoclonal antibodies),
- Performance of the flow cytometer and
- Sample measurement, data acquisition and their interpretation.
- We would like to include laboratory staff competency

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IQA procedures consist of daily monitoring of instrument setup and performance of the operators. IQA is implemented as part of "laboratory maintenance" in order to identify potential problem and take immediate corrective action. Results of daily monitoring of instrument performance should be kept in computer logs so that trends and variations can be noted and action taken immediately. Normal peripheral blood, cell lines, blood standards or CD Chex® (Streck Omaha, NE USA) can be used for precision as a standard analytical parameter. CD Chex® is one frequently used example of an IQA sample and an excellent way to measure the reproducibility of a single sample stained and analyzed in duplicate and/or at frequent time schedules. CD Chex® samples are preserved white cell controls manufactured by Streck Laboratories and one example of such internal quality controls. Both CD Chex® Plus CD4 - Low (used as QC for low CD4 positive values) and CD Chex® Plus Normal (used to resemble a normal patient sample) are run daily at Synexa Life Sciences.

Quality control for the performance of the flow cytometer

The quality control procedure for the performance of the flow cytometer instrumentation must be designed to evaluate instrument settings. These settings affect the reliability, reproducibility and sensitivity of the flow cytometer directly. Quantitative means and standard deviations for each monoclonal antibody should be documented and established standard deviation ranges developed depending on the laboratory. For Synexa Life Sciences, a Mean +/- 2SD is considered as within the acceptable range. Furthermore, instrument precision is accomplished by running the sample with results falling within the 2SD range with every run time schedule.

When samples fall outside these reference ranges, they should be investigated, and a list of trouble shooting should be followed to eliminate the error factor. No patient samples should be accepted or tested until the problem is identified and eliminated. The run of the IQA samples should be included daily or as the instrument is used.

Control of monoclonal antibodies incorporated

If is preferable to use monoclonal antibodies purchased from well established companies. If this is impossible or in the case of new antibodies, specific attention should be paid to their origin and the literature already published. The best way to validate the use of a new antibody or the company from which the antibody is purchased is to run a well identified sample in parallel with the old and the new monoclonal antibodies and to compare the results obtained.

2.3.2 External Quality Assurance Programs (EQA)

Once the internal quality control procedure has been performed correctly, it is advised to participate in an external quality control program. The main purpose of external quality control schemes is to identify differences/shortfalls of the laboratory when compared to independent laboratories conducting the same test procedure using an established standard reference. Due to the global HIV epidemic, the requirement for routine enumeration of T-lymphocyte subsets resulted in a desperate need for external quality assurance (EQA) programs.

In 1993, United Kingdom National External Quality Assessment Scheme (UK NEQAS) moved away from fresh blood samples and developed stabilized whole blood samples. UK NEQUAS incorporated these samples which today enables the accurate monitoring of laboratory performance. External quality control schemes play a major role in quality control (QC) and quality assurance (QA). It has greatly helped to reduce the variation seen on an inter-laboratory basis and enabled greater standardization. EQA programs can either be the evaluation of laboratory performance on a regional, national or international basis. Previously EQA programs have made use of frozen cells or fresh whole blood [Paxton *et al*. (1989), Van't Veer *et al.* (1992) and Homburger *et al*. (1993)]. The instability of these materials demanded that samples be shipped by express courier. This is one of the most taxing concerns in EQA, not only being extremely costly, but also not being able to guarantee that the sample arrives in a suitable condition. The unsatisfactory EQA sample integrity might result in great difficulty for identifying poor performing or inadequately performing laboratories [Barnett *et al.* (1996)]. The use of a fresh whole blood as EQA created a wide range of results (high CV and SD values) due to sample circumstance described by Schonwald and Jilch (1994). To this end, a whole blood stabilization procedure has been developed to overcome the problem of sample stability or in other words sample integrity [Barnett *et al*. (1996) and Barnett *et al*. (1998)]. These stabilized samples act as a full process control, because the matrix is similar to that of "fresh" blood. The use of stable whole blood preparations eliminate the need for express deliveries, and thus reduce the deviation around the consensus mean caused by sample deterioration.

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Studies done by Barnett *et al*. (1996) have shown by using stabilized whole blood, individual laboratory results for each issue are tightly distributed about the mean, as defined by a low standard deviation (SD) value. It was also shown that the majority of the CV values were less than 10% for each of the three parameters (CD3, CD3/CD4, and CD3/CD8). There are several EQA schemes for the routine measurement of CD3, CD3/CD4, CD3/CD8 and other B cell subsets available to date. What follows is a brief discussion on commercially available External Quality Control assessment schemes:

a. United Kingdom National External Quality Assessment Scheme (UK NEQAS)

As the largest provider of EQA for flow cytometric assays worldwide, UK NEQAS have 296 participants in 40 countries using the long-term stabilized whole blood samples for leucocyte immunophenotyping surveys i.e. CD4 positive T cell enumeration. Studies were done and laboratories using less sophisticated gating strategies (e.g. CD45, CD14-based backgating or simple light scatter-guided gating on lymphocytes) were up to 7.4 times more likely to fail the EQA exercise [Gratama *et al*. (2002)]. The adoption of single-platform methods by the participants resulted in a drop of the CV values from 56% (before advent of single-platform counting) to less than 10%. All participating laboratories receive a 1.5ml sample from each stabilized donation. They are requested to enumerate the percentages and absolute values for example CD3, CD3/CD4, CD3/CD8 for T lymphocytes, using their daily laboratory protocol. Laboratory results are returned to UK NEQAS, full data analysis are performed and returned to the participants shortly thereafter.

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The importance of EQA programs have been highlighted over the years, where for example UK NEQAS has pointed out important problems, such as the inappropriate use of fluorochromes and antibody titre, and the identification of effective gating strategies. Together these factors all contributed directly to the high inter-laboratory variations seen in cellular immunophenotyping. UK NEQUAS for leucocyte immunophenotyping programs have strongly indicated differences that occur between single and dual platform flow cytometric technologies, particularly in absolute counting of lymphocyte subsets. If a laboratory performance falls outside the desired score, remedial action is undertaken, either by the program organizers, or if this course of action fails, by the National Quality Advisory Panel for Immunology (NQAAPI). Evidence of adequate performance in an accredited EQA program is currently required for laboratory accreditation by Clinical Pathology Accreditation (UK) Ltd.

b. College of American Pathologists (CAP)

The College of American Pathologists (CAP) is recommended worldwide as EQA program to ensure accurate patient results in today's challenging healthcare environment. External quality assurance programs have consistently improved results during the last decade. For example, the 2003 survey of approximately 500 laboratories by the CAP, using 3 samples of stabilized whole blood with CD4 percentages ranging from 17 to 52, showed that the variation between laboratories ranged on average between 4% and 8% for CD3 and CD4 positive cells expressed as proportion of lymphocytes (using CV as a measure).

In the same survey, absolute cell counts of 220 laboratories, measuring CD4 positive, without including CD3 positive cells in the data analysis for lineage gating of T cells, the variation between the laboratories were between 7% and 16%. CD3-CD4dim monocyte contamination in the gated lymphocytes contributed to the results. Monocyte contaminating the gated lymphocyte population results in false CD4 positive T cell values. This is a common phenomenon in poorer African countries, where affordable methodology and gating strategies are incorporated, but may not deliver the most accurate results especially on older EDTA samples.

c. Additional Quality Assessment schemes

Some additional examples of external quality control programs available today are listed. Canadian Quality Management Program – Lab Services (QMP-LS) and the Canadian HIV trails Network (CTN) have adopted dual-anchor gating and single platform techniques. Consistent results of 5% and 10% for CD4 positive T-cell count were obtained from this smaller group of laboratories.

Quality Assessment and Standardization for Immunological measures relevant to HIV/AIDS (QASI) was established in 1997. It is an addition to the EQA schemes to meet performance assessment for immunophenotyping laboratories in countries where such services is not available [(Mandy *et al*. (2002)]. In this survey, 47 countries participated, including many from Africa, South America and the Asia Pacific region. More than two thirds of the participants used relatively simple two-color methods based on the selection of lymphocytes as $CD45^{bright}$ Side $Scatter^{low}$ [Glencross *et al*. (2002)].

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Since the start of this program, the between-laboratory CV for absolute and percentage CD4 positive T-cells decreased from 7.2% to 4.7% and from 14.2% to 8.8%, respectively. The general consensus was made that "pan-leucocyte gating" approach significantly increases the reliability of absolute CD4 positive counts [Glencross *et al*. (2002) and Mandy *et al*. (2002)].

2.4 IMPACT ON STANDARDIZATION ON CLINICAL CELL ANALYSIS BY FLOW CYTOMETRY

With the increased dependence on flow cytometry for clinical decision making, various efforts to establish standardized testing and to reduce the variability between laboratories have been undertaken by many governmental and professional bodies. The increased need for standardization approaches and quality control procedures in the clinical laboratory motivated the development of guidelines for CD4 positive T-cell enumeration. These guidelines also cover topics related to laboratory safety, specimen collection, specimen transport, maintenance of specimen integrity, specimen processing, flow cytometry quality control, sample analysis, data storage, data reporting, and overall internal quality assurance. Traditionally, the absolute T-cell subset counts were assessed using the dual platform technique. Single platform techniques were introduced late 1990s; the absolute T-cell values are directly assessed on the flow cytometer in precisely determined volumes of blood sample. As previously mentioned, single platform techniques can be based either on counting beads [Nicholson *et al*. (1997) and Schlenke *et al.* (1998)] or volumetric [Connelly *et al*. (1995)]. The big advantage is the elimination of a need for the secondary instrument and the denominator.

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The use of single-platform techniques was already recommended by the British Committee for Standards in Haematology (BCSH) guidelines and was incorporated in the 2003 update of the CDC guidelines [Barnett *et al*. (1997) and Mandy *et al*. (2003)]. The goal of the EQA exercise has been primarily educational, but the results will increasingly serve as a basis for laboratory accreditation. It is essential that the EQA programs themselves meet careful quality demands, which include the quality of distributed samples and the procedure for evaluating results. International relationships should facilitate the development on uniform and statistically appropriate method for evaluation of results. At the same time, this should allow monitoring of individual laboratory performance in order to allow and help in providing corrective action where needed. Instrument maintenance will be improved and errors or defective equipment will be identified immediately. Retraining can be provided where the need is identified. This will ensure that more accurate results will be released and this will result in better patient care.

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CHAPTER 3

STABILIZATION OF ANTI-COAGGULATED, WHOLE BLOOD SAMPLES

3.1 INTRODUCTION

Peripheral whole blood contains several cell populations: the erythrocytes and platelets are the most abundant and their functions are the exchange and transport of carbon dioxide and oxygen while the platelets play a role in blood coagulation. The least abundant cell populations (minority) are the leucocytes which are involved in the control of the immune system. By microscopic and flow cytometric analysis, three subpopulations of leucocytes can be distinguished: the polymorphonuclear cells having multi-lobed nuclei, mononuclear cells, having one large nucleus and lymphocytes being much smaller and having a single nucleus. These populations can be distinguished by flow cytometry in a so-called "scatter plot": the cells can be differentiated by differences in their forward scatter (FSC) and / or side scatter (SSC). The forward scatter parameter differentiates the relative cell sizes while the side scatter differentiates the relative granularity of the cells.

As the cells pass in single file past the laser beam, detectors will detect and measure the FSC and SSC of each individual cell: cells of the same size and granularity will be grouped into a common population and this is plotted into a "dot plot". **Figure 3.1.1** illustrates a single neutrophil as it passes the laser beam and it can be seen that it contains a complicated three-lobed nucleus with granules in the cytoplasm and result in loads of SSC.

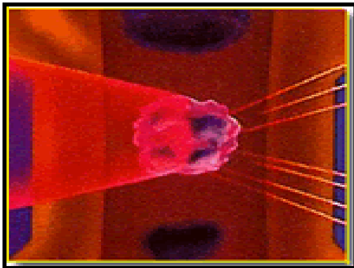

Figure 3.1.1 Illustrates a granulocyte passing in front of the laser with the granules causing high amounts of side scatter. (Figure adapted from www.wistar.upenn.edu/service.html).

Within the leucocyte population, approximately 60% are polymorphonuclear cells (neutrophils, eosinophils and basophils), 30% are lymphocytes and 10% monocytes. As previously described, the cells are separated via homogeneous morphological discrimination using the display of forward scatter versus orthogonal side scatter on the x and y-axes respectively. **Figure 3.1.2** demonstrates this phenomenon.

It is therefore possible to use the scatter properties of different cell populations to "gate" the cells of interest in order to conduct specific analyses within this chosen electronic gate. However, it is not very accurate since any degranulation of cells from a population having higher granularity (for example, granulocytes) into a population having lower granularity (for example, monocytes) can yield inaccurate results if one has no measurement of the purity of the chosen gate.

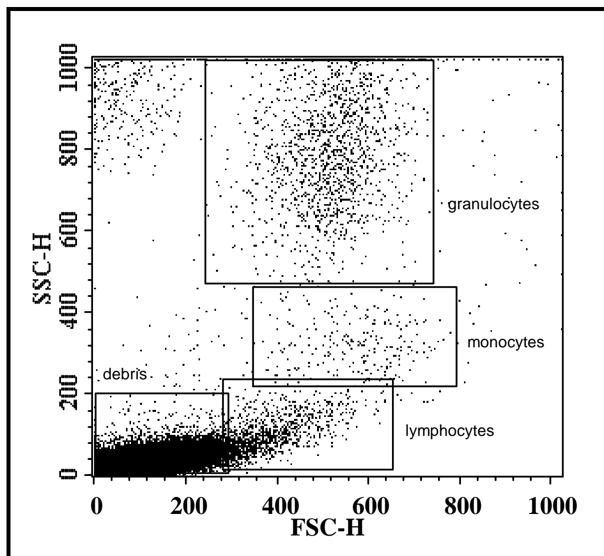

Figure 3.1.2 Different cell populations are visualized on the flow cytometer using their relative size or FSC (x-axis) versus their relative granularity/complexity or SSC (y-axis). This is an example of a dotplot obtained on the BD FACSCalibur™ using the CellQuest software.

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It is however more beneficial to abandon the use of homogeneous morphological discrimination (as described previously) and instead to adopt the heterogeneous "morphospectral" strategy for primary gating where one parameter is based on the reactivity of a fluorochrome-conjugated antibody (e.g. CD45-PerCP or CD3-phycoerythrin) used together with the side scatter [Mandy *et al.* (1992), Nicholson *et al.* (1996), Bergeron *et al*. (2002) and Glencross *et al*. (2002)]. The cell populations can now be distinguished from one another using both side scatter and CD45-PerCP: all lymphocytes are CD45 bright and will express high levels of the fluorochrome (shift much more to the right on the x-axis) when compared to the rest of the leucocyte populations as illustrated in **Figure 3.1.3.**

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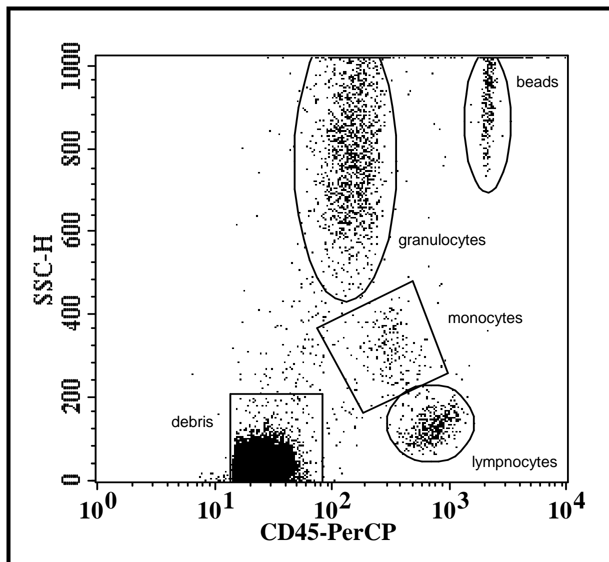

Figure 3.1.3 This dot plot demonstrates the separation of leucocyte populations using CD45 monoclonal antibody (on the x-axis) versus SSC (on the y-axis): all leucocytes express the CD45 epitope but this differs according to their cellular densities, hence intensities. This is another way of gating to obtain more accurate results on the BD FACSCalibur™ using the CellQuest software.

The variation in the percentages of the leucocytes or lymphocyte subpopulations is a good indication of the state of health of an individual. For example, as discussed by Maekawa (1995) an increase in the number of neutrophils is associated with such diseases as inflammation, myocardial infarction and leukemia, and a decrease in their number might be associated with viral diseases, hypoplastic anemia and agranulocytosis. An increase in the number of eosinophils is found in parasitosis, Hodgkin's disease and allergic conditions. Then again, monocytic leukemia may result in an increased number of monocytes [Maekawa (1995)].

By staining the blood cells with monoclonal antibodies conjugated to a fluorochrome (fluorescent marker), and after analysis on the flow cytometer, one can distinguish blood cell populations more precisely. For instance one can with CD4 and CD8 markers distinguish T cell sub populations having helper function or a suppressor / killer function respectively. As previously mentioned in Chapter one, the identification of lymphocyte subclasses is important for HIV monitoring, management and treatment of other diseases of the immune system.

Aim of stabilizing anti-coaggulated, whole blood

In the past, artificial latex particles and erythrocytes prepared in fixatives such as glutaraldehyde and formalin have been used as standard cell suspensions in hematology laboratories. With these preparations, several disadvantages such as agglutination of the fixed red blood cells and distortion in the shape and volume of the red blood cells occurred. Many modified whole blood control samples for the use of monitoring electronic counters were marketed by several commercial companies. But there was still the need for improved stability of reference control material for maintaining accuracy of red cell counts and other parameters when employing electronic counting methods.

The aim of standardizing whole blood controls is to stabilize or “fix” the cells without substantially destroying the cellular properties such as the cell’s surface molecules, morphology and the light scatter properties. The objective of this thesis is to provide methods to stabilize blood components, particular white blood cells, without damaging the white blood cells’ surface antigens as well as their activation markers.

The stabilized sample should still consist of red blood cells (RBCs) which can be lysed and not interfere with the other cell populations in the flow cytometric analysis. The stabilization process of the whole blood is achieved by using a series of stages where the additions of both organic and inorganic compounds are incorporated. The fixing components act by attaching to the cell proteins and cementing their structure. The fixed cells, although dead, frequently demonstrate properties during cellular analysis that are very similar to those demonstrated by "live cells". For example, the differentiation of cell types based on their light scatter properties is still unharmed. The morphology of the stabilized cells is very similar to that of live cells in that it shows much less swelling or shrinkage as typically displayed by fixed cells.

Stabilization protocols were initially developed to create biological standards (long-term stabilized cells) for evaluation of cytometer performance in different immunofluorescence assays as well as the training of new technicians [Barnett *et al.* (1996, 1998a, b, 2000a), Whitby *et al.* (2002)]. Today whole blood samples that are stabilized are used for quality control programs (IQA and EQA) for clinical laboratory performance evaluation and standardization.

These stabilized IQA reference samples can be applied quantitatively and qualitatively. In other words:

i. They should first display the ability for the various populations of cells to be identified based on their phenotypic characteristics.

ii. Secondly, they should consist of the particular populations of cells of interest which can be repeatability quantified over a period of time.

The use of fresh blood on a daily basis fails to provide the information on a day to day variation of the technique and/or equipment because the samples give unpredictable results after 48 hours due to the aging of the sample. There are some quality control samples available, for example fluorochrome coated microspheres, which can be used to monitor day to day flow cytometric performance, but they cannot provide quality control information concerning the performance of the labeling techniques of leucocytes. On the other hand, the use of stabilized samples can be utilized to monitor various immunofluorescence techniques and equipment. Quality control (QC), precision, repeatability and accuracy are extremely important in order to give the correct diagnosis and to monitor effective therapeutic regimes of patients. Stabilized whole blood are potential reference preparations that can act as a full process control and provide valuable means for monitoring quality control systems especially with respect to important steps when evaluating immunofluorescence staining patterns in qualitative and quantitative manner, for example antigen definition and absolute cell counting procedures [Barnett *et al*. (1996, 1998a, b, 2000a, b), Jani *et al.* (2001), Reilly and Barnett (2001), Bikoue *et al*. (2002), Whitby *et al*. (2002)].

The aim of this chapter therefore is to demonstrate and validate the best possible stabilization procedure for whole blood. We investigated the possibility of identifying various routine immunological phenotypic cell surface markers using the BD FACSCount™, BD FACSCalibur™ and BD FACScan™ flow cytometers.

We investigated the quantification of cell populations and the stability of whole blood cell suspensions where the red blood cells are still capable of lysing for the lyse-no wash flow cytometry technique. We investigated various temperatures at which these stabilized whole bloods will produce optimal results. Furthermore, we analyzed the correlation of the results obtained using two different flow cytometers (BD FACSCount™ and BD FACSCalibur™). We investigated the likelihood to use this stabilized whole blood for the calibration of the BD FACSCount™ flow cytometer, where a fresh blood sample is needed with every morning for calibration purposes.

As part of the aim to stabilize anti-coaggulated whole blood, we will demonstrate in the chapters to follow, activated and stabilized preparations of activated leucocytes. In particular use, for these blood controls for more specialized IQA controls in clinical immunology, for example in clinical studies where a new drug is utilized to deplete such activated cells from the circulation and the outcome of the drug is based on the reduction of such cells.

3.2 STABILIZING AND FIXING OF BLOOD CELLS

Various methods of preparing fixed cells have been investigated and compared: Connelly *et al.* (1995) described the fixation of white blood cells infected with viruses. They made use of agents such as 2,4-Dinitrobenzene sulfonamides, Dinitrophenols, 3,5-Dinitrosalicylic acid, 2,4-Dinitrobenzoic acid, 5-Sulfosalicylic acid, 2,5-Dihydroxy-1,4-benzene disulfonic acid, 3,5-Dinitrobenzoic acid, 8-Hydroxyquinoline-5-sulfonic acid, 4-Nitrophenol, 3,5-Dinitrosalicylaldehyde, and 3,5-Dinitroaniline as first fixative compound. The second fixative compound was alcohol-free agents such as formaldehyde, paraformaldehyde, gluteraldehyde etc. together with a fusogenic compound selected from a group consisting of dimethylsulfoxide, sulfolane, polyethylene glycol and ethylene glycol. The aim of Connelly's experiment was to fix cells without substantially destroying cellular properties such as the cell's surface markers, morphology, and light scatter properties.

In 1988 Hill and Winfrey described a process by which whole blood control samples were processed. Whole blood samples from a donor were separated each into red blood cells and plasma. The red blood cells were fixed (with gluteraldehyde), mixed with the plasma again to produce a suspension and quickly-freezing the suspension before the red blood cells could settle. However they did not mention stabilization of the leucocytes as part of their study. Fischer *et al.* (2005) contributed to the method described by Bakaltcheva *et al*. (2000) for stabilizing red blood cells, the red blood cells were treated with a reversible chemical cross-linker dimethyl 3,3-dithiobispropionimidate followed by the freezing the cells.

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The cells can then be lyophilized and re-hydrated to restore their fragility and deformability. However, the methods described above are very clearly more for cryopreservation of red blood cells rather then for the stabilization of white blood cells.

Another method for fixing cells was the use of the following agents: diazolidinyl urea, imidazolidinyl urea, dimethylol-5, 5-dimethylhydantion, dimethylol urea, 2-bromo-2-nitropropane-1, 3-diol and quaternary adamantine, but these were for tissue fixative purposes [Granger *et al*. (2001)]. Most of the formulations used here are free of aldehydes but contain among others zinc, strontium, calcium, barium and chromium salts. It was never suggested that any of these salts have stabilizing properties although it has been described as blood diluents and lysing agents for differential determination of white blood cells. Such leucocyte preparations have not been suggested as flow cytometric preparations, possibly because they have insufficient stability and lack certain specific antigenic activity for those routine quality control procedures.

Furthermore, as another method of "cell fixation", it has been discovered that a wide range of cells can be stabilized by the addition of a compound comprising a heavy metal [(Barnett *et al.* (1995)]. If an effective amount of the heavy metal compound is added to the cell preparation, this will stabilize the cells and remain active for much longer periods than those known until now. The heavy metal compound of choice was described as chromium (III) chloride hexahydrate solution. In addition to the previous method, red blood cells stabilization was with the use of aldehyde-based chemical cross-linkers.

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Many aspects of the native function of aldehyde-based chemical cross-linkers were added and used in U.S. Patent 5651966 by Read *et al.* (1995) as their advanced method whole blood stabilization. It was demonstrated that with a mild aldehyde cross-linking, re-hydrated, lyophilized platelets have near normal ultra-structure by electro-microscopy and retain many of the surface membrane function of fresh platelets. It is however a total different scenario using aldehydes in lymphocyte fixation for immunofluorescence techniques. It was mentioned that the paraformaldehyde would result in less autofluorescence than formaldehyde [Barnett *et al.* (1999)]. As part of this chapter this will be investigated further. For quality control purposes, the entire procedure of the whole blood lysing technique requires a sample preparation which would still allow the lysing of the red blood cells. One would not want to fix the red blood cells and defraud them of their lysing capability. As described by Barnett *et al*. (1999), the methods used to fix leucocytes until then inhibited the lysing procedure, resulting in a significant increase in debris that interferes with the tests. In theory to overcome this challenge we will investigate the removal of the erythrocytes from the leucocytes and to treat them with different concentrations of various aldehydes. The stabilized leucocyte preparation can then be added back to the leucocyte depleted whole blood (red blood cells) to form a stabilized whole blood preparation. This chapter describes this challenge in more detail.

3.3 METHODOLOGY AND MATERIALS

Chapter three is divided into seven phases:

i. The first phase was choosing an optimum concentration of chromium chloride as first stabilizing agent: 0.25% chromium chloride was compared with 0.1% chromium chloride in combination with 0.35% w/v formaldehyde. (At this stage there was no paraformaldehyde available)

ii. Once the optimum concentration of chromium chloride was established, ten individual stabilized samples were investigated for reproducibility over a period of 40 days.

iii. These ten individual stabilized samples were analyzed and compared on the BD FACSCount™ and BD FACSCalibur™ flow cytometers.

iv. Thereafter the use of stabilized whole blood for calibration material was investigated. Three individual stabilized blood samples were used as calibration material for the BD FACSCount™ instrument until day 84.

v. The effects of storage temperature were investigated on stabilized whole blood samples.

vi. The use of paraformaldehyde versus formaldehyde as second stabilizing agent was investigated for even better stability. This was in combination with 0.25% chromium chloride (the first stabilizing agent).

vii. The use of polyethylene glycol as additional stabilizing agent was investigated.

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3.3.1 Blood samples

After giving informed consent, peripheral blood samples from healthy donors were collected into K_2 EDTA [purchased from Becton Dickinson (BD), South Africa] tubes following venipuncture at Synexa Life Sciences, Tygerberg Hospital. All bloods were processed within 2 hours of draw. For all practical purposes, aseptic techniques were applied throughout the process.

3.3.2 Reagents for flow cytometry

To lyse erythrocytes, an NH_4Cl-based lysing solution (Pharmingen, BD, San Diego, CA, USA) was used. **Table 3.3.2** indicates the monoclonal antibodies used, their respective fluorochromes and their manufacturer. BD TruCOUNT™ tubes (purchased from BD, South Africa) were used for single platform flow cytometric techniques. BD MultiTEST™ monoclonal antibodies were used for phenotypic cell surface staining to determine the cell absolute counts and various percentage cell populations.

Table 3.3.2: Monoclonal antibodies used for specific immunofluorescence evaluation

Monoclonal Antibody	Fluorochrome	Manufacturer
Anti-CD3	PE	Becton Dickinson
Anti-CD4	PerCP	Becton Dickinson
Anti-CD25	FITC	Becton Dickinson
Anti-HLA-DR	FITC	Pharminogen®
Anti-CD8	PerCP	Becton Dickinson
Anti-CD40Ligand/ CD154	APC	Pharminogen®
MultiTEST™		
(CD3/ CD8/ CD45/ CD4)		Becton Dickinson
BD FACSCount™ Reagent		
CD3/CD4 and CD3/CD8		Becton Dickinson

3.3.3 Flow Cytometers

Before each batch of samples was run, calibrations of the flow cytometric instruments were performed in accordance with the manufacturer's instructions. Commercial IQA samples: both CD Chex® Low (used as QC for low CD4 positive values) and CD Chex® Plus Normal (used to resemble a normal patient sample) were run daily for instrument precision and accuracy. For the acquisition and analysis of the samples, either and/or both the FACSCalibur™ (BD, South Africa) and the FACSCount™ (BD, South Africa) flow cytometers were used. The BD FACSCount™ instrument is less complicated and more affordable than the BD FACSCalibur™ flow cytometer.

Less maintenance is required with the BD FACSCount™ instruments; they are also used more often in poorer African Countries at the rural clinics: no specialized expertise is required as compared to a BD FACSCalibur™ flow cytometer. For the BD FACSCount™ instruments; the stabilized whole blood samples were stained with BD FACSCount™ Reagent (CD3/CD4 and CD3/CD8) and analyzed according to manufacturer's guidelines.

The stabilized whole blood samples were stained and analyzed only once at various test dates. The stabilized samples were analyzed by using the single platform technique with BD TruCOUNT™ tubes (please refer to Chapter 2**,** section 2.2.2 for the calculations). The cell labeling was done after stabilizing the whole blood: 50µl well mixed blood was added to 20µl MultiTEST™ (CD3/CD8/CD45/CD4) monoclonal antibody. Cells were incubated for 20 minutes and lysed thereafter by adding 450µl BD Lysing solution (NH_4Cl-based lysing solution). The samples were analyzed after 15 minutes incubation in the dark. The BD Multiset**™** software was used throughout this thesis to calculate the CD3, CD4, CD8 absolute and percentage values. Please refer to **Figure 3.1.3** for an example of the scatter plots. BD CellQuest™ software was used to calculate the neutrophil, monocyte and lymphocyte percentages throughout this thesis. This was accomplished by using the same data (SSC versus CD45-PerCP) acquired with the BD Multiset**™** software. A gate was set around all the Leucocytes (granulocytes, monocytes and lymphocytes). Three gates were drawn around the granulocyte, monocytes and lymphocytes distinctively. Calculations were done in the following manner:

$$\frac{\text{cell population (e.g. monocytes)}}{\text{total leucocyte cell population}} \times \frac{100}{1} = \text{\% cell population (e.g. monocytes)}$$

3.3.4 Heavy metal compound as first stabilizing agent

The most suitable heavy metal compounds for this assay are those with complex properties, for example transition metals like chromium [Granger *et al.* (2001)]. It is preferred that the compounds include water-soluble salts of such metals, especially inorganic acid salts such as chlorides. The chromium salts such as chromium chloride (CrCl) subgroup 3 (or $CrCl_3$) is employed and is preferably used in the form of a solution.

Two concentrations, 0.1% and 0.25% w/v chromium chloride (III) hexahydrate (purchased from Sigma Aldrich, South Africa) where investigated:

i. Solutions of 0.1% and 0.25% chromium chloride were made up separately in (PBS) [Dulbecco's PBS without calcium chloride and magnesium chloride, purchased from Sigma Aldrich, SA].

ii. The pH was adjusted between 6.5 and 7.0 and allowed to stand / age at least 2 weeks (preferably one month) before it was used . The chromium chloride can be stored and used up to six months and after which it should be discarded. This solution was stored in the fridge (2 – 8°C).

    The reason why the performance of the solution improves with ageing is not completely understood, but may be due to the formation of hydrated metal hydroxyl ionic species in the solution.

iii. The pH was adjusted again before it was used. It has been observed that the pH drops upon ageing.

iv. The chromium chloride (III) solution was filtered through a sterile 0.22 - 0.45µm filter (purchased from Millipore, South Africa) and stored in the dark (wrapped in foil) until use. The formation of a precipitate will lower the concentration of the heavy metal ions in the solution, and it is preferred that the concentration of the first stabilizing agent is taken into account once the precipitate has formed: the final concentration of the chromium chloride should be less than 1% w/v. (The preferred concentration is 0.25% w/v in PBS).

v. Samples of blood drawn from the same individuals were treated with either the 0.1% or the 0.25% (w/v) in order to determine the effects of the concentration on the sample integrity.

The leucocytes were exposed to the chromium chloride for a period of approximately one hour at temperatures 2 to 6°C. Barnett *et al.* (1999) and Granger *et al*. (2001) stated that the presence of only the first stabilizing agent can indeed prevent the leucocytes from exhibiting excessive autofluorescence and at the same time can stabilize the leucocytes for periods of longer than 25 days. The mechanism of action and influence of the chromium chloride on blood cells is not an aim of this thesis and was therefore not investigated.

3.3.5 Paraformaldehyde as second stabilizing agent

Paraformaldehyde is used because it is an alcohol-free and excellent cellular fixative component (cells are fixed without destroying the cell's cellular properties such as surface markers), whereas agents such as methanol and other alcohols destroy the characteristics of cell surfaces. The cementing mechanism of action according to these alcohol-free compounds may vary, and includes reaction of free amines, reaction with lipids, or cross-linking of the cell proteins. It was said by Barnett *et al.* (1999) that the fixation of normal leucocytes, using compounds such as paraformaldehyde, although giving stability for 5-7 days, increases their autofluorescence. This unfortunately makes the preparation inappropriate for the use as long-term quality control samples. Paraformaldehyde still gives rise to autofluorescence but much less than formaldehyde. When making the working solution for paraformaldehyde it is preferred to keep the temperature below 60°C, in order to avoid the reversion of paraformaldehyde to formaldehyde and the formation of formic acids. The formic acid is extremely toxic to human cells and will completely distort the cell membranes. The method followed in this thesis is as follows: Paraformaldehyde was purchased in a powder form from Merck, (South Africa).

i. A 3.5% w/v stock solution of paraformaldehyde was made up in PBS. The paraformaldehyde was heated until fully dissolved.

ii. This stock solution was stored in the fridge (2 – 8°C) and allowed to age (preferably more than one week, but less than two weeks). The pH was adjusted (pH 6.5 - 7.2) before use.

iii. Paraformaldehyde was used in combination with chromium chloride. To prepare a working solution one volume of the 3.5% paraformaldehyde stock solution was mixed with 9 volumes of 0.25% (or 0.1%) chromium chloride solution to give a final concentration of 0.35% paraformaldehyde and 0.25% (x 0.9) chromium chloride.

iv. This mixture was stored in the dark at 2 – 8°C until use.

v. The paraformaldehyde / chromium chloride solution mixture was incubated with the cells for between 2 hours and up to 24 hours at 2 to 6°C.

vi. The time interval between the treatments with the first and the second stabilizing agent is preferably at least 30 min, more preferably at least one hour. In United States Patent 5858699 [Granger *et al.* (1999)] mentioned that the intervals between step one and two should be 12 - 24 hours.

3.3.6 Polyethylene glycol as additional stabilizing agent

Polyethylene glycol (PEG) decreases the surface potential of the lipid monolayers that facilitate the transport of components across cell membranes. PEG is a compound that may make the cell surface more permeable to low molecular weight compounds and facilitates entry of such compounds into the cell cytoplasm and at the same time prevents the cell from swelling [Connelly *et al.* (1995)]. The previously described fixative components can enter rapidly into the cell and fix the content of the cell before they spill out in any substantial amount of cellular content. It has been described that PEG protects biologically active polypeptides from inactivation or denaturation [Davis *et al.* (1979)] and is a well known polymer having advantageous properties such as its solubility in water and in many organic solvents and the lack of immunogenicity.

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Connelly *et al.* (1995) described the use of PEG as a compound that facilitated the transport of components across the cell membrane and decreased the surface potential of the lipid monolayers. PEG renders the cellular membrane more permeable to low molecular weight compounds, allowing them to enter the cytoplasm and in the same time it prevents the cells from swelling. Connelly *et al.* (1995) have used PEG in combination with dimethylsulfoxide (DMSO) and preferred concentrations ranging from 1 – 20% (w/v). In this thesis, we investigated different concentrations of PEG in combination with chromium chloride hexahydrate to see if we could stabilize the whole blood cell suspension for prolonged periods.

Polyethylene glycol (PEG, 4000) was purchased from Fluka BioChemika (South Africa) and was used at various concentrations:

i. 0% (where no PEG was added), 1%, 2% and 3% (w/v).
ii. These concentrations were added to the PBS.
iii. These solutions of PBS-PEG were used for the washing steps. They also served as a buffer for the various samples.

3.3.7 Formaldehyde versus paraformaldehyde for blood stabilization

i. Fresh 37% formaldehyde solution was purchased from Merck, (South Africa).

ii. This was diluted to yield a 0.35% solution using the 0.25% aged, filtered chromium chloride.

iii. The formaldehyde solution was stored in the dark at 2 - 8°C until use.

iv. A fresh working solution of formaldehyde / chromium chloride solution was compared to a working solution of paraformaldehyde / chromium chloride solution in three individual (n=3) stabilized whole blood samples.

3.3.8 Preparation of stabilized whole blood

i. After consent had been obtained, donor peripheral blood samples were collected from healthy individuals, into disodium ethylene-di-amine-tetra-acetic acid (K_2 EDTA) tubes.

ii. All procedures were conducted using aseptic techniques.

iii. Bloods were centrifuged at 650 g for 20 minutes at 4ºC and the plasma was removed and placed in a sterile tube and stored in the fridge (2 – 8ºC).

iv. The cells were washed twice (centrifuge cells at approximately 800 g for 20 minutes) using sterile PBS.

v. Two concentrations of chromium chloride solution were compared: one batch was put up with the 0.1% concentration and the other with the 0.25% solution: 3ml of the filtered working solution was added for every 5 ml of whole blood.

vi. The chromium chloride-treated cells were incubated for one hour in the dark at 4ºC on a roller mixer. Mixing is very important, the stirring of the cells prevent clumping of cells (cross-linking between individual cells).

vii. The cells were then centrifuged at 650 g for 20 minutes (at 4ºC).

viii. After gently removing the supernatant, the cells were re-suspended in the second stabilizing agent: a 0.35% w/v aldehyde (the first phase was formaldehyde and in a further phase it was paraformaldehyde) in combination with chromium chloride.

ix. The cells added to the second stabilizing agent were incubated at 2 - 8ºC in the dark for 16 - 22 hours on the roller mixer.

x. The next morning, the cells were centrifuged (650 g for 20 minutes at 4ºC), the supernatant was removed and the cells were washed twice using sterile PBS.

xi. The final supernatant was removed carefully and the cells were resuspended in their own donor plasma (or in heat-inactivated human AB sera purchased from the Tygerberg Blood Bank). The final stabilized bloods were stored in the fridge between 2 and 4ºC in the dark until use.

xii. As additional feature it was found that at the washing steps, one could deplete any sample of some of its lymphocytes, by taking off the supernatant and including some of the lymphocyte layer. These lymphocytes were then discarded. It was not investigated in detail and no statistics or raw data was included for this chapter. However it was found useful to utilize this stabilized blood sample for a quality control sample having lower cell absolute values.

3.3.9 Statistics Software

All statistical analyses were conducted using the GraphPad Prism 2.1 and GraphPad Prism 5.1 software. For the nonparametric test where we compared more that three paired groups, the One-way ANOVA Friedman test was used. The Friedman test first ranks the values in each matched set (each row) from high to low. Each row represents one of ten stabilized whole blood samples and is ranked separately. It then sums the ranks in each group (column) representing the various days the same ten blood samples were analyzed. If the sums are very different, the P value will be small. The whole point of using a matched test is to control for experimental variability between parameters (the days in this case), thus increasing the power of the test. Since the Friedman test ranks the values in each row, it is not affected by sources of variability that equally affect all values in a row. If the results obtained on each day are similar (indication of stability), the p values are non significant ($P > 0.05$). Significant changes in the results where $P < 0.05$ would indicate the instability of the samples [GraphPad.com Software Inc. (1999)].

The Levey Jennings chart was used in a number of experiments to visualize the stability of various whole blood samples. On the x-axis, the time the samples were analyzed was plotted. A Levey Jennings chart is a graph that quality control data is plotted on to give a visual indication whether a laboratory test is working well. On the y-axis, a mark was made indicating stability (how far off the actual result was from the mean). The distance from the mean is measured in standard deviations (SD). A line run across the graph at the mean, as well as one, two and sometimes three standard deviations either side of the mean [Guenet *et al.* (2006)]. This make it easy to see how for off the result was.

For this thesis, we took 2SD (for the percentage) and 3SD (for the absolute) values on either side of the mean as acceptable ranges / limits for the prepared IQA samples. These standard deviations or limits were not always indicated on all the graphs (especially Chapter 4).

The spearman correlation was used to compare the results obtained from the BD FACSCount™ (on the y-axis) to those obtained from the BD FACSCalibur™ (on the x-axis) flow cytometers. Correlation calculations do not discriminate between X and Y, but rather quantify the relationship between the two variables.

3.4 RESULTS

3.4.1 Determination of the optimal chromium chloride concentration as primary stabilizing agent

Blood samples from five individuals obtained on day 0 were divided into two groups: each group was prepared separately using either the 0.1% or 0.25% chromium chloride solutions (in combination with 0.35% w/v formaldehyde). Samples were kept at 4ºC and an aliquot were stained once a day (at random days) for various routine immunological phenotypic cell surface markers. The samples were analyzed from day 1 up to day 26 to validate the optimum concentration of chromium chloride solution needed to stabilize whole blood. In **Figure 3.4.1a** the two Levey Jennings plots illustrate a representative example of one of the stabilized whole blood samples. Statistical analysis of the data generated was conducted using the One-way ANOVA Friedman test. No SD bars were indicated in this figure due to fact that it is only one sample of the five stabilized preparations used as an example. This figure illustrates neutrophil, monocyte and lymphocyte percentages obtained with a 0.1% (top) and 0.25% (bottom) chromium chloride solution used as first stabilizing agent. Stability was obtained for both concentrations up to day 26. No statistical significant differences were measured for any of the parameters illustrated ($p > 0.05$), indicating that both concentrations induced stability over this period of analysis.

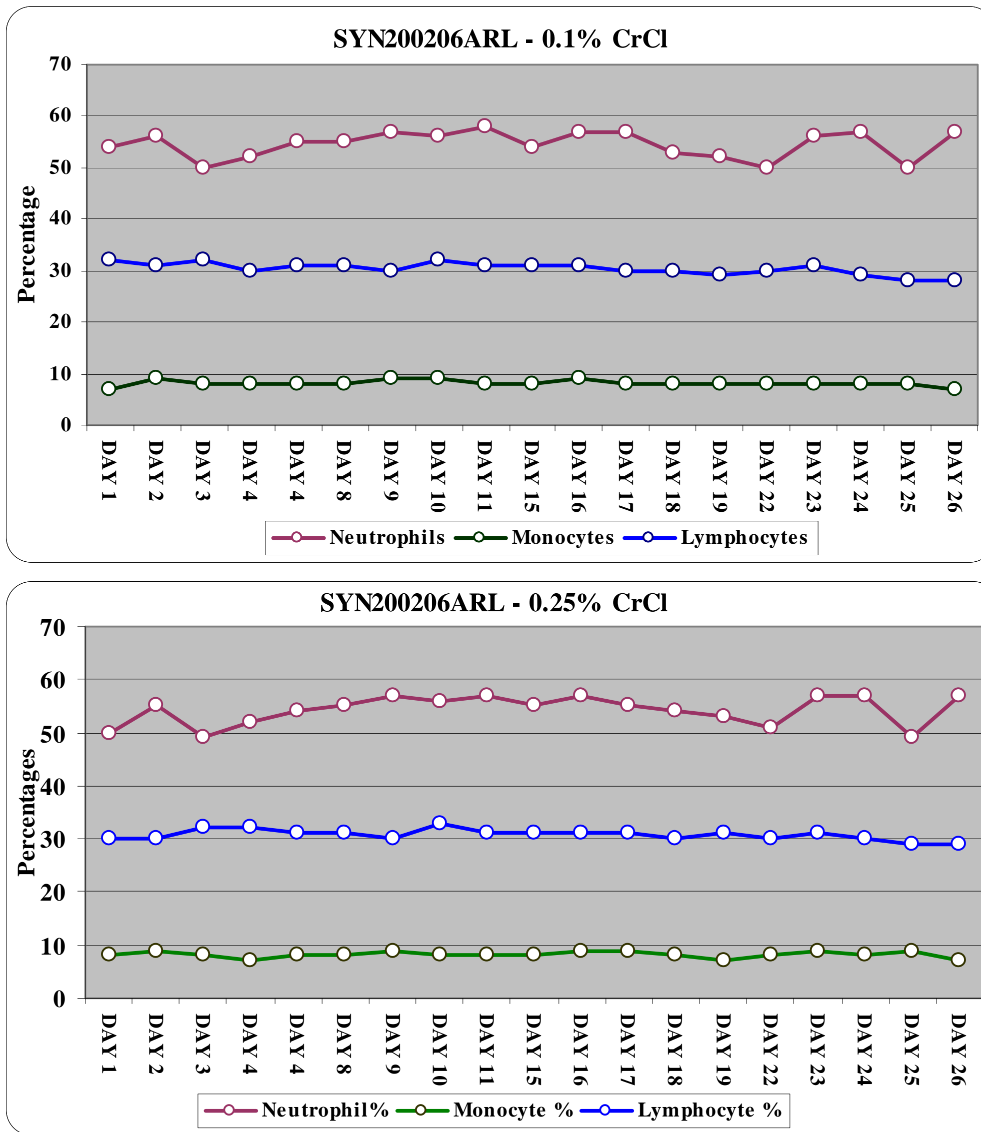

Figure 3.4.1a: Two Levey Jennings Plots illustrating only one example with one part stabilized whole blood with 0.25% and one part with 0.1% chromium chloride solution (in combination with formaldehyde). The upper graph indicates the results using a 0.1% chromium chloride concentration and the bottom graph indicates the results using a 0.25% chromium chloride concentration preparation. For both concentrations the percentages of neutrophils, monocytes and lymphocyte cell populations are plotted over a period of 26 days.

In **Figure 3.4.1b,** the same example of stabilized whole blood sample where the absolute values for the following phenotypic surface markers of lymphocytes CD3, CD4 and CD8 is shown. No SD bars were indicated in this figure as this is only one stabilized sample example (of n=5). The top illustrates the absolute values for the 0.1% and the bottom illustrates the 0.25% chromium chloride analysis. Once again, no statistical significant differences ($p > 0.05$) were measured indicating total stability of the samples expressing these phenotypic markers. However, after scrutiny of the scatter dotplot (data not shown) on the flow cytometer, it was concluded that the 0.25% concentration of chromium chloride yielded the better long term option: the scatter properties of the cells remained clearer with no overt changes over time. This concentration of chromium chloride was subsequently used throughout following experimentation.

Please refer to **appendix 6** (for one example of stabilized blood run on day 4) and **appendix 5** (for the same stabilized sample in appendix 6 that was analyzed on day 77) to visualize the data plots obtained from one sample stabilized with 0.25% chromium chloride concentration in combination with 0.35% w/v formaldehyde as secondary stabilizing agent.

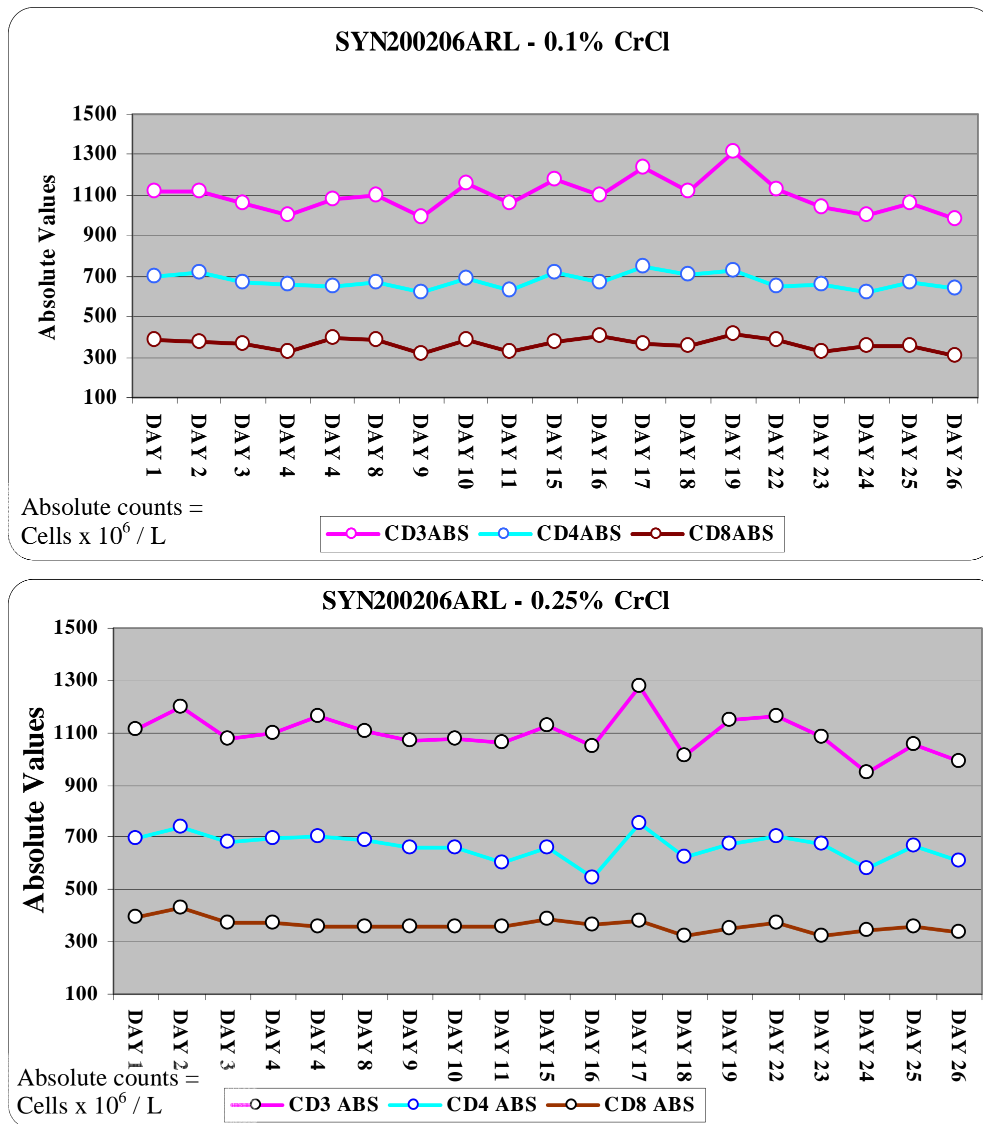

Figure 3.4.1b: Two Levey Jennings Plots illustrating only one example of stabilized whole blood with two concentrations of chromium chloride solutions. The upper graph indicates the results using a 0.1% chromium chloride concentration while the bottom graph indicates the results using the 0.25% chromium chloride concentration preparation. For both concentrations the absolute values for CD3, CD4 and CD8 cell populations is plotted over a period of 26 days. ABS = absolute cell values (cells x 10^6 / L). No SD values were indicated as this is one example of stabilized whole blood.

3.4.2 Stability of the whole blood preparation

Once we had determined the optimal concentration of chromium chloride to use, we investigated the reproducibility of this method to yield stabilized samples. Ten healthy individuals donated blood and each sample was stabilized as described above (i.e. using 0.25% chromium chloride in combination with 0.35% formaldehyde). The samples were monitored for the various routine immunological phenotypic cell surface markers from day 3 up to day 40 to validate the stability of the cell surfaces. An aliquot of each sample was stained and analyzed once a day (at random days) on two BD Flow cytometers. Unfortunately the sample volumes were depleted by day 40 for some of the stabilized samples; hence no further monitoring could take place for these ten individuals post day 40 and statistics performed only until day 40. Stability was obtained for more than six weeks before the scatter dotplots started to change: cellular debris increased and populations started to merge with each other, indicating instability from that time point onwards. [Please refer to **appendix 1** which illustrates a Levey Jennings graph of only one example stabilized sample (SYN030507B) analyzed until day 77].

Statistical analysis of the data generated was conducted as above using the One-way ANOVA Friedman test. **Table 3.4.2a** indicates the mean values of the ten individuals for the various phenotypic cell surface markers for the time period day 3 until day 40. The bulk of the surface markers are used for routine HIV monitoring. Significance was set at $p < 0.05$ if the results varied day to day compared to the baseline measurement done on day 3. It is evident from this data that the routine phenotypes measured did not show any significant changes over the 40 day period: however, significant changes were observed in the neutrophil and lymphocyte populations.

Table 3.4.2a: Mean values of various cell population and sub-populations of lymphocytes over time determined in stabilized whole blood samples (n = 10). Significant p values ($p < 0.01$) are highlighted in yellow and indicated =☆☆. Significant p values ($p < 0.001$) are highlighted in yellow and indicated =☆☆☆. Non-significance ($p > 0.05$).

MEAN	**DAY 3**	**DAY 10**	**DAY 13**	**DAY 18**	**DAY 24**	**DAY 32**	**DAY 40**
CD3 %	73	72	74	71	72	74	73
CD4 %	45	43	45	44	44	44	45
CD8 %	25	26	25	24	25	24	24
CD3 ABS	709	659	722	698	659	658	676
CD4 ABS	417	379	415	401	381	378	394
CD8 ABS	268	254	265	264	238	229	242
NEUT %	62	☆☆ **65**	☆☆ **64**	63	64	63	☆☆ **65**
MONO %	7	6	6	7	6	7	7
LYMPHO %	24	23	24	23	23	☆☆☆ **22**	☆☆☆ **21**

ABS = absolute cell values (cells x 10^6 / L), NEUT % = percentage neutrophils, MONO % = percentage monocytes, LYMPHO % = percentage lymphocytes

Although the neutrophil, monocyte and lymphocyte populations are not part of routine HIV monitoring, it was interesting to include this data. **Figure 3.4.2a** illustrates a Levey Jennings Plot to monitor stability of these populations: the stability of the mean values of the ten stabilized whole blood samples were recorded over a period of 40 days. The upper and lower limits for all the parameters are included in this figure. Unfortunately it is not that clear on this graph, but statistical data indicated the significant changes started to occur over time in the neutrophil and lymphocyte populations, especially closer to day 40. The neutrophil mean values indicated significant changes ($p < 0.01$) on day 10, day 13 and day 40. The lymphocyte cell population indicated a significant change ($p < 0.001$) for both day 32 and day 40.

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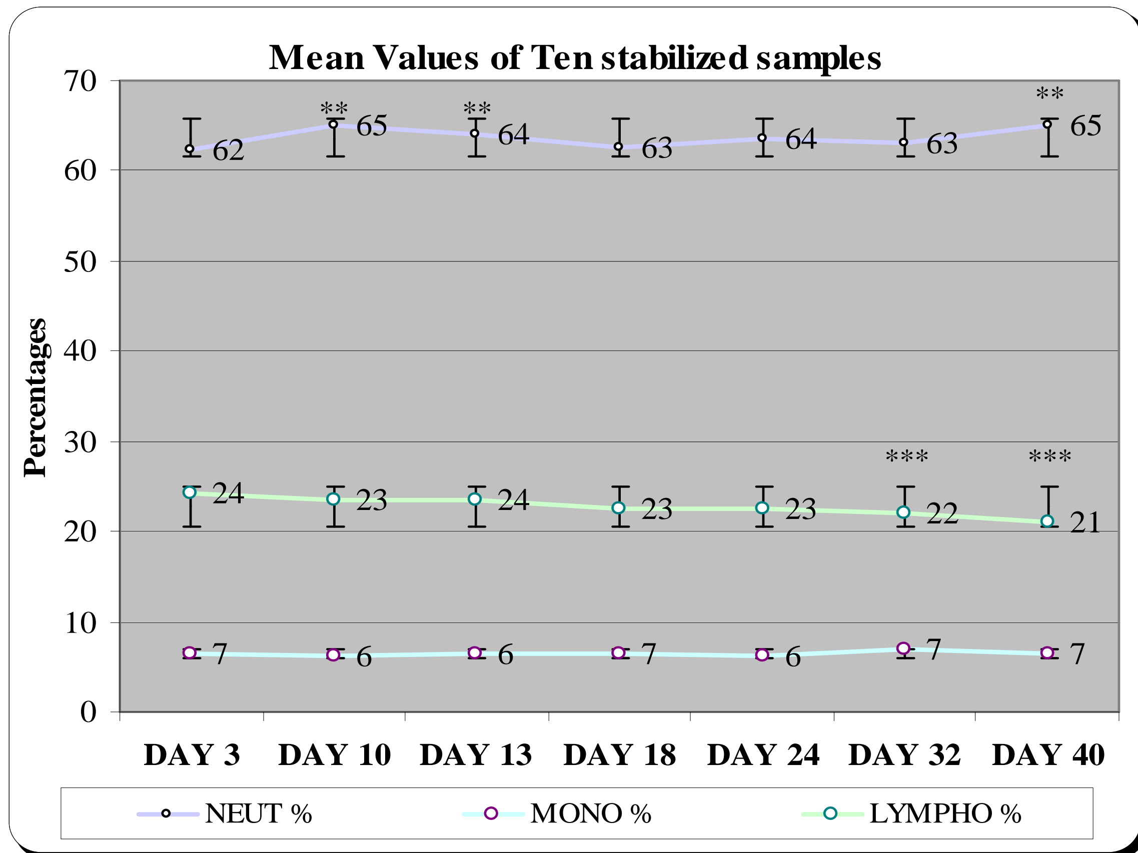

Figure 3.4.2a illustrates the Levey Jennings Plot to monitor stability of leucocyte subsets. This is the mean values obtained from ten stabilized whole blood samples indicating the stability of monocytes, lymphocytes and neutrophil percentages (including 2SD bars). The neutrophil cell population showed significant changes ($p < 0.01$) on day 10, day 13 and day 40 (this is indicated with two stars) and similarly, the lymphocyte cell population indicated a significant change ($p < 0.001$) for both day 32 and day 40 and indicated with three stars.

In the next two figures, **Figures 3.4.2b 3.4.2c,** the Levey Jennings plots illustrates stability of the ten samples mention above expressing routine CD3, CD4 and CD8 phenotypic markers. Both the percentages (including 2SD) and absolute values (including 3SD) for these significant phenotypic cell surface markers are indicated. The 3SD bars are an indication of upper and lower limits as described previously. **Figure 3.4.2b** illustrates the mean values of the ten samples recording the percentages for CD3%, CD4% and CD8% from day 3 up to day 40. All p-values > 0.05 and perfect stability was achieved up to day 40. On the graph error bar represents the 2SD over the mean and it is very clear that all values measured were within the 2SD range. Similarly, the mean absolute values of these sub-populations of lymphocytes are illustrated in **Figure 3.4.2c.** On the graph error bar represents the 3SD over the mean and it is very clear that all absolute parameters measured (CD3, CD4 and CD8 absolute values) fall within the 3SD range. Once again, with ONE-way ANOVA, Friedman statistics done on the ten stabilized samples indicated $p > 0.05$ for the entire 40 day period, therefore indicating reproducible stability.

To visualize the dataplots as it were obtained from the two cytometers, please refer to **appendix 2, 5 and 6. Appendix 2** illustrates one example of the stabilized whole blood analyzed on the BD FACSCount™. **Appendix 6** illustrates an example of stabilized whole blood analyzed on day 4 with the BD Multiset™ software on the BD FACSCalibur™. **Appendix 5** illustrated the same sample as analyzed on day 77; and illustrates the scatter plot as it was obtained on the BD FACSCalibur™.

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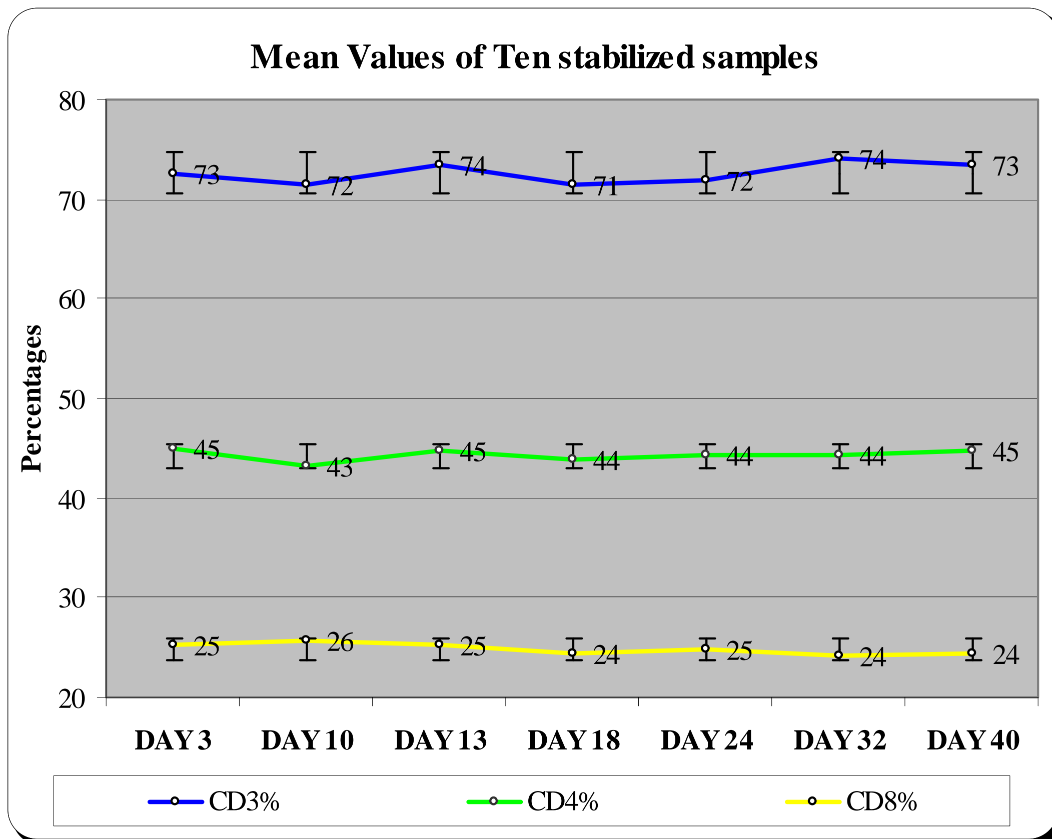

Figure 3.4.2b illustrates the Levey Jennings Plot to monitor stability of T cell subsets for six weeks. This is the mean values of ten stabilized samples indicating stability of CD3%, CD4% and CD8% starting at day 3 until day 40. These percentages resulted with $P > 0.05$ indicated no significance even until day 40 using the Prism 2.1 ONE-way ANOVA, Friedman statistics. For this thesis purpose for percentage values, the 2SD range limit was the measurement of stability within which the samples should fall.

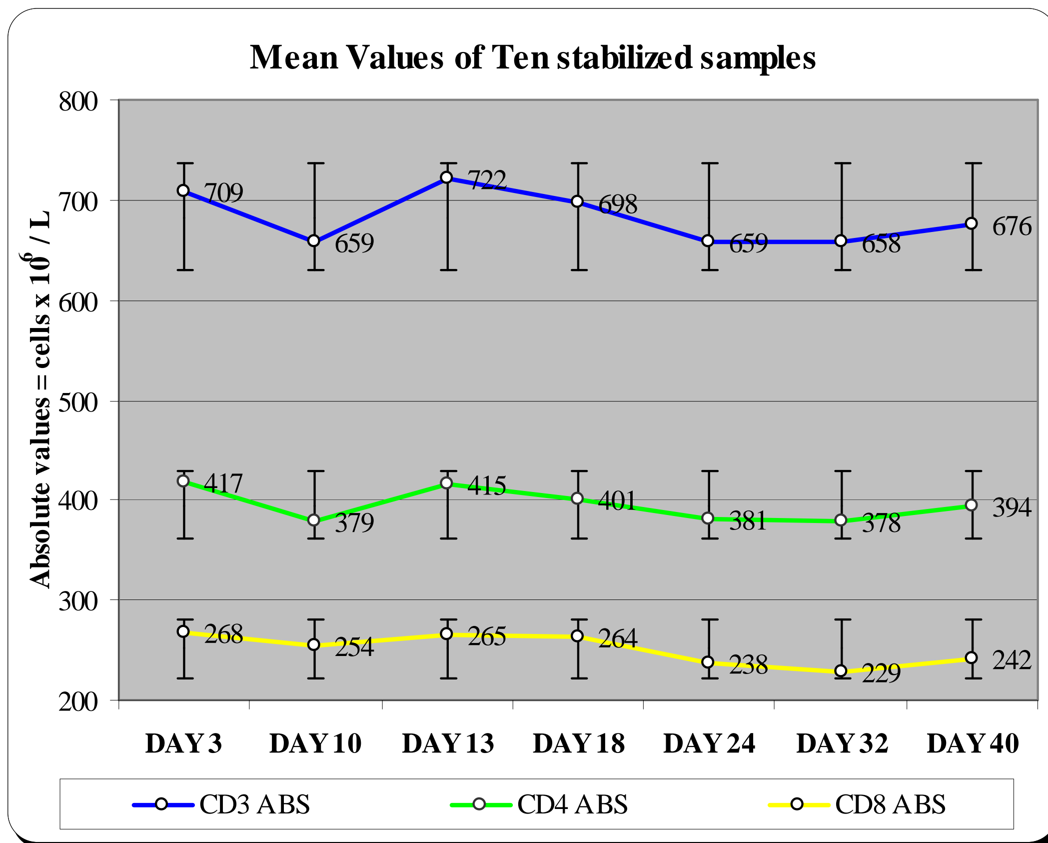

Figure 3.4.2c illustrates the Levey Jennings Plot to monitor stability over 40 days. This plot demonstrates the stability of the absolute values for CD3, CD4 and CD8. CD3, CD8 and CD4 absolute values with $P > 0.05$ indicated no significance even until day 40 using the Prism 2.1 ONE-way ANOVA, Friedman statistics. All the results obtained fall within the 3SD upper and lower limit as illustrated in this figure. ABS = absolute cell values (cells x 10^6 / L).

Taking a closer look on an individual level at these ten stabilized samples (mentioned above); mean values ± 2SD within each sample are illustrated in **Table 3.4.2b**. The mean values for each parameter were calculated over time (day 3 until day 40) and the SD was calculated for each sample individually. In brackets, the CV% values varied from 1 - 17%. The highest CV (17%) was found in the CD8 absolute values of sample 2 and sample 3. On average the CV for all these parameters measured was 7%.

Table 3.4.2b illustrates the mean values ± 2SD obtained for ten individual stabilized (0.25% chromium chloride in combination with 0.35% w/v formaldehyde) IQA samples. An aliquot of these samples were analyzed at 7 time point (once per day) from day 3 until day 40. Major phenotypic markers such as CD3, CD4, CD8 percentages and absolute values were indicated as well as leucocyte subsets such as neutrophil, monocyte and lymphocyte percentages. In brackets, the CV% is indicated and varies from 1 – 17% and average of 7%.

SAMPLE	% Lymphs	% Mono	% Neutro	CD3 %	CD4 %	CD8 %	CD3 ABS	CD4 ABS	CD8 ABS
1	17±3 (8)	7±1 (7)	67±4 (3)	62±5 (4)	36±3 (4)	24±4 (8)	504±127 (13)	287±78 (14)	197±45 (11)
2	24±3 (6)	6±1(10)	62±14 (11)	59±5 (4)	31±5 (7)	23±3 (6)	760±165 (11)	398±76 (10)	297±99 (17)
3	40±5 (7)	8±1 (7)	44±5 (5)	62±7 (6)	32±3 (5)	27±3 (6)	893±219 (12)	438±129 (15)	393±131 (17)
4	19±2 (4)	6±1 (9)	65±3 (2)	83±9 (5)	54±5 (4)	26±2 (3)	694±114 (8)	453±82 (9)	217±36 (8)
5	11±1 (6)	3±1 (15)	81±2 (1)	76±3 (2)	52±3 (3)	20±2 (5)	315±49 (8)	217±33 (8)	82±15 (9)
6	15±1 (5)	7±1 (8)	73±1 (1)	83±7 (4)	66±4 (2)	16±2 (3)	422±35 (4)	335±22 (3)	79±4 (3)
7	15±1 (5)	6±1 (9)	73±1 (1)	76±7 (5)	49±4 (4)	24±2 (4)	400±58 (7)	256±27 (5)	126±19 (8)
8	26±4 (8)	8±1 (7)	59±2 (2)	64±5 (4)	39±3 (4)	23±2 (5)	529±79 (7)	319±47 (7)	185±34 (9)
9	24±2 (4)	6±1 (8)	61±14 (12)	84±7 (4)	39±5 (6)	42±2 (2)	1116±255 (11)	520±99 (9)	558±144 (13)
10	40±4 (5)	8±1 (4)	45±3 (4)	74±6 (4)	44±3 (3)	25±3 (5)	1303±225 (9)	785±157 (10)	439±83 (9)

ABS = absolute cell values (cells x 10^6 / L), NEUT % = percentage neutrophils, MONO % = percentage monocytes, LYMPHO % = percentage lymphocytes

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3.4.3 Comparison of the stabilized blood reference control between the BD FACSCount™ and the BD FACSCalibur™

Blood of ten individuals were stabilized with 0.25% chromium chloride and 0.35% w/v formaldehyde. The aim of this experiment was to see if the cell populations in the stabilized whole blood could be identified and quantified on BD FACSCount™ instruments and then to compare the absolute results with the BD FACSCalibur™. The BD FACSCount™ instruments are less complicated and are usually the technology platform used in the poorer countries since it does not require the expertise and training required which would be necessary in order to operate a flow cytometer. The BD FACSCount™ is a closed cytometer ultimately developed for the routine monitoring of CD4 cell counts in HIV-infected patients. For the FACSCalibur, the MultiSet™ software were used in order to obtain the absolute values. Significant Spearman R^2 correlation values were obtained for both CD4 and CD8 absolute counts as determined between the two instruments: R^2 = 0.9848 for the CD4 cells a R^2 value = 0.9636 was obtained for the CD8 absolute count (**Fig 3.4.3a** and **Fig 3.4.3b** respectively). To visualize the dataplots as it were obtained from the two cytometers, please refer to **appendix 2** for an example of the stabilized whole blood analyzed on the BD FACSCount™ – this sample was set up and acquired on day 77. **Appendix 5** illustrated the same sample but with the scatter plot of day 77 as it was obtained on the BD FACSCalibur™.

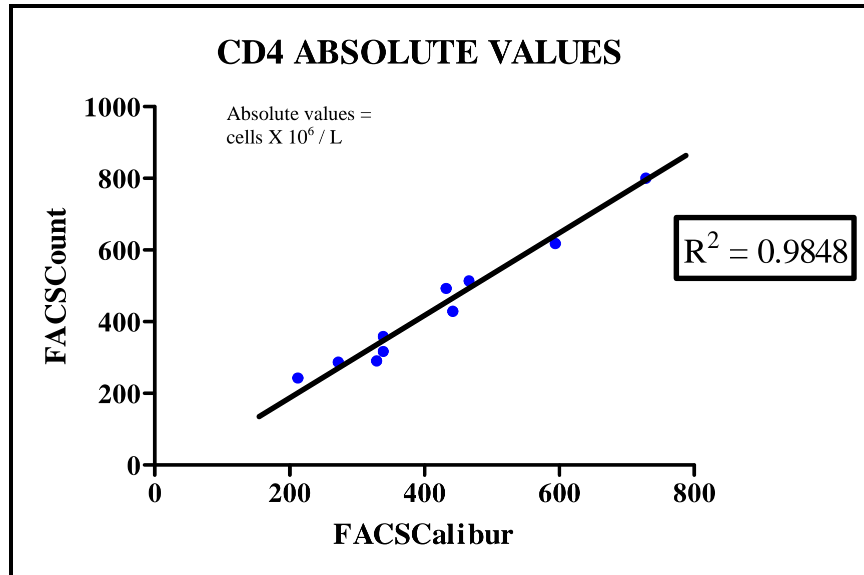

Fig 3.4.3a: Spearman correlation of stabilized whole blood (n=10) analyzed with FACSCalibur™ (x-axis) and the FACSCount™ (y-axis); R^2 = 0.9848. Absolute cell values are calculated as cells x 10^6 / L.

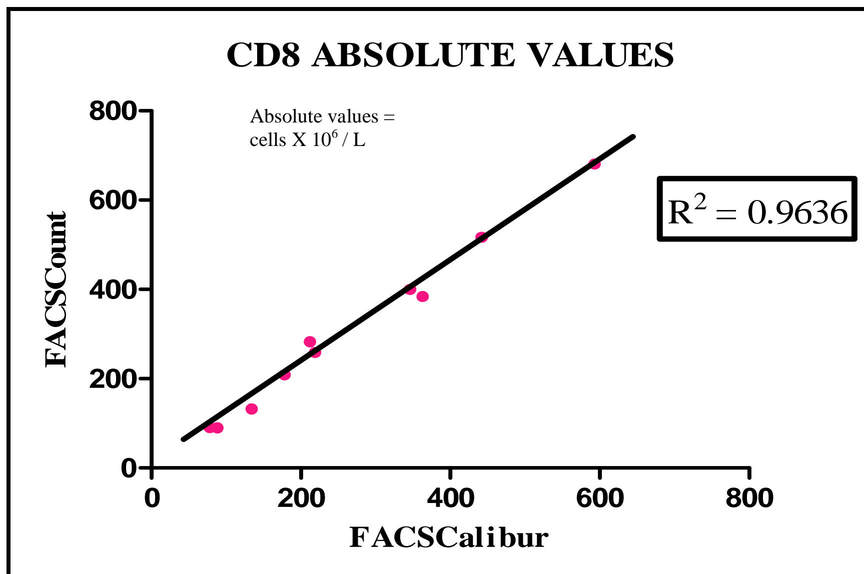

Fig 3.4.3b: Spearman correlation of stabilized whole blood (n=10) analyzed with FACSCalibur™ (x-axis) and the FACSCount™ (y-axis); R^2 = 0.9636. Absolute cell values are calculated as cells x 10^6 / L.

3.4.4 Stability of whole blood for BD FACSCount™ calibration

Based on the above data, it was decided to determine whether the stabilized samples could be used for the calibration of the BD FACSCount™ which is a pre-requisite as a daily routine before these instruments can be used. For this, blood samples were stabilized as above (0.25% chromium chloride as first stabilizing agent and 0.35% w/v formaldehyde as secondary stabilizing agent) from three healthy individuals and used daily over an extended period of time: to our surprise, the calibration passed without any error reporting from the instrument on day 40, day 52, day 59 and up to **day 84.**

3.4.5 Effects of storage temperatures on the stability of the Samples

Blood was collected from one volunteer and was stabilized in a similar manner (0.25% chromium chloride as first stabilizing agent and 0.35% w/v formaldehyde as secondary stabilizing agent): this sample was subsequently divided into three separate sub-samples: each sub-sample was monitored after storage at a different temperature (4ºC, 30ºC and at room temperature) for 22 days. An aliquot of each temperature sample was stained and monitored once a day (on random days). The results were compared so as to establish the temperature at which the stabilized whole blood should be transported and stored. The samples were monitored for CD3, CD4, CD8 percentages and absolute values as well as the neutrophil, monocyte and lymphocyte percentages. **Figure 3.4.5a**, **Figure 3.4.5b** and **Figure 3.4.5c** are Levey Jennings plots indicating the stability of the IQA samples stored at room temperature, 4ºC and 30ºC.

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The parameters measured for each of these samples are the CD3, CD4 and CD8 absolute values. For **Figure 3.4.5b** (samples stored at 4ºC) the 3SD bars were not included as this is only one stabilized sample. The 3SD bars were also not indicated for the room temperature or 30ºC storage temperatures, this was only one sample for both storage temperatures. And no statistics was done due to the fact that these were only one sample. The Levey Jennings plots for the percentage values were very similar to these plots and therefore data was not shown. When comparing these three illustrated plots, the sample stored at 4ºC is clearly the preferred storage and transport temperature for the stabilized samples. The higher temperature influence the IQA sample in such a way that stability was not obtained at all (please refer to **Figure 3.4.5c**).

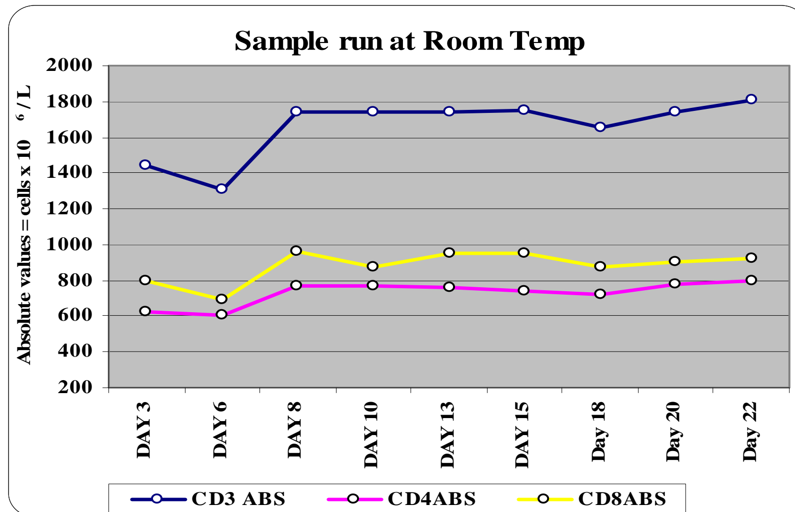

Figure 3.4.5a illustrates the one example of stabilized sample stored at room temperature and analyzed once on random days for 22 days. The absolute values for CD3, CD8 and CD4 were recorded on the Levey Jenning plot. A slight increase in absolute values was observed at day 8 with stability of the sample thereafter.

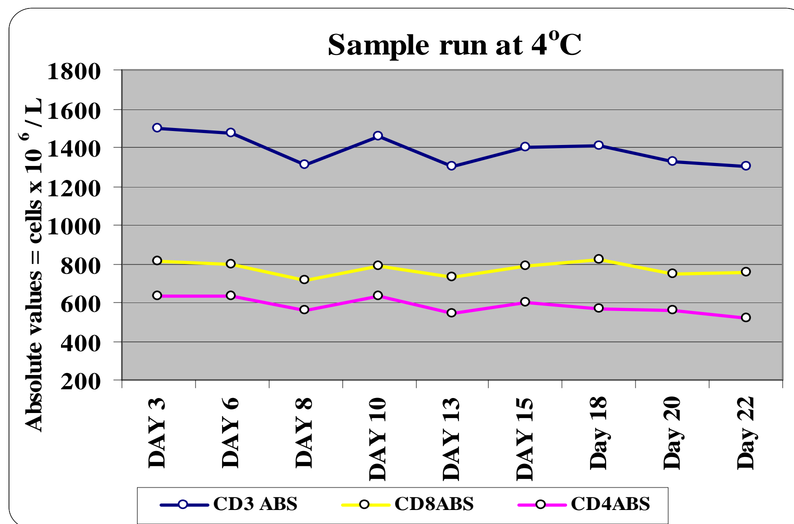

Figure 3.4.5b illustrates only one example of the stabilized sample stored at 4ºC and analyzed once on random days for 22 days. The absolute values for CD3, CD4 and CD8 were recorded on the same Levey Jenning plot. The SD bars was not indicated as this is only one sample. However stability can be observed of this sample stored for 22 days at 4ºC.

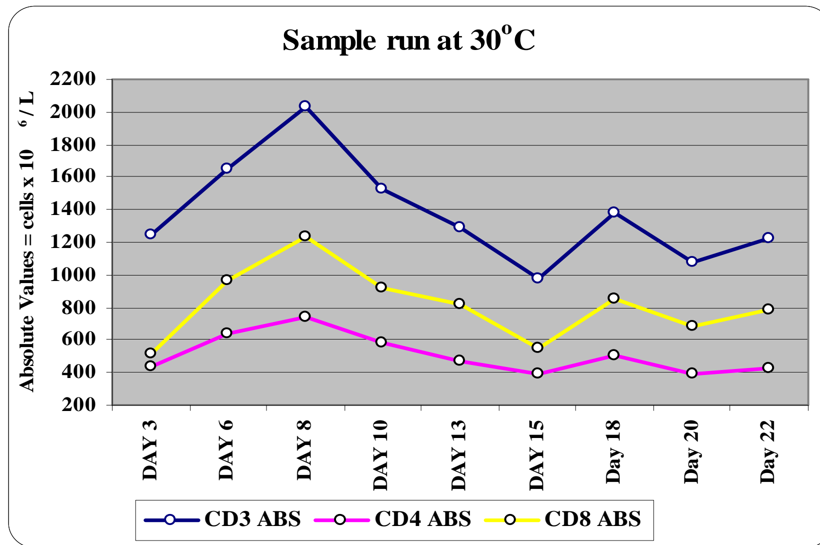

Figure 3.4.5c illustrates one example of stabilized whole blood sample stored at 30ºC and analyzed once on random days for 22 days. The absolute values for CD3, CD8 and CD4 were recorded on the Levey Jenning plot. The higher temperatures clearly influence the stability of these IQA samples early on in this experiment, the plots fall all over the graph. No SD bars were indicated as this is only one sample.

3.4.6 Comparison of the secondary stabilization step using paraformaldehyde versus formaldehyde

For this comparison between paraformaldehyde versus formaldehyde, blood was collected from 2 volunteers and on day 0, each sample was divided into two sub-samples: one sub-sample of each individual was stabilized as described previously using the 0.25% chromium chloride (as primary stabilizing agent) and 0.35% w/v paraformaldehyde (in combination with 0.25% chromium chloride) was used as a secondary agent. The other two sub-samples were stabilized using 0.25% chromium chloride as primary stabilizing agent and freshly purchased 0.35% w/v formaldehyde (in combination with 0.25% chromium chloride) as secondary stabilizing agent. The samples were monitored for the various routine immunological phenotypic cell surface markers from day 2 up to day 37 to validate which aldehyde (in combination with 0.25% chromium chloride) will be the better as a secondary stabilizing agent. These four samples were analyzed only once a day at random days. Statistical analysis was performed on these two samples using the One-way ANOVA Friedman test comparing the data of the paraformaldehyde (and likewise the formaldehyde) from day 2 until day 37 (including day 37). Non-significant changes in the parameters ($p > 0.05$) were measured for both the absolute and percentage values. The same was found for the two samples that were stabilized using freshly made up formaldehyde. A Levey Jennings graph illustrates the absolute values measured in one of these samples: **Figure 3.4.6a**. In the top graph paraformaldehyde was used and in the bottom graph formaldehyde was used as secondary stabilizing agent (in combination with chromium chloride). **Figure 3.4.6a** illustrates only the one example comparing the paraformaldehyde preparation versus the formaldehyde preparation and therefore no SD bars were indicated on these graphs.

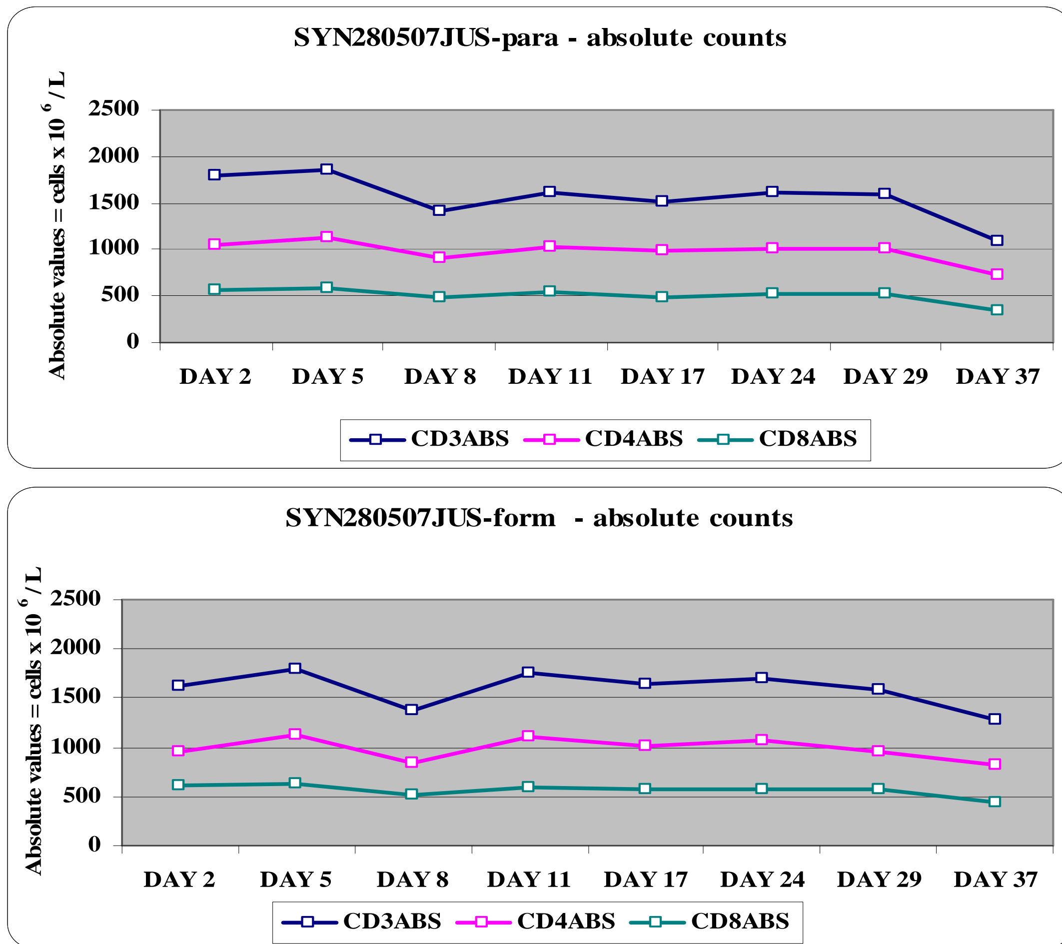

Figure 3.4.6a illustrates two Levey Jennings Plots to monitor stability of phenotypic markers: this is only one example of whole blood sample treated with paraformaldehyde (para, top figure) and one example stabilized sample treated with formaldehyde (form, bottom figure) as second stabilizing compound. One-way ANOVA Friedman test comparing the samples (n=2) indicated non-significant variations ($p > 0.05$) were obtained for both aldehyde preparations until day 37. Absolute cell values on the y-axis represent cells x 10^6 / L. The percentage data is not shown.

The following **Figure 3.4.6b** was the scatter plot on the BD FACSCalibur that illustrates the difference in formaldehyde versus paraformaldehyde preparation. This figure is an example of the two preparations analyzed on day 37. The diverse cell populations are still clearly distinguishable in both preparations. In the formaldehyde scatter plot (Right) the cell populations were separated and could easily be distinct from one another. The plot demonstrating the paraformaldehyde preparation (left), appear to be similar to the formaldehyde data plot. When looking at the debris gated area, it was evident that there was a slight increase in the debris in the formaldehyde preparation at this stage as the granulocytes started loosing their granules. As the samples are aged the debris increased in the formaldehyde preparation faster than in the paraformaldehyde preparations. It was therefore concluded that the paraformaldehyde preparation was the more stable preparation of the two. However, formaldehyde is not less of an option to use as second stabilizing agent should paraformaldehyde not be available for use.

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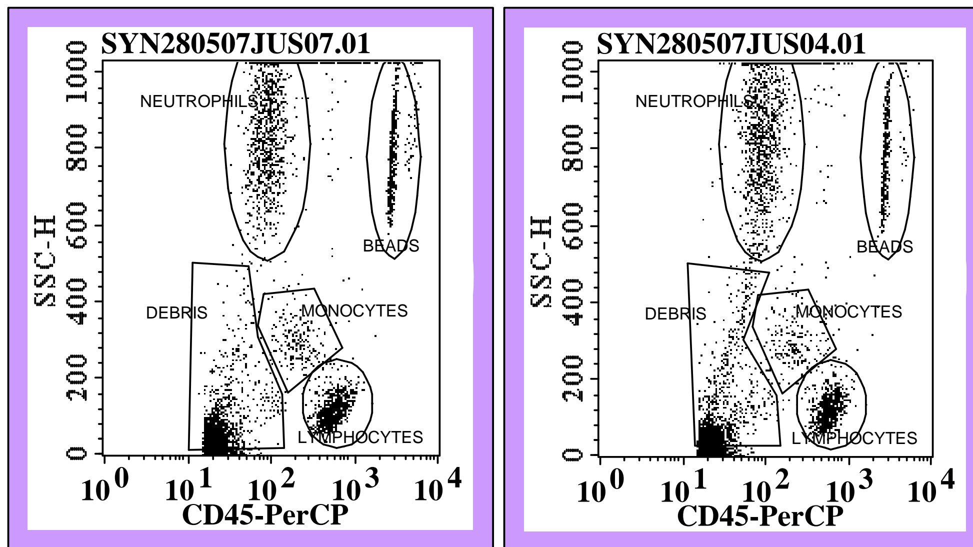

Figure 3.4.6b illustrates the scatter dotplot from the FACSCalibur when stabilized samples were run at day 37. On the left-hand side the plot illustrates the scatter of a sample prepared with paraformaldehyde. On the right-hand side the scatter plot demonstrates a formaldehyde preparation. The debris is slightly increased in the formaldehyde preparations on day 37.

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3.4.7 Use of Polyethylene glycol (PEG) as secondary stabilizing agent:

In order to evaluate the potential use of PEG as secondary stabilizing agent, blood was drawn from a healthy individual (on day 0) and divided into four sub-samples. The four samples were stabilized in the same manner as previously described (0.25% chromium chloride and 0.35% w/v paraformaldehyde stabilizing agents) but treated with various concentrations of PEG: 0% (were no PEG was added), 1%, 2% and 3% final concentrations of PEG. **Figure 3.4.7** illustrates the Levey Jennings plot of the data generated over a period of 22 days: both the stability of the percentage (top graph) and absolute values (bottom graph) of the diverse lymphocyte sub-populations is represented. The values obtained for the percentages (CD4 % and CD3%) were indicated in this graph: however SD bars are not shown due to this was only one sample analyzed. This applies also for the bottom figure where the plot for the absolute values (CD3, CD4 and CD8) was illustrated. No statistics was done due to the fact that these were only one sample. Looking at **Figure 3.4.7** it was evident that the sample preparations remained stable. However, severe lysing of the red blood cells in the stabilized blood preparations that contained the 1%, 2% and 3% PEG occurred from day 15 onwards and might have an influence in the drop of CD3 absolute values as seen in **Figure 3.4.7**. The presence of the PEG seemed to induce red blood cell fragility and it was uncertain whether this effect would impact on the white blood cells. It was therefore decided not to pursue this avenue but to rather use the paraformaldehyde or formaldehyde as secondary stabilizing agents. Due to the lysis of the red blood cells, we did not monitor the stability of the lymphocyte sub-populations any further and these preparations were discarded.

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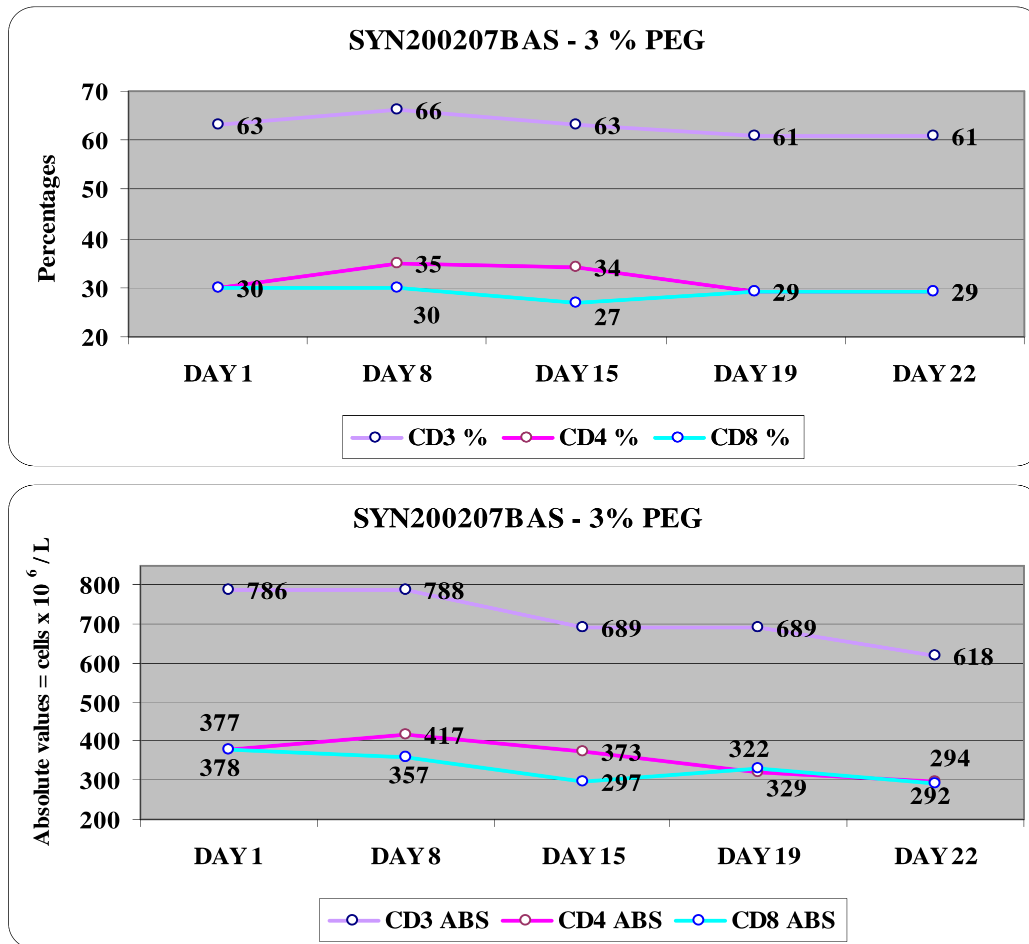

Figure 3.4.7: Levey Jennings Plots to monitor stability of absolute T cell values recorded for one example of stabilized blood that was treated with 3% polyethylene glycol (PEG). This blood sample showed stability up to 22 days for the percentage and absolute values of the CD4 and CD8 phenotypic surface markers. The absolute cell values on the y-axis represent cells x 10^6 / L. No SD bars were indicated due to the fact that this is only one sample.

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3.5 DISCUSSION

As previously described by Granger *et al.* (1999) in US Patent 5858699, the chromium chloride should be allowed to age and to form a precipitate: this precipitate is removed and the remaining chromium chloride solution should still contain approximately 0.1% chromium chloride. In this thesis it was shown that freshly drawn whole blood could be stabilized using both 0.1% and 0.25% chromium chloride as the first stabilizing agent. However after taking into consideration the scatter properties of the various cell populations on the flow cytometer, it was concluded that the 0.25% solution offered a better option for long term stability as determined using the routine phenotypic surface markers used for monitoring HIV infected individuals, namely CD3, CD4 and CD8 percentages and absolute values. The raw data analyzed for this on the flow cytometer and shown on the scatter plots is not shown. The higher concentration could represent a higher availability of the ions in solution after the precipitate was filtered off.

Stability of whole blood was described at two levels: level one was the identification of the various cell surface markers and level two was the ability to quantify the cells of interest. In this thesis it was demonstrated that whole blood could be stabilized for up to six weeks (and even longer) by using 0.25% chromium chloride as the first stabilizing agent and in combination with 0.35% w/v formaldehyde as secondary stabilizing agent. Reproducibility of this stabilizing technique was accomplishment in view of these ten stabilized samples over the 40 days. **Figure 3.4.2a, Figure 3.4.2b** and **Figure 3.4.2c** demonstrates the stability of the whole blood reference preparations. All ten samples were depleted on day 40 and no further analysis could take place. Lastly there were three samples monitored on the BD FACSCalibur™ and the BD FACSCount™ instruments: stability has been demonstrated until **day 84.**

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It is important to mention that the variation of the percentage values from various populations remain statistically non-significant over time whereas the absolute values of the populations decrease steadily over time. Please refer to **Appendix 1** (illustrating stability of the phenotypes) and **Appendix 5** (illustrating the scatter data plots for a sample run for 77 days). The scatter plot of these data displays clearly distinguishable populations even until day 77.

Great excitement was reached when these samples were run on the BD FACSCount™ and CD4 and CD8 populations of cells could be identified and quantified even until **day 84:** analysis of these samples on this less complicated flow cytometer resulted in only one error message. The samples were monitored on the BD FACSCount™ instrument and one out of the three samples had an error message (Error 8) which indicated "degraded reagent together with a very high threshold". The other two samples had still clearly distinguishable populations of cells. The third sample that resulted in the error message was the one sample that was manipulated (as mentioned at the bottom of section 3.3.8) to result in depleted lymphocyte counts. The data plot as obtained of the sample run on day 77 from the BD FACSCount™ can be found in **Appendix 2.**

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This thesis provides promising data in that it provides a relatively simple method for stabilizing whole blood reference material that could be used for training of newly qualified students and staff. These bloods can also be used as internal quality control samples to monitor variation in flow cytometric equipment on a daily basis to indicate possible instrument failure. This reference material can be used for staff competence evaluation in strictly clinical HIV monitoring laboratories for accreditation (SANAS) purposes.

The comparison of the data generated on the BD FACSCount™ versus that generated using the BD FACSCalibur™ yielded excellent correlations for both the absolute CD4 and absolute CD8 counts with excellent R^2 values (**Figures 3.4.3a** and **3.4.3b).** The first run of samples compared on these two instruments was disheartening because very different cell counts were measured: it was found that accurate pipetting was indeed crucial and that one cannot use a reverse pipette on the one instrument (as stipulated for the FACSCount) and a normal positive displacement micropipette for setting up the assay on the next flow cytometer (as is current practice in most clinical laboratories). However, when the reverse pipetting technique was used in both assays, this showed excellent correlations between the various samples. These reference samples can therefore be used for both instruments with total confidence.

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What is most promising is that these stabilized bloods can surely be used in poorer African countries as IQA reference material on the BD FACSCount™ system and might be helpful to indicate a possible instrument failure. These reference materials can be used to provide non-infectious blood samples for efficient training of staff where they are provided with reference ranges to practice their techniques. In poorer African countries, fresh whole blood from a healthy donor must be used to calibrate the instrument every morning before a batch of routine patient samples can be assayed. Bloods of healthy individuals are not always accessible at these clinics and result in staff forever donating their own bloods for the controls in the calibration processes. The stabilized blood can provide the clinical staff with “fresh normal bloods” for the morning run of controls.

Recently two staff members from BD, San Jose (USA) visited Synexa Life Sciences in order to validate new software upgrades for the FACSCount instrument. We requested them to test our stabilized blood samples in order to obtain an independent opinion. They agreed and after testing a 68 day old stabilized sample which was be pre-stained on day 1 and analysed on day 8 (more than one week later), the calibration passed on the BD FACSCount™. Stained samples were run over a period of 7 days and gave pretty much the same absolute values without showing error messages on this flow cytometer (see **Appendix 3**).

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The experimentation of the effects of storage temperature on the data slightly showed that the stabilized sample samples should be stored and transported at 4°C. As suspected, the 30ºC sample displayed degradation of the populations and lack of acceptable stability. The stabilized sample that was stored at room temperature showed a sudden increase of absolute values on day 8 and stability thereafter. This experiment was run in the middle of the winter and the sample monitored at room temperature, delivered promising results. It has been suggested that the cooler temperatures in the day might be an explanation for this phenomenon. Another observation that could not be neglected was the fact that the scatter dotplot of the room temperature stored sample starts to display significant scatter changes from day 18 and only suitably qualified and trained staff with experience would be able to gate accurately around the populations of cells. Therefore storage at room temperature would rather be discouraged.

The ultimate aim of an "ideal" reference material is to have a sample with the longest shelf-life with reproducible results over time. In order to address these requirements, we amended the methodology of stabilizing whole blood by including a secondary stabilizing step by using paraformaldehyde or formaldehyde. It has been said that the use of paraformaldehyde usually results in less auto-fluorescence than formaldehyde [Barnett *et al*. (1999)]. We investigated this phenomenon and found that auto-fluorescence was not such an issue while monitoring the routinely used HIV cell surface markers. Both aldehyde solutions resulted in stability until day 37 (see **Figure 3.4.6a**).

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However, after comparison of the scatter dotplots, it was found that paraformaldehyde was indeed the better aldehyde to use (see **Figure 3.4.6b**): in the formaldehyde prepared samples, the granulocytes tended to degranulate, they released their granules thereby decreasing in side scatter as the samples aged. This did not negate the potential use of formaldehyde because the contaminating cell population can still be excluded when using fluorochrome labeled antibodies. Taking into account the final SOP to follow for the activation studies which will be reported in the next chapter, paraformaldehyde in combination with chromium chloride was the second stabilizing agent of choice.

In the process of stabilizing the whole blood samples, we also investigated the potential use of PEG as a secondary stabilizing agent. We hoped that the PEG would result in longer stability of especially the white blood cells. Unfortunately it was observed that the red blood cells tended to lyse (visible heamoglobin in the supernatants) early on after treatment with PEG. We used 1%, 2% and 3% w/v PEG and none of these concentrations yielded acceptable sample preparations. The idea of using polyethylene glycol should not be discarded completely and might still be of value, but used at other concentrations. The use of PEG for stabilization was not further investigated in this thesis.

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The potential of manipulating whole blood samples to generate various reference ranges of important lymphocyte sub-populations:

It was thought of that if one should separate the white blood cells from the red blood cells and to treat them differently with two stabilizing techniques, maybe the red blood cells might still have the ability to lyse and the white blood cells being fixed more aggressively would result in longer stability. Hence the thought that one could perhaps stabilize the white blood cells separately and to add them back to an already stabilized whole blood sample in order to increase the white cell count (for increased reference values) was developed. An experiment was conducted whereby donor blood was divided into two: one portion was stabilized as described previously in this chapter and the other portion was manipulated in order to separate the red blood cells from the white blood cells (by Ficoll-Histopaque density centrifugation). The resulting white blood cell preparation was also stabilized as previously described (0.25% chromium chloride in the presence of 0.35% w/v paraformaldehyde) but the red blood cells were discarded. The first phase was to determine whether it was possible to stabilize the white blood cells separately. The stabilized white blood cells were then added back into the same blood donor's stabilized whole blood sample to investigate whether it was possible to produce a sample with increased lymphocyte counts. Initially it seemed as if it could be done, but after the first week of monitoring the routine white blood cell populations, it appeared that some neutrophils and/ or monocytes lost their granules and heavily contaminated the other cell populations until no difference could be distinguished between the cell populations by scatter properties. No statistics was performed on this raw data. Please refer to **appendix 4** for here the scatter plots of the "mixed" sample is illustrated; stabilized white blood cells of the same patient were added to investigate a possible increase the lymphocyte counts.

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The data shown here was run on day 4 and already degranulation of the granulocytes could be observed. Hence the idea of depleting stabilized whole blood, that is, removing some white blood cells leading to a sample with lower cells counts (and thus lower reference ranges) emerged. This was attempted: the samples were depleted slightly of lymphocytes during some of the washing stages. It was interesting to note that the samples with the lower (depleted) lymphocytes tended to be more stable than those preparations with "normal" lymphocyte levels. It was noticed, as previously described, that the depleted samples over a period of 77 days "failed" on the BD FACSCount™ instrument, but were still acceptable when run on the BD FACSCalibur™ instrument. This data is shown in **appendix 5 and 6.** It is suggested that if IQA control samples are to be prepared for the FACSCount instrument, that the whole blood should rather not be manipulated in this manner so that they contain too few absolute CD4 cells.

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CHAPTER 4

DEVELOPMENT OF STABILIZED WHOLE BLOOD SAMPLES EXPRESSING LYMPHOCYTE ACTIVATION MARKERS

4.1 INTRODUCTION

To date there are more than one *in vitro* technique to quantify the cellular immune response to various foreign agents. These techniques are based on the measurement of proliferating immune cells and the gold standard method to determine cell proliferation after cultured lymphocytes have been incubated with the microorganism or mitogen and/or specific antigen is the measurement of the incorporation of tritiated [^{3}H] thymidine into the DNA of the cells [Corradin *et al*. (1977) and De Francesco (1996)]. There are many disadvantages in using these laborious methods and these may include the use of radioactivity, the waste management of the radioactivity, the long incubation periods necessary, etc.

Another method for T cell activation determination is by using flow cytometry. Flow cytometry has the advantage above other techniques in that it is much more accurate, it is not subjective, is less labor-intensive and requires shorter incubation periods. Activated and/or proliferating lymphocytes are known to express several surface markers including CD25, CD69, CD71 and HLA-DR.

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Together with those already mentioned; other markers such as CD38, CD40L and CD122 antigens are up-regulated during lymphocyte activation. Lymphocyte activation status has not only pathogenic implications in HIV, but also in conditions such as autoimmunity and post-viral chronic fatigue syndrome [Landay *et al*. (1991) and Kestens *et al*. (1992), Liu *et al*. (1997) and Major *et al*. (1997)]. These surface markers are expressed minimally or are even absent on resting cells [Judd *et al.* (1980), Depper *et al.* (1984), Siegal *et al.* (1987), Nakamura *et al.* (1989), Fraser *et al.* (1993) and Caruso *et al.* (1997)] and thus received their name of "activation antigens". It is these activation antigens that are easily identified by immunofluorescence assay and flow cytometric analysis using monoclonal antibodies (Mabs) as specific reagents. For example it has been reported by Leroux *et al.* (1989), Maino *et al*. (1995) and Krowka *et al*. (1996), the usefulness of CD69 expression for rapid assessment of functional response by individual T-cell subsets to a variety of stimuli. Unless lymphocyte subsets are purified, the [^{3}H] thymidine incorporation technique fails to provide information on the cell subpopulations involved in antigen recognition or having functional defects. CD69 expression on stimulated lymphocytes was found in 1993 by Lopez-Cabrera *et al.* CD69 surface molecules appear early and seem to parallel, under certain conditions, the proliferative activity of the same lymphocytes as determined by [^{3}H] thymidine incorporation.

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It became clear to us that accurate and reliable flow cytometric measurements of cell percentages, fluorescence intensities and activation status of the cell preparations were becoming more important. A need was therefore identified in having an IQA control sample for the measurement of activated cell surface molecules. To date no such "specialized" activated IQA control sample exist. The aim of this chapter was therefore to investigate the possibility of providing such "specialized" control material: the investigation will start off in phase one by activating lymphocytes with various stimuli for optimum expression of selected activation molecules. The second phase will be to stabilize these activated lymphocytes.

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4.2 T-LYMPHOCYTE ACTIVATION MARKERS

In vivo, peripheral CD4+ T cells can be divided into two functional groups based upon the expression of distinct isoforms of the surface molecule CD45: isoforms containing exon A are termed CD45RA positive, while RA-depleted populations are called CD45RO positive [Trowbridge and Thomas (1994)]. CD45RA+ and CD45RO+ T cells subsets are believed to represent naïve or memory populations respectively [Young *et al*. (1997)]. Naïve cells show greater activation thresholds for effector function (the first encounter of the antigen) and proliferation. Antigen-induced recall responses appear to be greater in memory T cells [Bell *et al*. (1998)]. It has been indicated that TCR-induced responses in memory T cells may differ a great deal from those of naïve cells. They have smaller increased numbers of intracellular free calcium, reduced intracellular calcium stores [Nagelkerken & Hertogh (1992), Hall *et al*. (1994)and Sigova *et al.* (1999)] and decreased levels of TCR-dependent tyrosine phosphorylation [Patel *et al*. (1994)]. Differences in calcium mobilization between T cell subsets may be particularly applicable to the regulation of calcium-dependent genes including CD40L. Co-stimulatory molecules such as CD80 and CD86 on professional antigen presenting cells (APC) are involved in stimulating naïve T cells that express the co-receptor CD28. To achieve full T cell activation, the CD80 and CD86 co-receptors on the APC ligates with the CD28 on the T cell. The CD28 signaling enhances, but is not critical for memory-T cell responses.

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In both primary and secondary T cell activation, CTLA-4 serves as a second co-receptor for CD80 and CD86, and modulates CD28 signaling mostly in an inhibitory way [Chambers *et al.* (1999)], in other words, it dampens down the T cell response. This is illustrated in **Figure 4.2:** T cell interactions with a professional APC upon activation with a number of surface molecules.

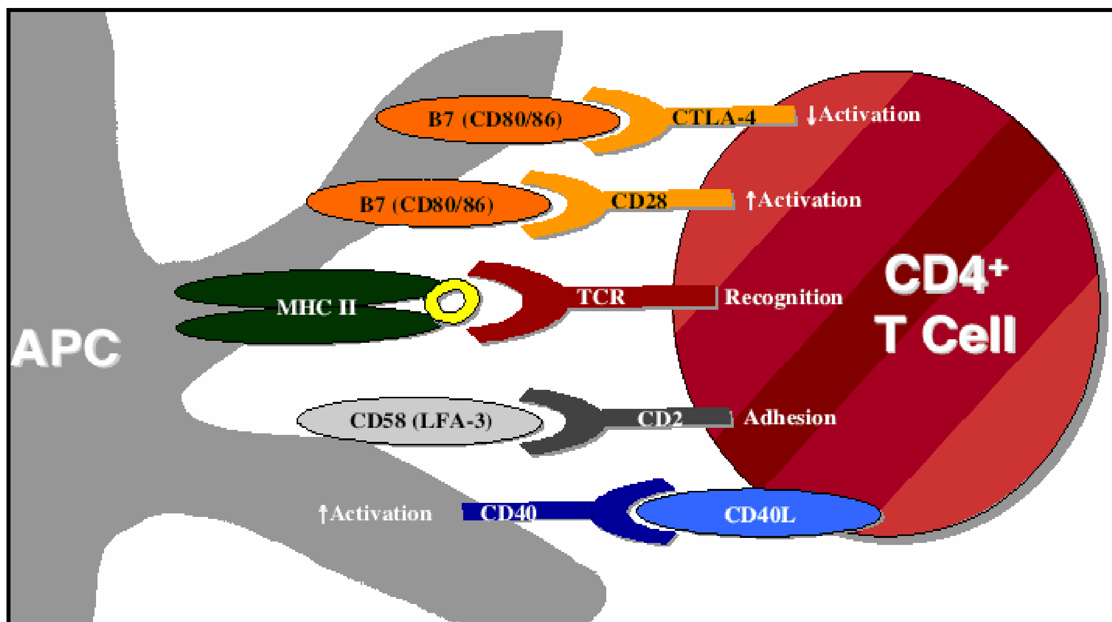

Figure 4.2 illustrates the cell surface molecules in T cell activation. Some of these are potential targets for immunotherapy. The interactions between the T cell receptor and the major histocompatibility complex (MHC) class II-peptide complexes are fundamental to T cell activation. T cells also provide activating signals to the APC such as CD40: CD40L interactions that result in immunoglobulin class switching in B cells and cytokine production in dendritic cells. (Figure adapted from www.uchsc.edu/misc/diabetes/oxch1. html).

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The inducible T cell co-receptor molecule (ICOS) is found on memory T cells and effector T cells. This molecule has an important role in T cell activation and differentiation, as it interacts with its co-receptor ICOS Ligand (ICOS-L) on professional APCs [Hutloff (1999) and Yoshinaga (1999)]. In addition to the B7 family, the CD40: CD40L signaling pathway increases the T cell activation after both primary and secondary encounters with antigens [Cayabyab *et al.* (1994)].

***In vitro* activation of T lymphocytes**

T-cell can also be activated *in vitro* for example and with antigen-induced T-cell activation, it may result in the expression of various activation markers such as CD38, CD40L, CD69, CD25 (also known as IL-2 receptor), CD71 and HLA-DR [Bass *et al.* (1992), Kestens *et al.* (1992 and 1994), Caruso *et al.* (1997) and Benito *et al.* (1997)]. For example when analyzing the expression of CD69, its *de novo* induction occurs within one to two hours after triggering the protein kinase C activation pathway and calcium mobilization. This occurs by engagement of the T cell receptor/CD3 complex or stimulation with phorbol esters according to Cebrian *et al.* (1988) and further studies in 1989. This was also discussed by Risso *et al.* (1991). Various stimuli can be utilized to activate T lymphocytes. In this investigation, we examined the activation of T lymphocytes using various stimuli: the phorbol ester, PMA (phorbol 12-myrisate 13-acetate) also called TPA, Ionomycin, Phytohemagglutinin (PHA, a lectin from *Phaseolus vulgaris* – derived from the red kidney bean), the anti-CD28 monoclonal antibody [Vandenberghe *et al*. (1993)], the anti-CD49 antibody and a combination of these two antibodies.

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Part of the aim of this chapter was to determine the optimal concentration of the different stimuli together with optimal incubation periods that are required to produce the most favorable activation marker expression. This was measured as the percentage positive cell population expressing the activation marker.

This chapter is therefore divided into three phases:

i. The optimization of the stimulant concentration, incubation periods and the stimulus of choice to activate our lymphocytes, not only CD40L but also the more robust surface activation markers such as CD25, HLA-DR and CD69.
ii. The second and more challenging phase of this chapter was to stabilize these activated lymphocytes to utilize as Internal Quality Assessment reference material.
iii. Then the final phase of this investigation was to monitor the stability of these activated IQA samples. Stability is defined in this chapter as the identification of the various phenotypic activated cell surface markers and the reproducible measurement and quantification thereof.

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4.3 SURFACE CD40 LIGAND (CD40L or CD154) AS A T-LYMPHOCYTE ACTIVATION MARKER

The CD40L (also named CD154) plays a major role in the regulation of cell mediated and humoral immune responses. It controls the balance of proliferation, maturation and apoptotic (cell suicide) processes [Banchereau *et al*. (1994)]. **Figure 4.2** illustrates the interaction of CD40L that takes place with its receptor (CD40) on B cells and other antigen presenting cells (APCs). The CD40 ligation on APCs induces the up-regulation of adhesion and co-stimulatory molecules. It increases APC activity and results in enhanced T cell co-stimulation. This in turn promotes APC induction of cytokines and also promotes dendritic cell growth and survival [Alderson *et al.* (1993), Kennedy *et al*. (1994), Caux *et al.* (1994) and Shinde *et al.* (1996)]. CD40L promotes increased B cell growth and survival and it also triggers antibody isotype class switching *in vivo* [Krockzek *et al.* (1994)]. As previously mentioned, CD40L expression is controlled by the immune responses. In fact, preventing CD40: CD154 interactions can prevent or decrease the severity of a number of T cell-mediated autoimmune diseases. **Figure 4.2** also illustrates the interaction between the T cell receptor (TCR) and the major histocompatibility complex (MHC) class II-peptide complexes which are fundamental to T cell activation. Not only in autoimmune diseases, but also interactions that are of particular interest in transplantation tolerance are those between CD80/CD86 and CD28, CD154 and CD40, ICAM-1 (intracellular adhesion molecules) and LFA-1 (leukocyte function antigen -1).

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4.3.1 The importance of CD40 Ligand

The importance of CD40L has been demonstrated in that the expression of defective CD40L protein causes an immunodeficiency state characterized by high IgM and low IgG serum levels. Together they indicate faulty T cell-dependent B cell activation [Allen *et al.* (1993), Korthauer *et al*. (1993), Ramesh *et al.* (1993) and Aruffo *et al*. (1993)] and may lead to recurrent bacterial infections. A lack of functional CD40L gene in individuals suffering from X-linked Hyper-IgM syndrome results in immunodeficiency. There is a general defect in cell-mediated immunity, abnormal lymph-node architecture and the inability to produce IgG, IgA and IgE antibodies. Patients with this immunodeficiency have normal numbers of circulating B and T cells, but predominantly IgM antibodies in response to T-dependent antigens and fail to undergo isotype switching. There is another group of primary immunodeficiency known as common variable immunodeficiency CVI that may be caused in part by defective CD40: CD154 signaling (Farrington *et al.* 1994). This suggests that the cross-linking of CD40 also fails to activate B cells. Some patients with Hyper-IgM appear to have normal expression of CD154: in this subset of patients, the B cells fail to respond to anti-CD40 cross-linking [Conley *et al.* (1994)]. In addition, isotype switching (class switching) in B lymphocytes isolated from such patients can be rescued by treatment, *in vitro*, with either CD40 monoclonal antibody [Fuleihan *et al.* (1993)] or wild-type CD40L [Allen *et al.* (1993)] showing directly that the interaction of CD40L with CD40 is functionally non-redundant for the production of IgG, IgA, and IgE.

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Furthermore, CD40L is found in a small population of activated CD8+ T cells, purified NK cells, monocytes, basophils, mast cells, activated eosinophils and activated platelets [Gauchat *et al.* (1993 and 1995) and Henn *et al.* (1998)].

4.3.2 CD40L: membrane-bound versus soluble form

Reports documented that a soluble, biologically active form of CD40L can be released into medium of cultured cells [Graf *et al.* (1995) and Pietravalle *et al.* (1996)]. The identification of soluble CD40L and Fas ligand broadens the mechanism of these ligands to diffuse cytokine-like activity. In the case of CD40L, it has been well documented by Parker in 1993 that the helper function of T cells is contact-dependent indicating that the membrane anchored form is physiologically important. In addition of the full length protein, two shorter forms of CD40L are present on the surface of T lymphocytes [Yen-Ming *et al.* (1997)]. It was indicated that the cell surface CD40L molecules are primarily heterotrimers consisting of the full-length and truncated forms of CD40L. CD40L are naturally expressed as both membrane-linked and soluble forms. In its full length it has an apparent molecular mass of 33 kDa [Jones and Rose-John (1992)]. The soluble CD40L appears to be the truncated form (15 – 18 kDa) cleaved at the methionine 113 portion and lacking the transmembrane domain. CD40L is a member of the tumor necrosis factor (TNF) family. It is predominantly expressed on mature activated CD4+ T cells [Spriggs *et al*. (1992) and Banchereau (1994)] and can be expressed on Th0, Th1 and Th2 cells [Roy *et al.* (1993)].

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Elevated levels of sCD40L are associated with Arherosclerosis, unstable angina, hypercholesterolemia, Systemic Lupus Erythematosus (SLE) and inflammatory bowel disease [Danese *et al.* (1993)]. Soluble CD40L is speculated to play a role in the pathogenesis of these diseases by acting on the CD40 receptor bearing bystander cell in a cytokine-like fashion. In these circumstances, the sCD40 may regulate distinct signaling pathways or complement the biological activity of membrane bound CD40L. It has been hypothesized that proteolytic cleavage of membrane CD40L on T cells rise to the 18 kDA protein [Hsu *et al*. (1997) and Hirohata (1999)]. Shedding is the proteolytic release of the extracellular domains of the cell-surface proteins. For CD40L expression, the membrane bound form of CD40L is of interest and not the soluble form of CD40L (sCD40L). As previously investigated by Wingett and Nielson in 2002, no significant expression of sCD40L could be observed at any ionomycin concentration tested: they therefore concluded that calcium signaling appears sufficient to induce membrane CD40L expression yet insufficient for sCD40L.

4.3.3 CD40 Ligand molecule therapy

Promising results are being obtained from animal models of RA and SLE with anti-CD40L MAb treatment. Cho *et al.* (2001) studied the role of CD40: CD40L interaction in cross talk by administering a MAb against CD40L. Another way is by inhibiting CD40L expression from within by using small molecules which are able to enter the cell and inhibit specific pathways responsible for CD40L expression.

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Desai-Mehta *et al.* (1996) have reported that peripheral blood mononuclear cells (PBMC) from patients with active lupus exhibit a 21-fold increase in the percentage of CD40L+ CD4+ cells compared to healthy subjects. A report studying the functional role of sCD40L concluded that sCD40L can induce B-cell activation and differentiation. Plasma sCD40L levels were significantly higher in active-SLE patients than in healthy donors [Santana-Sahagun *et al.* (1999)]. The authors of this study proposed that sCD40L levels could serve in the future as a predictive marker of SLE disease flares. Later a study done with lupus nephritis patients who received anti-CD40L therapy showed that a short course of this treatment leads to a reduction in the number of IgG anti-DNA antibody-producing B cells. These changes persisted for several months after treatment was stopped [Huang *et al*. (2002)]. A similar study of patients with active lupus nephritis who received CD40L treatment showed B cells expressed B cell differentiation markers (CD38, CD5 and CD27) disappeared from the periphery during this treatment [Grammer *et al*. (2003)].

4.3.4 The role of CD40 Ligand in HIV

B cell abnormalities described in HIV disease include hypergammaglobulinemia [Lane *et al*. (1983) and Morris *et al.* (1998)], increased expression of activation markers [Forster *et al.* (1997)], increased levels of auto-antibodies [Horvath *et al.* (2001)], increased risk of developing B cell lymphomas [Martinez-Maza and Breen (2002)], and decreased responsiveness to *in vivo* vaccination and *ex vivo* stimulation [Steinhoff *et al.* (1991) and Conge *et al*. (1998)].

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Van Kooten and Banchereau (2000) stated that one of the most important contact-mediated interactions between B cells and CD4+ T cells involves CD40 expression of B cells and its membrane-bound ligand CD154 (CD40L) expressed on activated CD4+ T cells. The expression of CD40L on activated CD4+ T cells is influenced after T cell receptor triggering by the APC and requires co-stimulatory interactions between CD80/CD86 on mature antigen presenting cells (APC) and CD28 on CD4+ T cells [Grewal and Flavell (1998)]. These interactions induce CD4+ T cells to secrete cytokines, which together with expression of CD40L provide the CD4+ T cell with full helper function. As mentioned previously the T helper cells can be divided into Th1 and Th2 subsets, depending on the cytokines in the surrounding environment. B cells respond to either subset, although Th2 responses involving IL-4, IL-10 and IL-13 have been more widely described among B cells than Th1 responses involving IL-2, IFN-γ, and IL-12 [Paul and Seder (1994)]. More recently, Johnson-Leger *et al*. (1998) and Smith *et al*. (2000) suggested that both the Th1 and Th2 CD4+ T cells provide help to the B cells.

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4.4 METHODS AND MATERIALS

4.4.1 Blood samples

After giving the necessary informed consent, donor peripheral blood samples from healthy subjects were collected into K_2 EDTA tubes following venipuncture at Synexa Life Sciences, Tygerberg Hospital. Bloods were kept at room temperature and processed within 2 hours of draw. All procedures were done aseptically.

4.4.2 Reagents and equipment

Histopaque-1077 Ficoll was purchased from Sigma Aldrich (South Africa) and used for lymphocyte separation in the gradient density centrifugation method. Foetal bovine serum (Gibco™ EU approved origin, heat inactivated) was purchased from Scientific Group (South Africa). Sterile RPMI 1640 (Roswell Park Memorial Institute medium) with GlutaMAX (Gibco® 61870) was purchased from Laboratory Suppliers (South Africa). This in combination with the foetal bovine serum was used for the complete media for the cell suspensions. PBS (Dulbecco's PBS without calcium and magnesium chloride) was purchased from Sigma-Aldrich (South Africa). Ionomycin was purchased from Synexa Life Sciences Ldt (South Africa). The phorbol ester, PMA (Phorbol 12-Myrisate 13-Acetate) also called TPA, was purchased from Sigma Aldrich, (South Africa). Trypan blue was purchased from Sigma-Aldrich, (South Africa) for cell viability determination.

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The concentration of stock solution of TPA that was used was 1mg/ml (in DMSO). Phytohemagglutinin (PHA, a lectin from *Phaseolus vulgaris* – derived from the red kidney bean) was purchased from Sigma Aldrich (South Africa) and stock solution of 1mg/ml (in complete media) was used.

The CD28 pure and CD49 pure antigen reagents were purchased from BD, (South Africa). The anti-CD40L - APC (allophycacyanin) monoclonal antibodies from Pharmingen, the anti-CD3 - PE monoclonal antibodies, the anti-CD4 - PerCP monoclonal antibodies, the anti-CD4 - APC monoclonal antibody, the anti-CD25 - FITC monoclonal antibodies and the anti-HLA-DR - FITC monoclonal antibodies from Pharmingen were purchased from BD, (South Africa). A cocktail for the monoclonal antibodies; Anti-CD3 – PerCP, anti-CD4 – FITC and anti-CD69 - PE (FASTIMMUNE) was purchased from BD. To lyse existing erythrocytes, an NH_4Cl-based lysing solution (BD, South Africa) was used.

The flow cytometers of choice were the FACSCalibur™ (BD) and the FACScan™ instruments (BD). BD CellQuest™ software was used for analysis. For the acquisition and analysis of each activation marker, different instrument settings were employed.

Sterile 12x75mm polystyrene round bottom BD Falcon™ tubes (5ml) for the BD FACSCalibur™ and BD FACScan™ equipment were purchased from BD, (South Africa). K_2 EDTA coagulated tubes were purchased from BD, (South Africa). Pooled human AB sera was purchased from the blood blank, Tygerberg Hospital, (South Africa).

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4.4.3 Phase I: *In vitro* stabilization of lymphocytes

Preparation of peripheral blood mononuclear cells (PBMCs) for stimulation

Peripheral blood mononuclear cells were isolated from whole blood samples using density gradient centrifugation with Histopaque-1077 Ficoll (Sigma Aldrich, South Africa). The bloods were double diluted with PBS, layered on Histopaque-1077 Ficoll and centrifuged at 650 g for 25 minutes at approximately 4ºC. The cells were recovered from the interface and washed gently in PBS to get rid of the platelets. Cell viability was confirmed by Trypan blue exclusion. One million cells per milliliter complete media (10% FCS in RPMI) was the final cell concentration that was prepared and were kept in the fridge (2 to 6ºC) until the next morning.

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(a) Stimulation of PBMCs for CD40 Ligand expression

The isolated PBMCs were stimulated by using various concentrations of phorbol ester, PMA, Ionomycin, PHA, anti-CD28, anti-CD49 and a combination thereof. Cell concentrations of 1 X 10^6 cells/ml were incubated with the various stimulants for 2, 4, 6, 8 and 24 hours in a 5% CO_2 environment (at 37ºC). The percentage of activated T helper cells expressing sCD40L was measured once the cells had been stained for flow cytometry:

- 10µl anti-CD40L-APC, 10µl anti-CD3-PE and 10µl anti-CD4-PerCP were added in one BD Falcon™ tube.
- 50µl of the activated lymphocytes post culture were added to the monoclonal antibodies in the BD Falcon™ tube.
- This was incubated in the dark for approximately 15 minutes.
- BD FACSLyse™ lysing solution was added and the samples were analyzed using CellQuest software on the flow cytometer.
- T helper cell expression of CD40L was calculated by gating on the CD3+CD4+ cell populations: at least 5000 gated cells were acquired and analysed in each experiment. The threshold was set on FL-2 and the primary gate was on the CD3 events.

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(b) Stimulation of PBMCs for CD69 surface activation markers

Similar to the experimental set-up described above, PBMCs were stimulated by using various concentrations of PMA, Ionomycin, PHA and a combination thereof:

- Isolated PBMCs were incubated with the various stimulants for different time intervals (2, 4, 6, and 24 hours) in a 5% CO_2 environment (at 37ºC).
- After incubation, the cells were stained with the respective FASTIMMUNE cocktail of monoclonal antibodies (anti-CD3 – PerCP, anti-CD4 – FITC and anti-CD69 - PE) purchased from BD Biosciences.
- 10µl of the antibody cocktail was added in a BD Falcon™ tube together with 50µl of the activated lymphocytes.
- The cells were incubated for 15 minutes in the dark and FACSLyse™ Lysing solution was added. The cells were acquired and analysed on the flow cytometer.
- T helper cell expression of CD69 was calculated by gating on the CD3+CD4+ cells population. At least 5000 gated events were acquired and analyzed in each experiment using the CellQuest™ software (BD, South Africa). The threshold was set on FL-3 and the primary gate was set on the CD3 events.

For the set-up of this experiment the full panel with the incubation times and the various stimuli used is illustrated in **Table 4.4.3.** This table also included the other activation markers to follow.

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(c) Stimulation of PBMCs for CD25 and HLA-DR surface activation markers

Once again, similar experimental set-up was used to induce the expression of CD25 and HLA-DR on isolated PBMCs (**Table 4.4.3).** The threshold was set on FL-2 and the primary gate was set on the CD3 events.

Table 4.4.3 illustrates the panel for the set-up of the range of stimuli used and incubation time intervals. For each stimulated test sample, a non-stimulated control sample was included at these time intervals.

CD3-PE/CD4-APC/HLA-DR-FITC	**2 Hours**	**4 Hours**	**6 hours**	**24 hours**
UNSTIM				
IONO				
PHA				
IONO/PHA				
IONO/TPA				
TPA				
CD3-PE/CD4-APC/CD25-FITC	**2 Hours**	**4 Hours**	**6 hours**	**24 hours**
UNSTIM				
IONO				
PHA				
IONO/PHA				
IONO/TPA				
TPA				
CD69-PE/CD3-PerCP/CD4-FITC	**2 Hours**	**4 Hours**	**6 hours**	**24 hours**
UNSTIM				
IONO				
PHA				
IONO/PHA				
IONO/TPA				
TPA				

UNSTIM = unstimulated tubes, IONO = Ionomycin stimulated tube, PHA = Phytohemagglutinin stimulated tube, TPA = phorbol 12-myrisate 13-acetate stimulated tube, IONO/PHA = Ionomycin and Phytohemagglutinin stimulated tube, IONO/ TPA = Ionomycin and phorbol 12-myrisate 13-acetate stimulated tube

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4.4.4 Phase II: Stabilization of the activated lymphocyte preparations

(a) Stabilizing of activated surface marker CD40Ligand

One million cells were stimulated with 4uM Ionomycin for 4 hours: this proved to represent the optimum conditions in order to get the cells to express the highest percentage CD40L on their surfaces. After incubating the cells with the stimulant, they were divided into eighteen Falcon tubes™ BD Biosciences, (South Africa). Each tube was treated with an aged 0.25% chromium chloride (III) hexahydrate (purchased from Sigma Aldrich) solution (pH approximately 6.7) for one hour at 4ºC. The cells were centrifuged at 450 g for 10 minutes and the supernatant was removed. Centrifugation at this stage was much gentler the whole blood stabilization (see Chapter 3) because active lymphocytes tend to clump together more easily in the presence of left-over platelets. The activated cells were then treated with various concentrations of a second stabilizing agent (paraformaldehyde, in combination with chromium chloride), at pH of approximately 6.7 and incubated overnight on the roller mixer (2 – 8ºC). Different concentrations of paraformaldehyde (1.4%, 0.7%, 0.35%, 0.175%, 0.0875% and 0% - where no paraformaldehyde was added) in combination with chromium chloride were added to the cells. **Table 4.4.4a** illustrates the panel of the concentrations and incubation periods with paraformaldehyde for these 18 sample tubes.

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The next morning the cells were washed twice with sterile PBS. Gentle speeds of 450 g were used and the samples were centrifuged for 20 minutes at 4ºC. After the supernatant was removed, the cells were made up at 1 x 10^6 cells per ml of their own plasma or in decomplemented (pooled) human AB sera. After stabilization, 50µl of the samples used to stain at each time interval and analyzed to identify the various surface activation antigens. The remaining of the stabilized samples was stored at 4ºC. Once the activation markers could be identified on these stabilized samples; an aliquot of each sample were stained and analyzed on each of the following days; day 4, day 7, day 11, day 15, day 18 and day 23 to establish the stability thereof.

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Table 4.4.4a Illustrates the 18 cell sample tubes of 1 x 10^6 cells per ml that was incubated at three time slots and six concentrations of paraformaldehyde.

TUBE	CONCENTRATION PARAFORMALDEHYDE	INCUBATION TIME
1	**1.4% paraformaldehyde**	**1 hour**
2	1.4% paraformaldehyde	4 hours
3	1.4% paraformaldehyde	24 hours
4	**0.7% paraformaldehyde**	**1 hour**
5	0.7% paraformaldehyde	4 hours
6	0.7% paraformaldehyde	24 hours
7	**0.35% paraformaldehyde**	**1 hour**
8	0.35% paraformaldehyde	4 hours
9	0.35% paraformaldehyde	24 hours
10	**0.175% paraformaldehyde**	**1 hour**
11	0.175% paraformaldehyde	4 hours
12	0.175% paraformaldehyde	24 hours
13	**0.0875% paraformaldehyde**	**1 hour**
14	0.0875% paraformaldehyde	4 hours
15	0.0875% paraformaldehyde	24 hours
16	**0% paraformaldehyde**	**1 hour**
17	0% paraformaldehyde	4 hours
18	0% paraformaldehyde	24 hours

(b) Stabilization of activated lymphocytes expressing the surface molecules CD69, CD25 and HLA-DR

One million of cells per ml were stimulated with stimuli (as previously described in **section 4.4.3**) and incubated for expression of various T cell surface activation molecules. The T helper cells were stimulated to express optimum percentages of CD25, CD69 and HLA-DR on their cell surfaces. After incubation with the stimulant, the cell samples were divided into BD Falcon tubes™. Each tube was treated with aged 0.25% chromium chloride (III) hexahydrate solution (pH approximately 6.7) for one hour at 4ºC. The cells were centrifuged at 450 g for 10 minutes and the supernatant was removed. The samples were then treated with 0.35% or 0% (where no paraformaldehyde was added) paraformaldehyde in combination with 0.25% chromium chloride. The pH of this second stabilizing agent was adjusted to approximately 6.7. The cell suspensions were incubated for 2 hours on the roller mixer at 2 to 8ºC. The cells were washed twice with sterile PBS. For the washing steps, gentle speeds of 450 g were used for 20 minutes at 4ºC. After the supernatant was removed, the cells were adjusted to 1 x 10^6 cells per ml either within their own plasma or in decomplemented (pooled) human AB sera. The cells were then ready to be stained with the monoclonal antibodies (as previously described in **section 4.4.3**) and analyzed on the BD FACSCalibur™ using CellQuest™ software. These activated, stabilized samples were run over a time period to identify their stability.

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(c) Spiking of stabilized whole blood with stabilized activated lymphocytes

Together with the activated PBMC stabilization, whole blood of the same individual was stabilized. The whole blood was stabilized as previously described in Chapter 3 with 0.25% chromium chloride in combination with 0.35% w/v paraformaldehyde. After stabilizing the PBMCs, 75% v/v of these activated lymphocytes were added with 25% v/v stabilized whole blood. Another sample was created where 50% v/v of the activated PBMCs were added to 50% v/v of the stabilized whole blood. We called these samples "spiked samples" since they contained stabilized whole blood as well as stabilized activated PBMCs. The aim of this "spiking" was to generate controls expressing the desired activation markers, similar to what would be expected from a patient sample. **Table 4.4.4.b** illustrates the panel put up for these activation markers. In parallel with these spiked samples, "unstimulated" whole blood was stabilized and monitored for the same phenotypic activation molecules. These "unstimulated" samples served as negative controls for baseline with each run. There were samples containing only stimulated PBMCs and they were monitored in parallel with these spiked and control samples. For each activation marker (CD25, CD69 and HLA-DR) these four samples were monitored over a period of time.

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Table: 4.4.4b illustrates the panel for the spiked whole blood samples. These samples were previously stimulated with various stimuli to express CD25, CD69 and HLA-DR. Thereafter they were stabilized and spiked back into the stabilized whole blood of the same donor blood.

Activation Marker	Control	Test	Spiked (v/v)	Spiked (v/v)
CD25	**UNSTIM WBL**	**PBMC STIM**	**75% PBMC/ 25%**	**50% PBMC/ 50%**
HLA-DR	**UNSTIM WBL**	**PBMC STIM**	**75% PBMC/ 25%**	**50% PBMC/ 50%**
CD69	**UNSTIM WBL**	**PBMC STIM**	**75% PBMC/ 25%**	**50% PBMC/ 50%**

UNSTIM WBL = unstimulated stabilized whole blood, PBMC STIM = peripheral blood mononuclear cells that are stimulated (and stabilized).

4.5 RESULTS

The first phase of this chapter was to identify a stimulus to activate isolated lymphocytes in order that they express optimal levels of the activation surface markers. The second phase was the stabilization of these activated lymphocyte samples. The third phase was to monitor the stability of these activated stabilized samples.

4.5.1 Activation: Surface CD40L expression

All the CD40L samples were analyzed using the BD FACSCalibur™ and CellQuest™ software. For the CD40L analysis, specific instrument settings were selected with the threshold on FL-2 and the primary gate was set around the CD3 positive events. Total events of 150 000 were acquired for the CD40L determination. **Figure 4.5.1a** illustrates an example of the scatter plots and dotplots as captured on the flow cytometer. Whenever cells were stimulated, an unstimulated control sample was included and incubated under the same condition as the stimulated samples. After activating T helper cells with various stimuli, Ionomycin was the stimulus of choice for the expression of surface CD40L. Other stimuli such as pure CD28 and pure CD49 were investigated but due to the expense of these co-stimulatory factors, they were not further considered (data was not shown). TPA and in combination with Ionomycin were to aggressive and "over-stimulated" the samples in that the CD4 cells were down-regulated. This made this stimulated samples not suitable for further investigation in this thesis i.e. stabilization for IQA purposes. **Figure 4.5.1b** illustrates the data obtained showing these optimal incubation time and concentrations of Ionomycin and it was found that the optimum concentration of Ionomycin was 4µM. Cells should be incubated for a period of four hours at 37ºC in a 5% CO_2 environment. The activated T lymphocytes expressed approximately 25 to 35% membrane bound CD40L.

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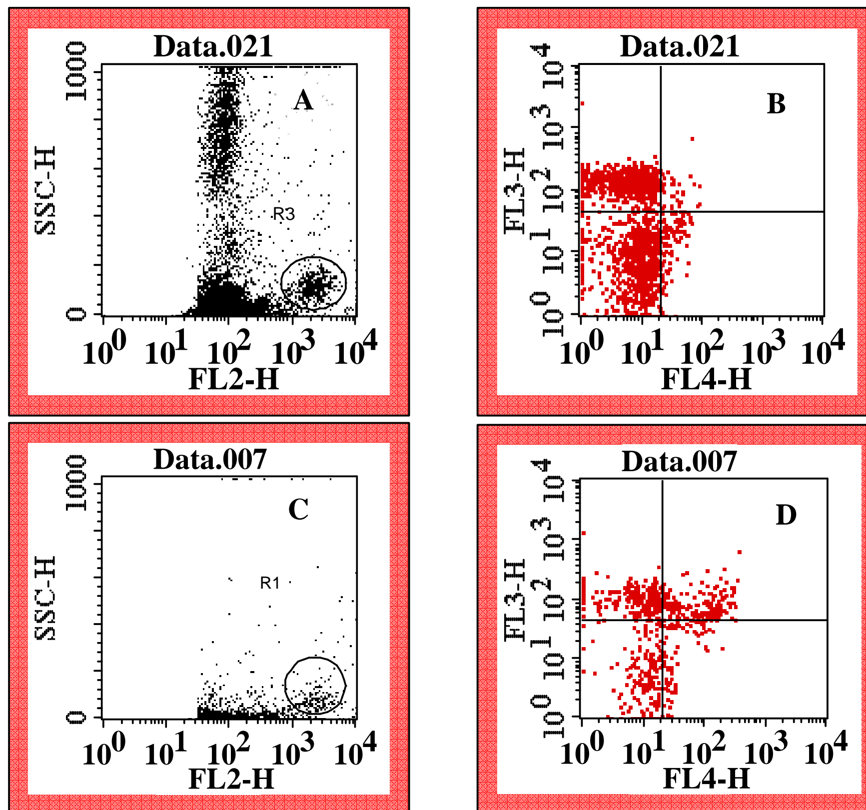

Figure 4.5.1a: Data.021 (labeled A and B) illustrates for CD40L expression an example of the dotplot for the unstimulated control sample and Data 007 (labeled C and D) illustrates the dotplot for the sample stimulated with Ionomycin. In A and C, the primary gate (e.g. R3 and R1) was on the CD3 positive events (FL-2 in this case). The analysis of CD4+CD40L+ cell population was determined within R3. Panel B show very little CD4 (FL-3) cells expressing CD40L (FL-4) in the upper right quadrant, since these are unstimulated cells. Panel D illustrates the data generated from stimulated cells; the CD4 (FL-3) positive cells that express CD40L (FL-4) are found in the upper right quadrant.

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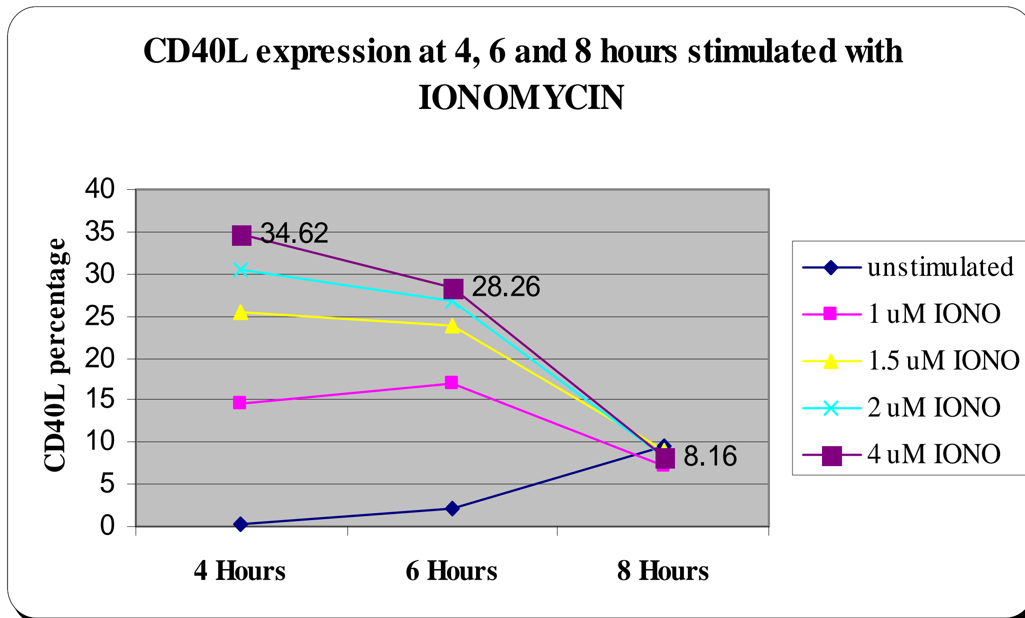

Figure 4.5.1b: PBMCs were incubated for 4, 6 and 8 hours with 1µM, 1.5µM, 2µM and 4µM Ionomycin. On the x-axis is time plotted against CD40L percentage (CD4+/CD3+ cells expressing CD40L%) on the y-axis. After incubation, the CD3+/CD4+/CD40L+ cell percentages were analyzed using the BD FACSCalibur™. The 4µM ionomycin concentration appeared to be optimal at 4 hours of incubation with 34.62% activation. And this final concentration was used throughout the rest of this assay. IONO = Ionomycin

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4.5.2 Activation: CD69 surface expression

All CD69 samples were analyzed using the BD FACSCalibur™ and the CellQuest™ software. For the CD69 analysis, specific instrument settings were selected with the threshold on FL-3. 150 000 total events were acquired for each run. For all the stimulated tests unstimulated controls were included. **Figure 4.5.2a** illustrates the one example of the data plots obtained on the BD FACSCalibur™ for specific CD69 surface marker expression. For the investigation of CD69 expression, activation of T lymphocytes was accomplished by stimulating it with TPA, Ionomycin, and TPA in combination with Ionomycin, PHA, and PHA in combination with Ionomycin. CD69 was expressed on surface membranes of T helper cells after various incubation periods (2, 4, 6 and 24 hours) in a 5% CO_2 environment (at 37ºC). The CD69 expression on CD3 positive T helper cells is represented in **Figure 4.5.2b.** Although it is visible in this figure that the expression after 24 hours was the highest measured, the data on the scatter plots and long incubation periods discouraged this incubation time and no further analysis were done at 24 hours. The combinations of stimuli resulted in good scatter plots, but was discouraged on the basis of cost saving. The use of TPA on its own was discouraged; a down-regulation of CD4 cells were observed on the scatter plots of these populations from 2 hours onwards. This would not make the sample suitable for further IQA experimental purposes. An observation was made with the high unstimulated CD69 values of the 2 hour and 6 hour preparation which might be due to handling of samples while processing. Another explanation for the high unstimulated values might be due to endotoxins (for example LPS) in the fetal calf serum (FCS) used to resuspend the cells in after optimizing the cell concentrations in complete media.

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It was demonstrated by Tkachenko *et al.* (2005) that dendritic cells were influenced by culture media supplemented with FCS due to its potential immunogenicity whether it was growth factors or other substances (for example endotoxins). Dendritic cells are professional antigen presenting cells and activate T helper cells upon contact, which in turn display the activation cell surface markers such as CD69. It is most likely that T helper cells became activated in the unstimulated tubes due to endotoxins in the FCS rich culture media. Strong evidence for this explanation was in **Figure 4.5.6b,** human AB sera were used instead of FCS a much less CD69 percentages (less than 6.5%) were obtained within the unstimulated preparations. Therefore comparing the scatter of each dataplot, the 6 hour incubation was the optimum incubation period. The stimulus of choice in this case was Ionomycin which delivered 84.21% CD69 expression in the test samples.

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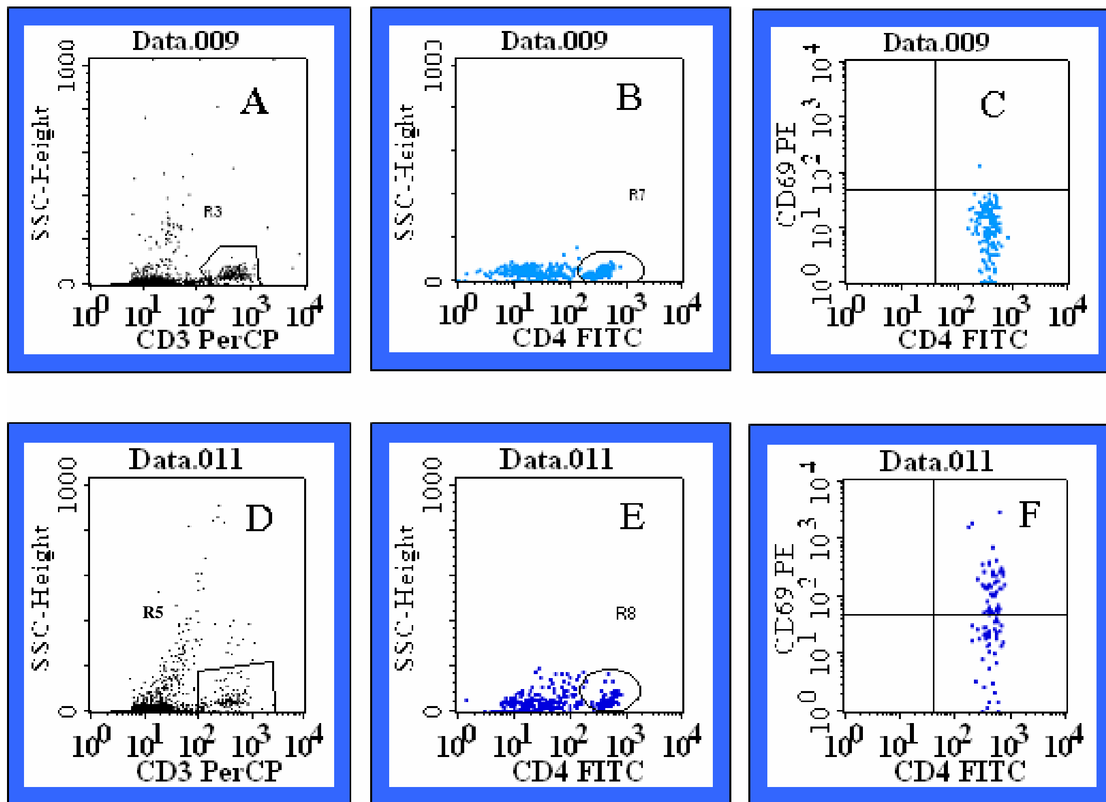

Figure 4.5.2a illustrates the difference for CD69 expression in an unstimulated (Plot A, B and C) and a stimulated (D, E and F) sample of isolated PBMCs. The cells were stimulated with Ionomycin. In plots A and D, the threshold was set on FL-3. From the primary gates R3 and R5, the next data plots B and E were described. From gates R7 and R8, the true CD3+CD4+CD69 population was analyzed (Plots C and F).

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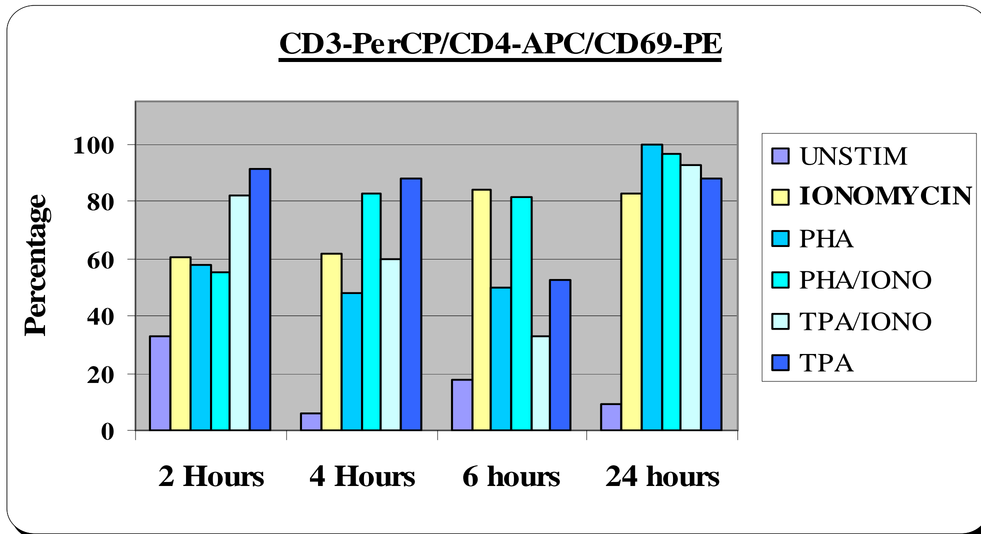

CD69-PE/CD3-PerCP/CD4-FITC PERCENTAGES				
	2 Hours	**4 Hours**	**6 hours**	**24 hours**
UNSTIM	32.73	5.63	17.65	9.09
IONO	60.38	61.76	**84.21**	82.91
PHA	57.89	48	50	100
IONO/PHA	55	82.61	81.25	96.84
IONO/TPA	82.35	60	33.11	92.8
TPA	91.67	88	52.86	87.89

Figure 4.5.2b: This diagram demonstrates that for the optimum CD69 expression, Ionomycin is the stimulus of choice. Furthermore it demonstrates that the incubation period with Ionomycin should take place for 6 hours. The percentage values are CD69 expression on CD3+/CD4+ cells.

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4.5.3 Activation: CD25 surface expression

All CD25 samples were analyzed using the BD FACSCalibur™ and CellQuest™ software. With specific instrument settings for CD25 expression, the threshold was set on FL-2. With acquisition, a total 150 000 events were acquired. The primary gate was set for the CD3 positive events. For all the stimulated tests unstimulated controls were included. **Figure 4.5.3a** illustrates the one example of the data plots obtained on the BD FACSCalibur™ for specific CD25 surface marker expression

For the investigation of CD25 surface molecule, isolated T lymphocytes were stimulated with TPA, Ionomycin, and TPA in combination with Ionomycin, PHA, and PHA in combination with Ionomycin. CD25 was expressed on these cells after activation by these stimuli and incubating for various incubation periods in a 5% CO_2 environment at 37ºC. The expression of CD25 on T helper cells is illustrated in **Figure 4.5.3b**. On this graph it might seem that at the 6 hour incubation time period, the other stimuli combinations give higher values of stimulation. But when one compared the scatter of the different data plots, it was concluded that the 6 hour incubation with PHA was the optimum stimulus resulting in approximately 43% CD25 expression with clearly distinct cell populations on the data plots. The six hour was much more convenient than the 24 hour incubation because of time lapse between setting up the experiment and the processing of the samples post-activation.

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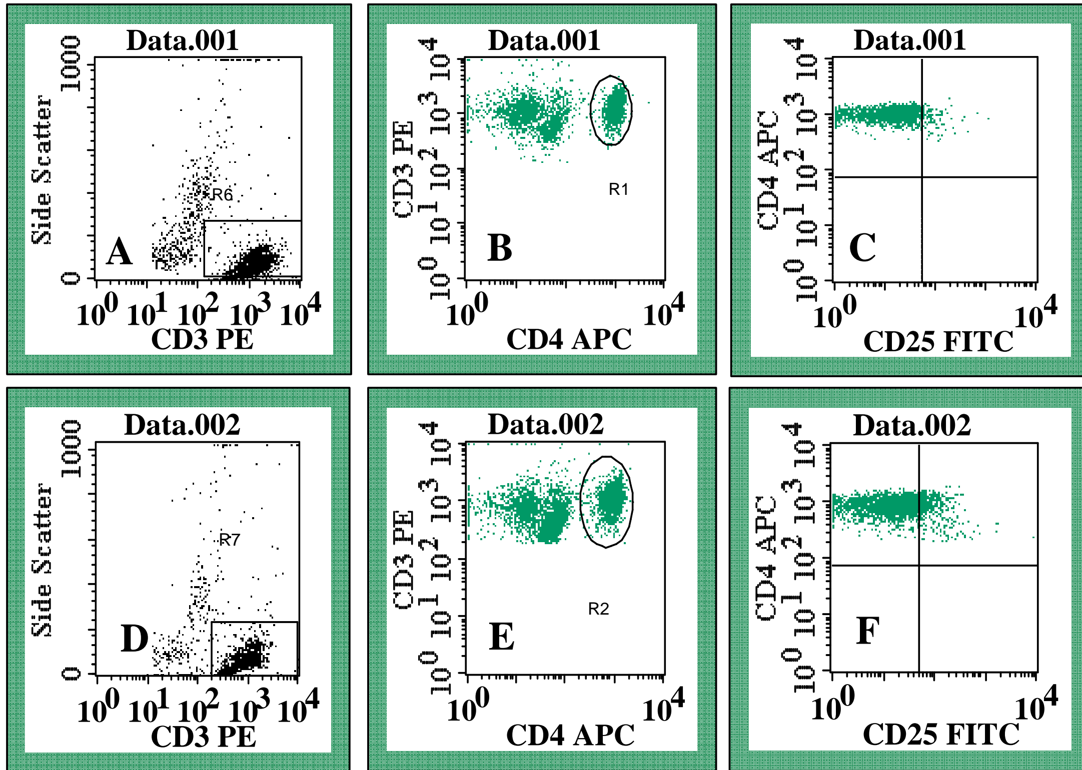

Figure 4.5.3a illustrates the difference in CD25 expression of an unstimulated (Plot A, B and C) and a stimulated (D, E and F) sample of isolated PBMCs. In Plot A and D the threshold was set on FL-2, and the primary gates were on the CD3 positive events (R6 and R7). From these gates the next data plot B and E was defined: R1 and R2 contained the true CD3+CD4+CD25+ population.

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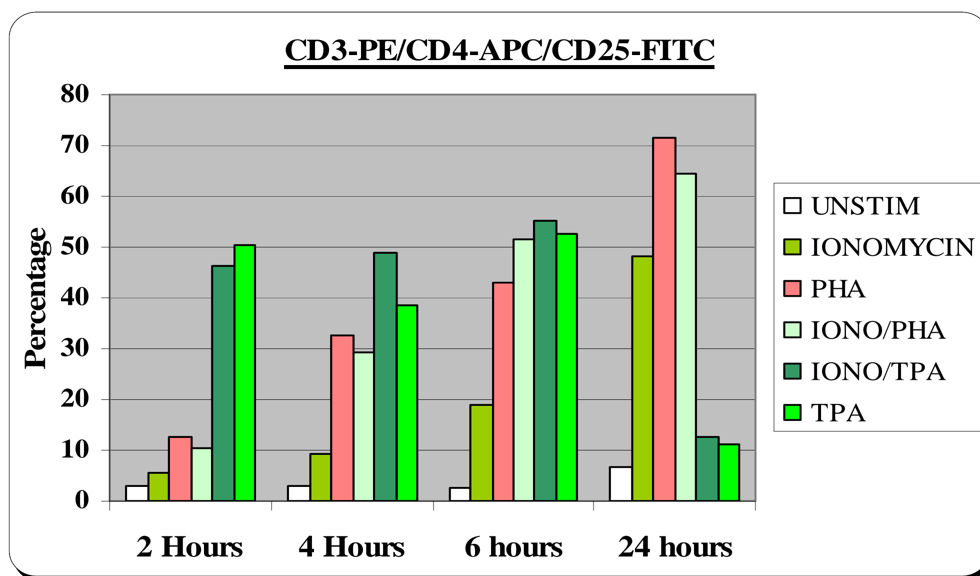

CD3-PE/CD4-APC/CD25-FITC PERCENTAGES				
	2 Hours	**4 Hours**	**6 hours**	**24 hours**
UNSTIM	2.96	2.95	2.54	6.78
IONO	5.53	9.08	18.81	48.16
PHA	12.54	32.73	**43**	71.55
IONO/PHA	10.37	29.22	51.35	64.3
IONO/TPA	46.18	49.04	55.35	12.41
TPA	50.23	38.69	52.53	11.15

Figure 4.5.3b: This diagram demonstrates that for the optimum CD25 expression, PHA was the stimulus of choice. Furthermore it demonstrates that the incubation period with PHA should take place for 6 hours in 5% CO_2. The percentage values are CD25 expression on CD3+/CD4+ cells.

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4.5.4 Activation: HLA-DR surface expression

All HLA-DR samples were analyzed using the FACSCalibur™ and CellQuest™ software. With specific instrument settings for HLA-DR expression, the threshold was set on FL-2. With acquisition, a total 150 000 events were acquired. The primary gate was set for the CD3 positive events. For all the stimulated tests unstimulated controls were included. **Figure 4.5.4a** illustrates the one example of the data plots obtained on the BD FACSCalibur™ for specific HLA-DR surface marker expression.

After incubation at various time intervals (2, 4, 6 and 24 hours) in a 5% CO_2 environment (at 37ºC), HLA-DR surface antigens were monitored. The percentage HLA-DR expression is illustrated in **Figure 4.5.4b**. In this graph it might seem at the 6 hour incubation time period, the stimuli in combinations gave higher percentages of activation. Considering cost saving, the PHA stimulant was the choice, resulting in approximately 43.32% of HLA-DR expression. The 6 hour was much more convenient than the 24 hour incubation because of time between the setting up and the analysis of the samples.

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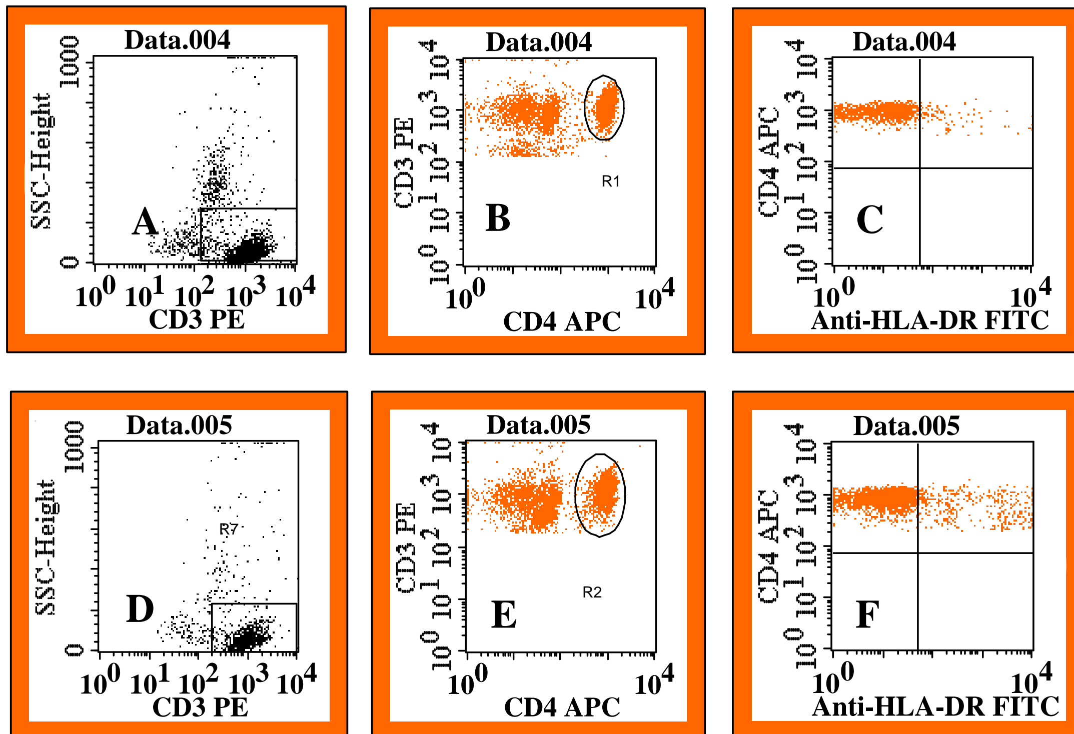

Figure 4.5.4a illustrates the difference in HLA-DR expression for an unstimulated (Plot A, B and C) and a stimulated (D, E and F) sample of isolated PBMCs. In Plot A and D, the threshold was set on FL-2. The primary gate (R6 and R7 respectively) was set on the CD3 positive events. Out of R6 and R7, plot B and E was conducted. In regions R1 and R2, the true CD3+CD4+HLA-DR+ population of cells were analyzed.

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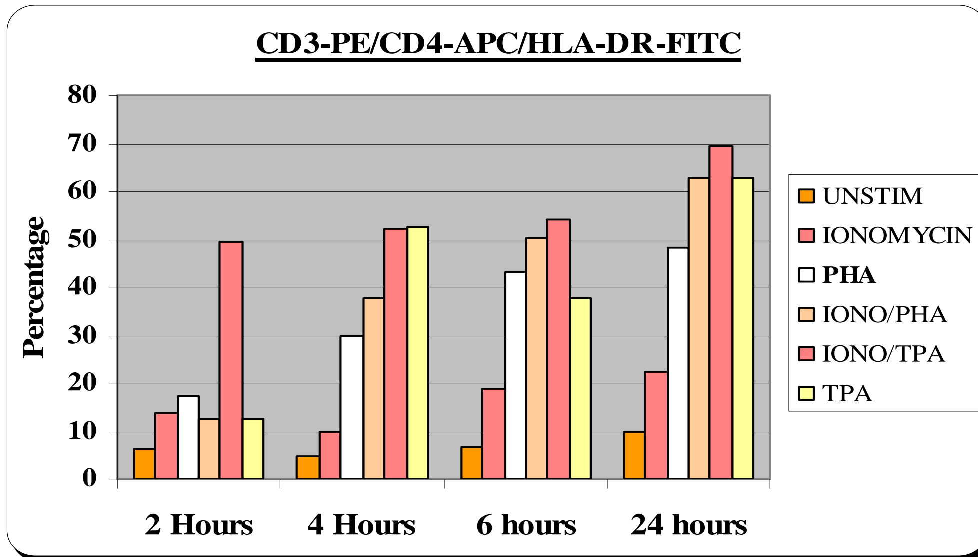

CD3-PE/CD4-APC/HLA-DR-FITC PERCENTAGES				
	2 Hours	**4 Hours**	**6 hours**	**24 hours**
UNSTIM	6.12	4.56	6.86	9.63
IONO	13.71	9.61	18.69	22.48
PHA	17.27	29.65	**43.32**	48.29
IONO/PHA	12.58	37.5	50.16	62.69
IONO/TPA	49.4	52.13	54.24	69.46
TPA	12.7	52.73	37.84	62.59

Figure 4.5.4b: This diagram demonstrates that for the most favorable HLA-DR expression, PHA was the stimulus of choice for this study. Furthermore it demonstrates that the incubation period with PHA should take place for 6 hours in 5% CO_2. The percentage values are HLA-DR expression on the CD3+/CD4+ cells.

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4.5.5 Stabilization: activated surface CD40Ligand

CD40 Ligand activated T helper cells were stabilized with various concentrations of paraformaldehyde for 1, 4 and 24 hour time intervals. Different concentrations of paraformaldehyde (1.4%, 0.7%, 0.35%, 0.175%, 0.0875% and 0%) were added to the activated cells as secondary stabilizing agent (in combination with 0.25% chromium chloride as first stabilizing agent). These samples were stained with anti-CD3-PE, anti-CD4-PerCP and anti-CD40L-APC monoclonal antibodies and analyzed on random days, once a day for 23 days. Statistics could not be performed due to the fact that this was only one sample that was prepared in the prescribed manner. However some important observations were made that was applicable for further use. What was interesting to observe was the morphology of the activated cell surfaces incubated with 1.4% paraformaldehyde: these cells degenerated extremely fast: the paraformaldehyde concentrations were possibly too high for the cells and the CD40L+ populations could no longer be distinguishable from the other cells populations and these samples were discarded by day 15. **Table 4.5.5** illustrates the percentage CD40L in the CD3+/ CD4+ population of T cells after these samples have been stabilized with various concentrations and incubation periods of paraformaldehyde. It was observed that the samples incubated for 24 hours with various paraformaldehyde concentrations did not show stability and these samples were therefore excluded from further analysis after day 15. By visual inspection we concluded that 0% paraformaldehyde at 1 hour incubation, meaning only 0.25% chromium chloride as stabilizing agent is the method of choice for CD40L IQA samples; stability can be obtained until day 23.

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Table 4.5.5 illustrates activated IQA samples that were stabilized with 1.4%, 0.7%, 0.35%, 0.175%, 0.0875% and 0% paraformaldehyde as second stabilizing agent. These samples were incubated for 1 hour, 4 hours and 24 hours with various concentrations of paraformaldehyde. These samples were monitored for CD40L percentage in the T helper cell population.

Concentration paraformaldehyde	Incubation time	DAY 1	DAY 4	DAY 7	DAY 11	DAY 15	DAY 18	DAY 23
1.4% paraformaldehyde	**1 hour**	30.9	50.75	48.89	not run	44.54	not run	not run
1.4% paraformaldehyde	4 hours	26.31	58.3	57.77	68.89	80.75	not run	not run
1.4% paraformaldehyde	24 hours	31.71	61.45	66.23	76.9	84.5	not run	not run
0.7% paraformaldehyde	**1 hour**	31.98	27.59	21.9	27.9	30.24	30.22	26.66
0.7% paraformaldehyde	4 hours	25.37	34.59	23.33	36.51	37.14	34.21	19.75
0.7% paraformaldehyde	24 hours	31.99	49.44	42.64	49.03	50.88	not run	not run
0.35% paraformaldehyde	**1 hour**	34.53	28.22	30.09	42.68	41.32	54.04	43.93
0.35% paraformaldehyde	4 hours	27.28	37.98	25.16	49.05	47.74	not run	not run
0.35% paraformaldehyde	24 hours	41.93	41.53	22.05	28.67	35.89	not run	not run
0.175% paraformaldehyde	**1 hour**	19.89	10.23	7.23	12.04	8.41	8.37	16.85
0.175% paraformaldehyde	4 hours	18.42	11.73	11.13	23.02	19.63	24.4	32.014
0.175% paraformaldehyde	24 hours	35.89	19.35	7.4	49.75	17.31	not run	not run
0.0875% paraformaldehyde	**1 hour**	22.97	10.49	8.54	22.17	17.12	20.83	24.84
0.0875% paraformaldehyde	4 hours	15.69	13.55	10.77	21.81	25.31	not run	not run
0.0875% paraformaldehyde	24 hours	42.86	18.78	12.03	34.9	27.68	not run	not run
0% paraformaldehyde	**1 hour**	26.8	22.27	20.04	24.29	23.03	21.68	25.55
0% paraformaldehyde	4 hours	22.1	29.44	22.16	41.42	32.24	35.15	40.05
0% paraformaldehyde	24 hours	35.82	25.62	28.45	40.27	27.08	33.11	50.94

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Only one stabilized sample was included in this experiment due to extensive reagent costs. Therefore no statistical analysis were performed on this sample but it was clearly visible considering the Levey Jennings plots and the scatter plots on the flow cytometer which concentration was more preferable. Please follow **Figure 4.5.5a** and **Figure 4.5.5b** (data obtained from **Table 4.5.5**), which illustrates it was clearly visible that the 1 hour incubation with paraformaldehyde produced much better results than the four hour incubation. The 2SD values were not indicated on these two graphs as the values overlap each other on the paraformaldehyde concentration levels. The scatter plots obtained from flow cytometry was not shown. The 24 hour secondary stabilization data was not very reproducible: the scatter data changed rapidly and the expression of the CD40L marker changed dramatically. For this reason, we did not include these data on the Levey Jennings plot and was excluded from any further experimentation.

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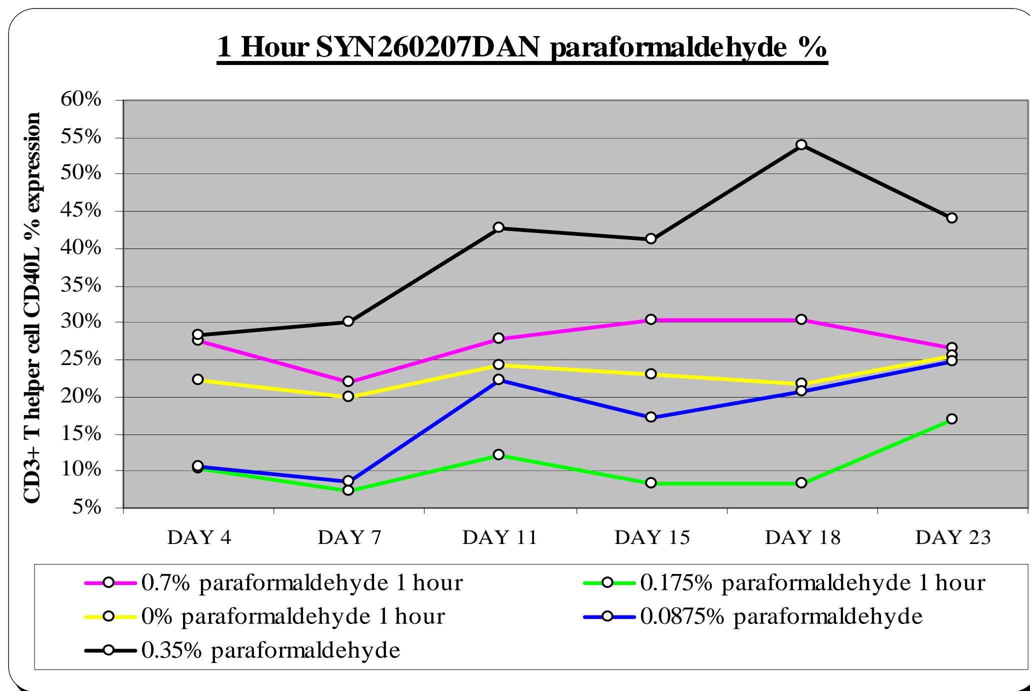

Figure 4.5.5a Data for CD40L expression represented on the Levey Jennings Plot to illustrate the stability in the various concentrations of paraformaldehyde that was used as second stabilizing agent. The samples were incubated for one hour with various concentrations of paraformaldehyde (in combination with 0.25% chromium chloride): the 0.7%, 0.175% and 0% paraformaldehyde were much more stable than 0.35% and 0.0875% paraformaldehyde concentrations. This is example of only one stabilized sample; therefore no SD bars were indicated.

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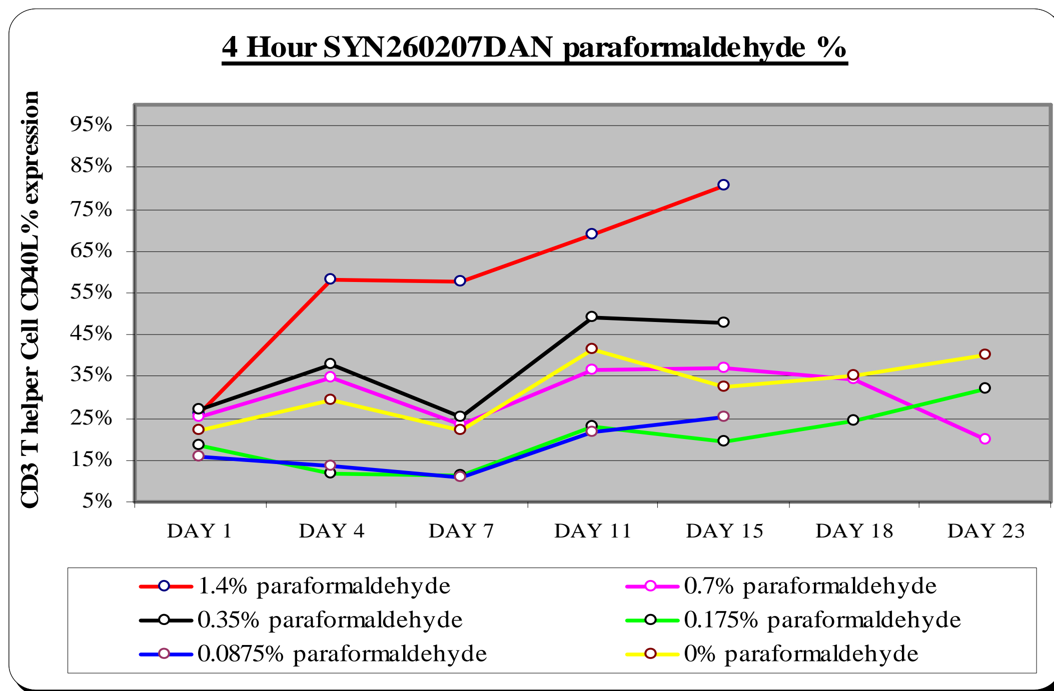

Figure 4.5.5b: the Levey Jennings Plot illustrating CD40L expression recorded for 4 hour incubation time with various concentrations of paraformaldehyde. The samples were monitored for 23 days. The 0.0875%, 1.4% and 0.35% paraformaldehyde concentrations were discarded after day 15 and excluded from any further experimentation. This is example of only one stabilized sample; therefore no SD bars were indicated.

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4.5.6 Stabilization: Activated samples expressing CD69, CD25 and HLA-DR

To stabilize activation markers such as CD25, HLA-DR and CD69, chromium chloride on its own was not sufficient. The samples stabilized with chromium chloride only showed a sufficient decrease in percentage values for the activation expression after treatment already (on day 1) and this method was not suitable for further investigation. The 0.35% w/v paraformaldehyde in combination with chromium chloride was the better option and was chosen as method of stabilizing these activation markers. Data for this experiment was not shown. After establishing the most suitable secondary stabilizing agent, further investigation took place. A batch was put up after the activated cells had been stabilized (with 0.25% chromium chloride in combination with 0.35% w/v paraformaldehyde) and resuspended in PBS and a second batch was resuspended in human AB sera. In batch one, as clearly visible in **Table 4.5.6a** the unstimulated stabilized whole blood samples had very high percentages of CD25, CD69 and HLA-DR expression for this batch, possibly due to non-specific staining. Another observation was the clumping of the activated cells even after stabilization. No further data analysis could be performed after day 11 already. It was then thought of blocking all the excess antibody binding sites by adding human antibodies: this could be done by using from Human AB serum. The second batch was prepared and the cells were re-suspended after activation and stabilization (0.25% chromium chloride in combination with 0.35% w/v paraformaldehyde) in heat-inactivated human AB serum: **Table 4.5.6b** illustrates the data obtained from this experiment. The unstimulated control samples had much less indication of the non-specific staining and these samples proved to be more promising for being able to be analyzed until day 16. Another interesting observation was the 75% versus the 50% spiked samples did not show major differences in the percentages of cells expressing the activation markers.

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As mentioned earlier the higher unstimulated values might be due to endotoxins in the FCS when used in the complete media to make up the final concentration of cells before stimulation. The second batch of activated cells was treated with human AB sera instead and yields much lower unstimulated values. No further investigations were performed to confirm this.

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Table 4.5.6a illustrates the percentage activation markers on CD3+/CD4+ cells. The CD25, CD69 and HLA-DR data was obtained from stabilized activated lymphocytes analyzed on day 2, day 8 and day 11. During stabilization at the final centrifugation step, the cells were re-suspended in PBS.

PBS	**CD25-FITC**	**UNSTIM WBL**	**PHA STIM 6 hrs**	**75% PBMC/ 25%**	**50% PBMC/ 50%**
DAY 2	05-Apr-07	11.33	19.56	Not run	Not run
DAY 8	10-Apr-07	11.86	19.79	26.33	11.86
DAY 11	13-Apr-07	15.44	33.99	15.44	17.76
	HLADR-FITC	**UNSTIM WBL**	**PHA STIM 6 hrs**	**75% PBMC/ 25%**	**50% PBMC/ 50%**
DAY 2	05-Apr-07	15.02	24.61	Not run	Not run
DAY 8	10-Apr-07	11.03	33.16	14.22	21.72
DAY 11	13-Apr-07	15.34	30.07	17.14	15.93
	CD69-PE	**UNSTIM WBL**	**PHA STIM 6 hrs**	**75% PBMC/ 25%**	**50% PBMC/ 50%**
DAY 2	05-Apr-07	4.95	90	Not run	Not run
DAY 8	10-Apr-07	6.28	92.89	36.18	20.94
DAY 11	13-Apr-07	6.44	86.67	24.11	20.24

PBS = phosphate buffered saline, UNSTIM WBL = stabilized whole blood that was not manipulated with a stimulus for activation, PHA STIM= Cells stimulated with Phytoheamagglutinin and Apr = April.

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Table 4.5.6b illustrates the percentage activation markers on CD3+/CD4+ cells. The CD25, CD69 and HLA-DR data was obtained from stabilized activated lymphocytes run on day 2, day 6 and day 16. During stabilization at the final centrifugation step, the cells were re-suspended in decomplemented human AB sera.

AB SERA	CD25-FITC	UNSTIM WBL	IONO STIM 4 hrs	75% PBMC/ 25%	50% PBMC/ 50%
DAY 2	19-Apr-07	4.1	8	7.13	5.11
DAY 6	23-Apr-07	4.69	41.36	7.84	7.01
DAY 16	03-May-07	4.94	37.01	10.55	9.11
	HLADR-FITC	**UNSTIM WBL**	**IONO STIM 4 hrs**	**75% PBMC/ 25%**	**50% PBMC/ 50%**
DAY 2	19-Apr-07	6.24	11.11	9.01	8.66
DAY 6	23-Apr-07	6.41	16.57	8.44	8.88
DAY 16	03-May-07	6.5	16.97	9.24	8.9
	CD69-PE	**UNSTIM WBL**	**IONO STIM 4 hrs**	**75% PBMC/ 25%**	**50% PBMC/ 50%**
DAY 2	19-Apr-07	3.92	73.35	29.81	26.28
DAY 6	23-Apr-07	4.4	71.53	24.83	22.17
DAY 16	03-May-07	5.8	65.02	21.27	17.19

AB SERA = decomplemented human AB serum, UNSTIM WBL = stabilized whole blood that was not manipulated with a stimulus for activation, IONO STIM = cells stimulated with Ionomycin and Apr = April.

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4.6 DISCUSSION

In this chapter, the activation of PBMCs using different agents was investigated: for example Ionomycin, PHA, TPA, CD28 pure and CD49 pure and a combination thereof were used in order to induce the optimal expression of the activation cell surface antigens. Sato *et al.* (2004) stimulated PBMCs with PHA for three days and thereafter, stimulated the same cells with 1 µg/ml Ionomycin for another three hours. According to the authors, the activated T cells expressed less than 15 percent CD154 (CD40L) after the first incubation of 3 days with only the PHA. The cells described here were between 70 – 90 percent CD154/CD4 positive after the three hour Ionomycin incubation. It was mentioned by Chambers *et al*. (1999) that CD28 enhanced memory T cell responses. The use of CD28 and CD49 in combination with Ionomycin was investigated and valuable results (higher amounts of CD40L expression) were obtained. Due to the expense of these co-stimulatory factors, they were not further investigated or considered.

TPA and/or in combination with Ionomycin activated the T helper cells aggressively early on after contact: the T cells were over-stimulated and down-regulation of the CD4 surface molecules were observed (data not shown). This down-regulation of the activated CD4 cells made the sample not suitable for further stabilization and quality control requirements. Protein shedding is enhanced by stimulation with protein Kinase C (PKC) activators such as TPA and it is feasible that what we observed (low levels of CD40L expression) can be accounted by the secretion of the ligand into solution.

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Phorbol ester stimulation enhances sCD40L production, but this process can be blocked by metalloproteinase inhibitors [Hsu *et al*. (1997) and Hirohata (1999)]. It has been suggested that PKC initially increases membrane CD40L and at later time points, it may promote CD40L processing and release of the soluble form. Shedding is the proteolytic release of the extracellular domains of cell-surface proteins. For IQA samples one needs a stabilized sample that could represent or mimic a fresh activated sample's characteristics. It has been hypothesized that proteolytic cleavage of membrane CD40L on T cells gives rise to the 18 kDA protein [Hsu *et al*. (1997) and Hirohata (1999)] which might be the soluble form of CD40L. That might be a reason why the CD40L decreases with extended incubation periods. (Please refer to **Figure 4.5.1b**).

Membrane bound CD40L was of interest and the production of soluble CD40L was thus not investigated. Wingett and Nielson (2002) documented that no significant expression of soluble CD40L could be observed at any Ionomycin concentration tested. For this reason the suggestion of utilizing only Ionomycin as stimulant to activate T helper cells was investigated. The use of Ionomycin only was confirmed to be the stimuli of choice for sufficient surface CD40L expression. Additionally after using various concentrations of ionomycin and at different time intervals, the incubation of the cells with 4μM ionomycin delivered for 4 hours 36% of all the CD4+ T cells.

For the CD69 expression on T helper cells, the 6 hour incubation appeared to be the optimum incubation period: Ionomycin stimulation had shown an increase over time and peaked at 6 hours, with a decline at 8 hours. The stimulus of choice in this case was Ionomycin which induced 84.21% CD69 expression in the test samples. The 24 hour incubation period gave higher CD69 expression, but was not considered because of the time between setting up the experiment and the processing of the samples.

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PHA in combination with Ionomycin gave excellent results of 96.84% CD69 positivity, but, due to cost saving, Ionomycin on its own was sufficient.

For the expression of CD25 on T helper cells, the comparison of the scatter data plots led us to the conclusion that 6 hour incubation with PHA was the optimum stimulus, resulting in approximately 43% CD25 expression with clearly distinct cell populations on the data plots. The 24 hour incubation period with the various stimuli gave higher CD25 expression, but again was not considered due to the time issue.

Likewise, for the HLA-DR expression, the 6 hour incubation time period for the combination stimuli (Ionomycin / PHA and Ionomycin / TPA) was not considered as optimal incubation period even though higher percentages of activation were obtained. Due to cost consideration, PHA was the stimulant of choice, resulting in approximately 43.32% HLA-DR expression at 6 hour incubation. The 6 hour incubation with PHA simplified the process of IQA samples; the same method is used for CD25 stimulation conditions. Therefore one IQA stabilized sample can be processed where both CD25 and/or HLA-DR activation markers can be monitored in the same sample.

Current formulations for stabilizing cells unfortunately contain one or more agents which are highly reactive with the proteins of the cells. They denature and insolubilize the cells' membranes and intercellular components, sometimes making it impossible to stain these proteins using monoclonal antibodies. For example, certain compounds like those used to stabilize whole blood cells may preserve some cellular antigens though certain antigens are more sensitive and lose their reactivity. Examples of such antigens are the CD antigens present on the surface of hematopoietic stem cells and other activation markers on stimulated T lymphocytes.

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Much of the clinical useful applications of surface marker analysis and the development of surface marker assay technology have focused on lymphocyte CD markers. It would be desirable to provide a formulation which stabilizes white blood cells without losing CD antigen reactivity. Activated lymphocyte cell populations were processed / stabilized in the same manner as whole blood preparations. Soon it was discovered that the lymphocytes are much more labile and not only tend to clump together but they lose their side scatter properties early on in the experiment. The concentrations of the second stabilizing agent had to be re-evaluated and this was one of the aims of this thesis.

After the evaluation of several secondary agents, it was found that paraformaldehyde was the second stabilizing agent of choice and this was evaluated at 1.4%, 0.7%, 0.35%, 0.175%, 0.0875% and 0%. What was interesting to observe was the morphology of the activated cell surfaces incubated with 1.4% paraformaldehyde: activated cells in this high concentration of the fixative disintegrated extremely fast and lost their expression of the CD40L.

It was established that samples that had been incubated for 24 hours with all the various paraformaldehyde concentrations could still be analyzed until day 15. Thereafter the cell morphology changed and the scatter plot on the flow cytometer was distorted; the samples were excluded from further experimental studies and discarded. It was thought that for the 24 hours incubation, the cells were too long in contact with the paraformaldehyde. This assumption was made on the fact that the sample without any paraformaldehyde contact (0% paraformaldehyde) and incubated for the 24 hours only in chromium chloride showed identifiable CD40L+ cell populations.

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These samples that were treated with 0% paraformaldehyde (only chromium chloride) were monitored and CD40L could be identified and quantified with repeatable results for more than three weeks (23 days). With whole blood stabilization studies, Barnett *et al.* (1999) and Granger *et al*. (2001) confirmed, the presence of only the first stabilizing agent can indeed prevent the leucocytes from exhibiting excessive autofluorescence and at the same time can stabilize the leucocytes for periods of longer than 25 days. It was not that straightforward when activated lymphocytes were stabilized: once activated, the lymphocytes are a lot more fragile and lose their membrane markers more easily than when not activated. The secondary agent therefore has to be evaluated carefully if such preparations are to be used long term. In the case of CD40L expressing cells, it appears that no secondary agent is better than using paraformaldehyde as an agent.

Similarly the stabilization of T helper cells expressing CD69, CD25 and HLA-DR, a combination of stabilizing methods was employed. After the first attempt, it was quickly discovered that the first challenge was to prevent the activated cells from clumping together. As with whole blood stabilization, the first stabilizing agent was aged filtered 0.25% chromium chloride (pH close to 6.7). The cells were incubated with this agent for approximately one hour. The second stabilization agent was paraformaldehyde in combination with chromium chloride. The use of 0.35% paraformaldehyde and 0% paraformaldehyde and incubated for 2 hours were investigated. The shorter incubation periods were utilized for a gentler fixation step. After the last wash, the cells were first resuspended in sterile PBS and monitored for the various activation molecules. In the early stages of the stabilization monitoring, there appeared to be a suspicious population of fluorescence that could not be explained. This might have been due to either auto fluorescence, or non-specific antibody binding.

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It was mentioned at an Immune Function BD training seminar (Nov 2007) that activated platelets tend to bind non-specifically especially to FITC (FL-1) when under stress which might be another reason for the suspicious fluorescent population due to stabilization of the remaining platelets in the cell preparations. Another reason might be due to the platelets themselves expressing CD40L upon activation. This was demonstrated by Henn *et al.* (1998), where human platelets were analysed by flow cytometry. They found that the activated platelets expressed CD40L on their surfaces, but no CD40L was detected on the unstimulated platelets. The same experiment was repeated, except the activated cells were this time re-suspended in decomplemented human AB sera. The addition of the human AB sera was thought to bind and block the various non-specific antibody binding sites. This process seemed to address the issue of non-specific fluorescence: longer stability was achieved in the preparations where the cells were resuspended in decomplemented Human AB sera. The human AB serum is rich in antibodies that bind to non-specific antibody binding sites. It should be brought to the attention of the reader that further investigations are needed in order to extend the shelf life of such preparations. We found that sometimes, clumping of the cells occurred and this could be due to the presence of some platelets remaining in the preparation and that these could still undergo their physiological functions. It was demonstrated by Read *et al.* (1995); with a mild aldehyde cross-linking (1.8% for human platelets and 0.68% paraformaldehyde for canine platelets), re-hydrated, lyophilized platelets have near to normal ultra-structure by electron-microscopy but more importantly, they retain many of the surface membrane function of fresh platelets. In other words, the platelets still retained their functions and clotting actions even if they were in contact with the paraformaldehyde. The percentage paraformaldehyde we use is only 0.35% which may not inhibit the clotting properties of the platelets in the stabilized blood preparations.

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Recommendation for future studies is to investigate a method where the platelets and their factors are removed completely. This can be done at the step where the whole blood is centrifuged and the plasma is removed. The plasma on its own can be spun down at fast speeds to pellet the platelets and clotting factors. Then the supernatant decomplemented and used as medium to re-suspend the final activated cell suspensions in.

The aim of spiking stabilized samples was that the sample can represent a “kit” containing three different levels of activated stabilized control samples. The IQA kit could represent or mimic fresh activated samples where the red blood cells are still capable of being lysed. These “activated reference material” should simulate the scatter data plots of fresh activated samples as they are acquired and analyzed on the BD FACSCalibur™. Samples were prepared where 75% of the activated lymphocytes were added to 25% stabilized whole blood (v/v) to serve as a “high” activation marker control. Another sample was created where 50% of the activated PBMCs were added to 50% of the stabilized whole blood to serve as a “lower” activation control. The whole blood that was not manipulated with a stimulant would serve as the “zero” activated control. Stability could be obtained for 16 days for the CD25, CD69 and HLA-DR sample preparations and 23 days for the CD40 Ligand stabilized preparations. When comparing the 75% versus the 50% spiked samples, where the cells were re-suspended in human AB sera, in most cases the 75% spiked samples did deliver higher percentages of activation than the 50% spiked samples in the entire phenotypic surface molecules monitored. The unstimulated whole blood preparations showed very low levels of activation marker expressions as suspected.

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These spiked samples are very promising for future IQA control kits. They can be used either for training of people that are involved in less routine and more specialized immunological clinical set up or alternatively, be used in a laboratory where the activated cell populations are being monitored in patients participating in clinical trials making use of biologicals targeting these activated cells (for example, Rituximab which targets the CD40L surface marker in Rheumatoid arthritis patients). It is critical to determine whether the activated cells are being depleted from the circulation of the patients and to determine clinical outcomes. Such stabilized samples can be used as IQA during the routine monitoring of patients samples.

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CHAPTER 5

GENERAL CONCLUSION

Flow cytometry today has become such a major diagnostic and prognostic tool. The importance to be subjected to quality control procedures and participation in internal quality assurance (IQA) program can not be emphasized enough. Together with previous research done by great scientists, we have improved the technique in stabilizing whole blood samples for so called IQA samples. The aim of this thesis was to stabilize whole blood with a shelf life of greater than 40 days. The improved method for stabilizing whole blood samples might just be the break clinical laboratory staff was looking for. In-house studies have confirmed stability of both the light scatter and staining characteristics of the preparations. The excellent light scatter patterns enable easy identification of lymphocytes, monocytes and granulocytes by using CD45/SSC gating. These cell populations can be quantified and implemented as IQA for daily inter laboratory processes up to 40 days. The IQA reference sample will then serve to monitor the major instrument settings that can affect the reliability, reproducibility and sensitivity of their cytometer. This should then be documented in order to ensure identical conditions of measurement on a daily basis. It is important to take note that these samples should be stored and transported at 4ºC. These stabilized blood samples show potential to meet the need for a more improved method for stabilizing not only whole blood, red blood cells, white blood cells, but further manipulated blood cell products. Complete compatibility was demonstrated with both FACSCalibur™ and FACSCount™ flow cytometers.

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These stabilized whole blood samples can be utilized as a "healthy/ normal control" for calibration applications with the FACSCount™ instruments where such bloods are not always accessible. As part of an external pilot study; this thesis improved the method of stabilizing whole blood for IQA samples and showed promise in the training of newly qualified students and laboratory staff in HIV clinical settings. This product has further developed as BD (South Africa) approached us to make use of these reference control samples. The goal of the IQA samples for BD was to serve as a guideline, to point out quantitative and qualitative differences when compared against an established standard reference. BD already made use of these IQA samples on a monthly basis to present workshops with practical training sessions. Bloods were drawn from healthy individuals after the necessary consent was attained. These bloods were stabilized, and monitored from day 3 for the various routine HIV phenotypic cell surface markers. Reference ranges were established with a mean, 2SD and 3SD values and provided to BD with sample collection or transport. The competency of the students as well as the training instructor's ability to communicate the methodology of flow cytometry was thoroughly evaluated. This is especially appreciated in the poorer African countries where language dialect barriers can be breached with teaching through interpreters jointly with practical demonstrations. Students received training as far as Namibia and up in poorer African countries. They have received training on their own FACSCount™ and those who had FACSCalibur™ cytometers and the results they obtained fall well within the reference ranges provided.

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Until today the field of clinical diagnosis has been rapidly expanding. New diagnostic techniques have been developed and older techniques have been improved. The equipment capable of making clinical measurements has also been enhanced in recent years. The instruments/ equipment used, methodology employed and staff competency require periodic verification of their ability to perform properly and accurately. The design of this thesis was related to the preparation of control samples (IQA) designed to simulate whole blood having defined properties in clinical laboratory situations. Verification is usually obtained using control samples having a predetermined property, typically a set value (reference ranges) for the property being measured. T cell stimulation is a complex event that is primarily detected by surface molecules on flow cytometers. These surface molecules stimulate different responses depending on the quantitative and qualitative differences in surface molecule composition.

We have noticed in early method development that the 0.35% w/v paraformaldehyde is one example of a reagent that results in down-regulation of CD40L on activated T lymphocytes and results in a complete loss of membrane bound CD40L is found over a short period of time. The mechanism by which the paraformaldehyde decreases the CD40L antigen expression is not fully understood. We hypothesize that too high concentrations of paraformaldehyde can cause the membrane bound CD40L to go into solution and they become soluble CD40L. This was not part of the aim of this thesis and further investigation was not implemented.

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Another challenge stabilizing activated cells is that certain critical antigens are present in small quantities and thus become undetectable, especially when even a small percentage of these antigens are destroyed and cellular detail is required. We found that to stabilize the CD40L expressing T lymphocytes, it is better to only use chromium chloride in absence of paraformaldehyde. The lymphocytes will express about the same amounts of CD40L on their surfaces for more than three weeks. After four weeks the cell membranes become totally distorted and the scatter plot of the primary gate becomes unclear to gate the desired population of cells.

In future kits can be developed with a zero, low and high activation control sample for the various activated surface molecules. These “activation IQA kits” can be implemented for training of newly qualified staff, competency testing of staff, method development, software testing, panel settings and instrument setting testing. Control samples ideally must have a number of properties in order to be effective. For instance stability during storage times, preferably lasting more than a few weeks, reproducibility and ease of handling. The use of a fresh blood on a daily basis fails to provide the information on day-to-day variation of the technique or equipment. Cells are considered stabilized if they are monitored via flow cytometric leucocyte immunophenotyping techniques for more than 8 days. It is not disregarded that with this invention of activated stabilized IQA samples, still further testing is required to reach stability of a month or longer. Although the heavy metal compound when used alone was found to have a significant stabilizing effect over a period of 15 days in some cases, it has in general not been found to give stability for 30 days, which might be regarded as a commercially desirable storage life for these activated IQA samples.

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Future recommendations

It might be useful to stabilize a larger volume of whole blood from healthy individuals together with a larger study group to validate more accurately the period these bloods can be stabilized. Stability is not only defined as the identification of various cell populations for routine HIV monitoring and the quantification of these cell populations but also includes the variations in percentage and absolute values obtained for each individual cell population.

Clinical application of flow cytometry implies that the results produced can lead to diagnosis and thus can influence the therapeutic decision. To date we still do not have adequate control/reference material which will mimic the great diversity of properties specific for leukemic blasts. An additional upcoming requirement identified is that an internal quality control sample must be established in the field of immunophenotyping for leukemia and lymphoma phenotyping. The procedure of stabilizing whole blood in Chapter 3 should be investigated in the use of known leukemic patient's blood. It would be profitable to investigate the possibility to stabilize the blood after consent was obtained. The first application will be to identify populations of the cells based on their phenotypic characteristics. The second application would include procedures which quantify a particular population of cells. The same can be applied for the development of CD34 positive (stem cells) quantification in peripheral blood or cord blood, if it was possible to stabilize these samples.

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CHAPTER 3:

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APPENDICES

APPENDIX 1:

Diagram A, B, C and **D** illustrates the Levey Jennings Plot for the stability of one example of the reference control sample monitored for 77 days. This sample was stabilized with 0.25% chromium chloride and 0.35% w/v paraformaldehyde. The sample was monitored for the routine phenotypic cell surface markers and was analyzed on the BD FACSCalibur™. The upper and lower limits (expected ranges) were indicated on each graph with a yellow line representing the mean values.

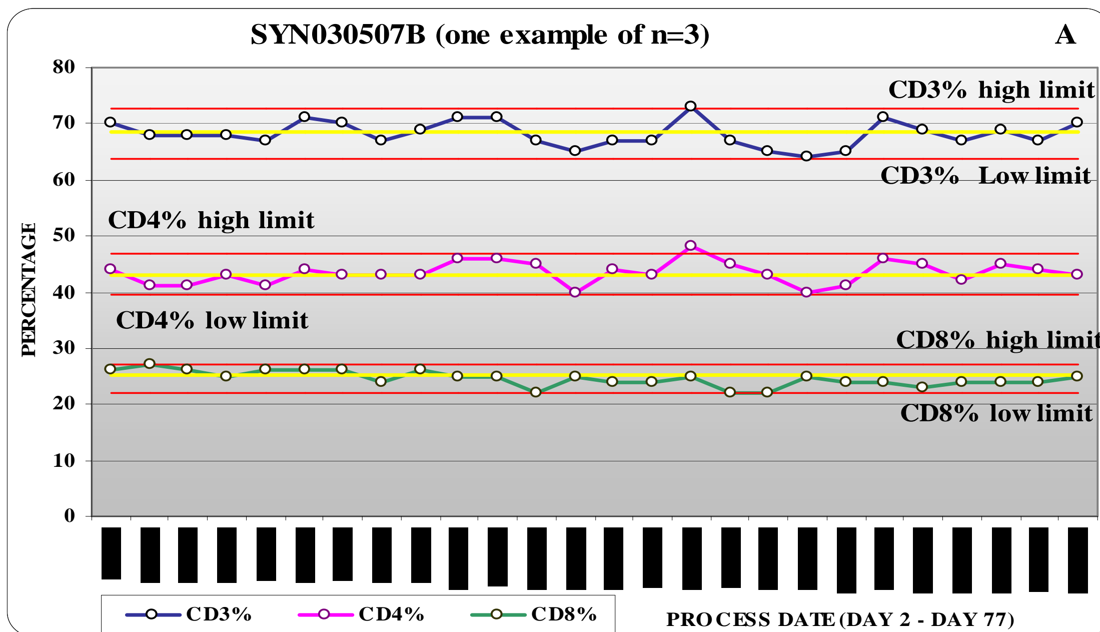

Diagram A illustrates the CD3, CD4 and CD8 percentages with the higher and lower expected ranges as indicated individually for with each parameter. The yellow lines indicated the mean value for each parameter Stability was obtained until day 77 for the percentage (CD3, CD4 and CD8) values.

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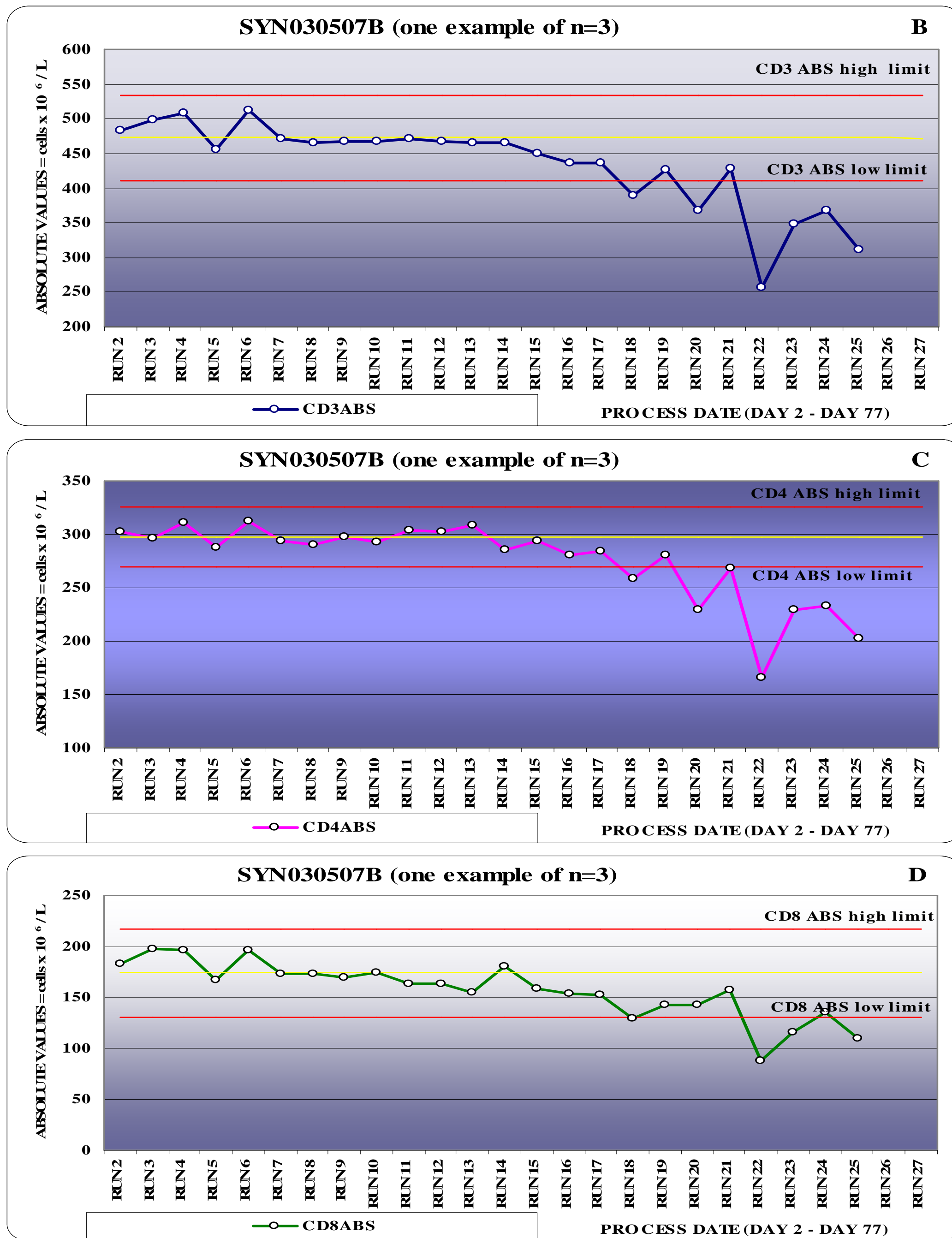

Diagram B, C, and D illustrates the absolute values for CD3, CD4 and CD8 with the upper and lower limits indicated until day 77 (run 27). Absolute cell values on the y-axis represent cells x 10^6 / L. In our thesis we can confidently say that these samples are stabile until day 40. Thereafter (as of run 18) the cell populations were still clearly identifiable and quantifiable, however the absolute values suddenly decreased.

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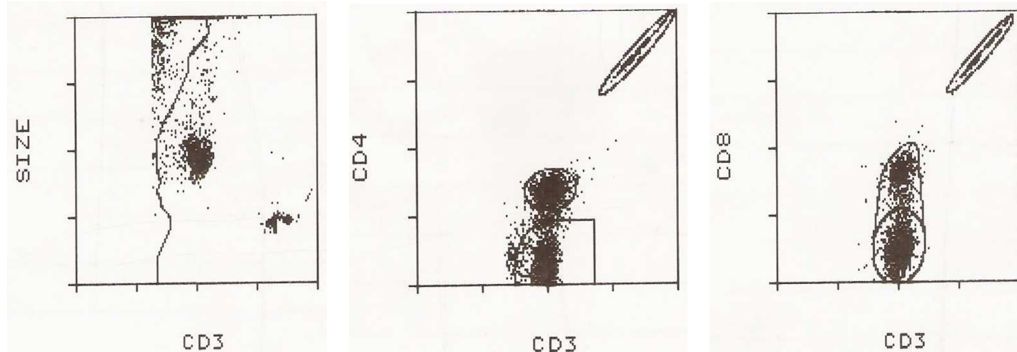
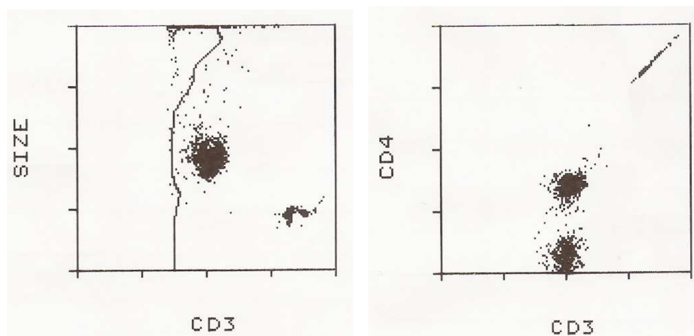
APPENDIX 2:



Appendix 2a: Illustrates the same example in appendix 1 (SYN050307B) of stabilized blood analyzed on day 77 on the BD FACSCount™ instrument. The blood was stabilized using 0.25% chromium chloride in combination with 0.35% w/v paraformaldehyde. The CD4 and CD8 cell populations are still clearly visible.



Appendix 2b: illustrates an example of stabilized whole blood (stabilized in the same batch as in appendix 2a) and analyzed on day 77 with the BD FACSCount™ instrument that resulted in an error message. The CD4 population is still clearly visible.

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APPENDIX 3:

E-mail sent on 19 July 2007 to Prof Bouic:

I also thank you for letting us test Synexa stabilized cells as control since we do not have normal blood daily , after working with it for a week, I know it is a very good product. The staining was stable after 7 days and the profile looked better than UKNEQAS, CD Check and Multi-Check. If the cells can be stabilized longer than 6 weeks then you have created a great product that was currently needed for these regions.

Best Regards,

Nga Bui
Scientist I / Reagent Application & Development

BD Biosciences - Immunocytometry Systems
2350 Qume Dr, San Jose, CA 95131 USA
tel: 408-954-2523 fax: 408-954-2156
E-mail: nga_bui@bd.com

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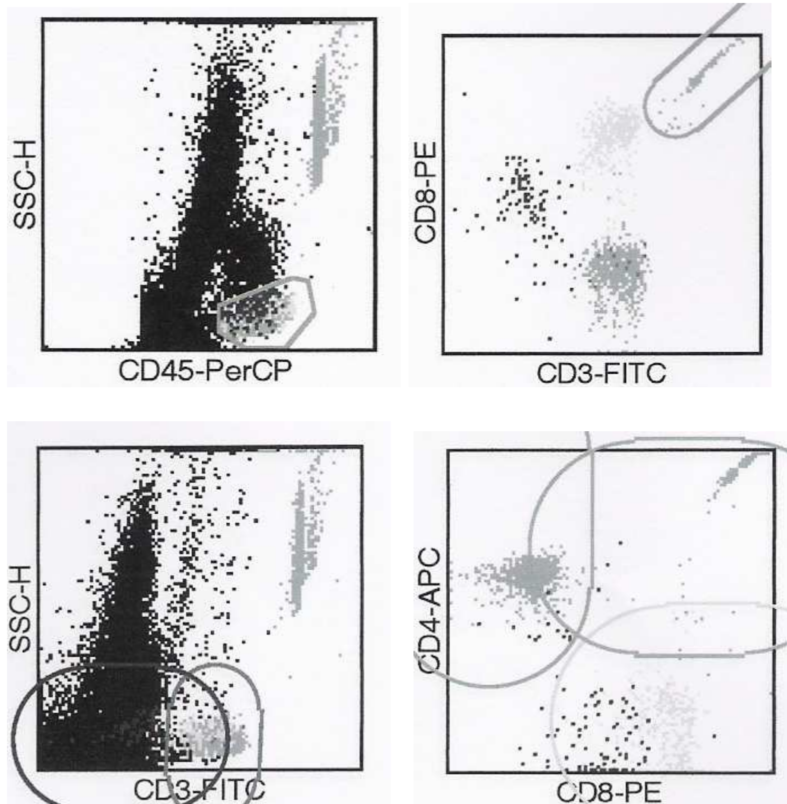
APPENDIX 4:



Illustrates the data obtained from one example run on day 4. This sample of stabilized whole blood contained an added amount of stabilized PBMCs to increase the lymphocyte count. In the two plots on the left hand side, the degranulation of the granlulocytes is clearly visible. There is a contamination of cell populations having lower side scatter: it was therefore considered that this sample was non- stabilized and therefore discarded. It was not possible to add PBMCs to a pre-stabilized sample in order to increase the lymphocyte cell counts.

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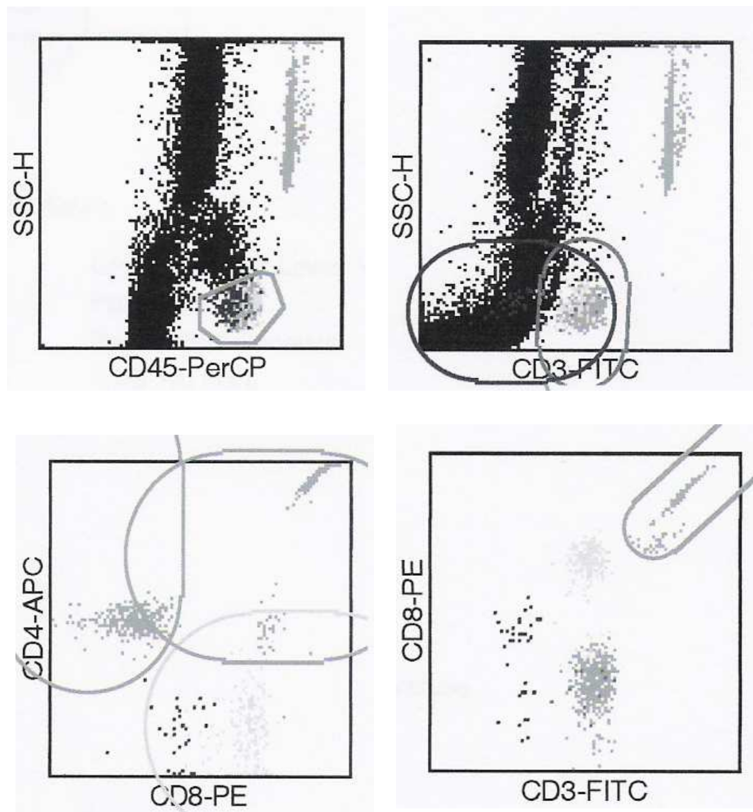
APPENDIX 5:



Illustrates the same example (SYN050307B) of a stabilized sample mentioned in appendix 1; here it was analyzed on the BD FACSCalibur™ on day 77. The Multiset™ software was used to monitor the routine phenotypic cell surface molecules CD3, CD4 and CD8. The populations of cells are still easily distinguishable on day 77.

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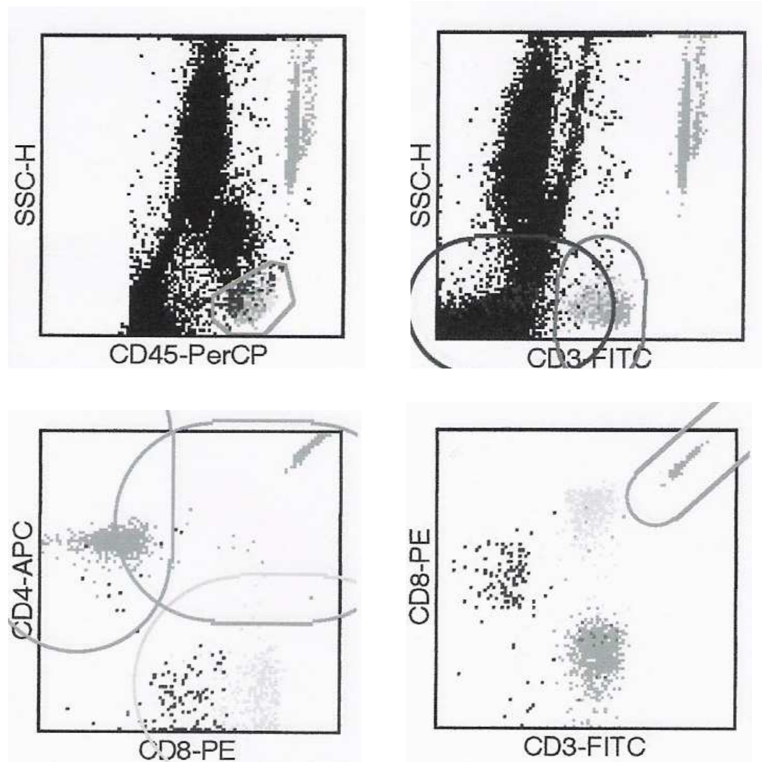
APPENDIX 6:



Illustrates the same example (SYN050307B) of a stabilized sample mentioned in appendix 1; here it was analyzed on the BD FACSCalibur™ on day 4. Comparing day 77 (appendix 5) with day 4, there is not much difference in the scatter dotplot, which is an indication of excellent stability.

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GLOSSARY

- **Agranulocytosis**- An acute disease marked by high fever and a sharp drop in circulating granular white blood cells. It may be drug-induced or the result of exposure to radiation.
- **AIDS (acquired immunodeficiency syndrome)—**life-threatening disease caused by the human immunodeficiency virus, which breaks down the body's immune defenses.
- **Allergen—**any substance that causes an allergy.
- **Allergy**—a harmful response of the immune system to normally harmless substances.
- **Antibody (Ab) -** an immunoglobulin molecule produced by B lymphoid cells with a specific amino acid sequence evoked in humans or other animals by an antigen (immunogen). These molecules are characterized by reacting specifically with the antigen in some demonstrable way, antibody and antigen each being defined in terms of the other. Antibodies may also exist naturally, without being present as a result of the stimulus provided by the introduction of an antigen; antibodies are found in the blood and body fluids, although the basic structure of the molecule consists of two light and two heavy chains, antibodies may also be found as dimers, trimers, or pentamers.

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- **Antigen (Ag)** - Any substance that, as a result of coming in contact with appropriate cells, induces a state of sensitivity and/or immune responsiveness after a latent period (days to weeks) and that reacts in a demonstrable way with antibodies and/or immune cells of the sensitized subject in vivo or in vitro. Modern usage tends to retain the broad meaning of antigen, employing the terms "antigenic determinant" or "determinant group" for the particular chemical group of a molecule that confers antigenic specificity.
- **Antigen-presenting cells (APC)** - cells that process protein antigens into peptides and present them on their surface in a form that can be recognized by lymphocytes. APCs include Langerhans cells, dendritic cells, macrophages, B cells, and in humans, activated T cells.
- **Antiserum**—a serum rich in antibodies against a particular microbe.
- **Appendix**—lymphoid organ in the intestine.
- **Autoantibodies**—antibodies that react against a person's own tissue.
- **Autoimmune disease** - any disorder in which loss of function or destruction of normal tissue arises from humoral or cellular immune responses to the body's own tissue constituents; may be systemic, as systemic lupus erythematosus, or organ specific, as thyroiditis or a disease that results when the immune system mistakenly attacks the body's own tissues. Examples include multiple sclerosis, type I diabetes, rheumatoid arthritis, and systemic lupus erythematosus.
- **B cells**—small white blood cells crucial to the immune defenses. Also know as B lymphocytes, they come from bone marrow and develop into blood cells called plasma cells, which are the source of antibodies.
- **Bacteria—**microscopic organisms composed of a single cell. Some cause disease.

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- **Basophils—**white blood cells that contribute to inflammatory reactions. Along with mast cells, basophils are responsible for the symptoms of allergy.
- **Biological response modifiers—**substances, either natural or synthesized, that boost, direct, or restore normal immune defenses. They include interferons, interleukins, thymus hormones, and monoclonal antibodies.
- **Blood vessels**—arteries, veins, and capillaries that carry blood to and from the heart and body tissues.
- **Bone marrow—**soft tissue located in the cavities of the bones. Bone marrow is the source of all blood cells.
- **CD** - Abbreviation for cluster of differentiation. Cluster of differentiation (CD) antigen - an antigen (marker) on the surface of a cell, usually a lymphocyte. Cell membrane molecules that are used to classify leukocytes into subsets. CD molecules are classified by monoclonal antibodies. There are four general types: type I transmembrane proteins have their COOH-termini in the cytoplasm and their NH2-termini outside the cell; type II transmembrane proteins have their NH2-termini in the cytoplasm and their COOH-termini outside the cell; type III transmembrane proteins cross the plasma membrane more than once and hence may form transmembrane channels; and glycosylphosphatidylinositol-anchored proteins (type IV), which are tethered to the lipid bilayer via a glycosylphosphatidylinositol anchor.
- **CD25** - a type I transmembrane protein present on activated T cells, activated B cells, some thymocytes, myeloid precursors, and oligodendrocytes that associates with CD122 to form a heterodimer that can act as a high-affinity receptor for IL-2; expressed in most B-cell neoplasms, some acute nonlymphocytic leukemias, and neuroblastomas.

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- **CD4** - a type I transmembrane protein found on helper/inducer T cells, monocytes, macrophages, and dendritic cells that is involved in T-cell recognition of antigens; expressed in mycosis fungoides, Sézary syndrome, and T-cell lymphomas.
- **CD8** - a type I transmembrane protein found on suppressor (cytotoxic) T cells, some natural killer cells, and most thymocytes that is involved in T-cell antigen recognition; expressed in some T-cell lymphomas and large granular lymphocyte leukemias.
- **Chemokines—**certain proteins that stimulate both specific and general immune cells and help coordinate immune responses and inflammation.
- **Clone—**a group of genetically identical cells or organisms descended from a single common ancestor; or, to reproduce identical copies.
- **Complement cascade—**a precise sequence of events, usually triggered by antigen-antibody complexes, in which each component of the complement system is activated in turn.
- **Complement—**a complex series of blood proteins whose action "complements" the work of antibodies. Complement destroys bacteria, produces inflammation, and regulates immune reactions.
- **Cytokine** - Any of numerous hormone like, low-molecular-weight proteins, secreted by various cell types, which regulate the intensity and duration of immune response and mediate cell-cell communication. See: chemokines, interferon, interleukin, and lymphokine. See also: interferon, interleukin, lymphokine or powerful chemical substances secreted by cells that enable the body's cells to communicate with one another. Cytokines include lymphokines produced by lymphocytes and monokines produced by monocytes and macrophages.

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- **Cytotoxic T lymphocytes (CTLs)—**a subset of T cells that carry the CD8 marker and can destroy body cells infected by viruses or transformed by cancer.
- **Denominator** - A common trait or characteristic. In this thesis the denominator is associated with a population of cells.
- **DNA (deoxyribonucleic acid)—**a long molecule found in the cell nucleus; it carries the cell's genetic information.
- **Enzyme—**a protein produced by living cells that promotes the chemical processes of life without itself being altered.
- **Eosinophils—**white blood cells that contain granules filled with chemicals damaging to parasites, and enzymes that affect inflammatory reactions.
- **Epithelial cells—**cells making up the epithelium, the covering for internal and external body surfaces.
- **Epitope** - The simplest form of an antigenic determinant, on a complex antigenic molecule, which can combine with antibody or T cell receptor.
- **Exon** - A sequence of DNA that codes information for protein synthesis that is transcribed to messenger RNA.
- **Flow cytometry** - is a method of sorting and measuring types of cells by fluorescent labeling of markers on the surface of the cells. It is sometimes referred to as FACS (Fluorescent Activated Cell Sorting) analysis.
- **Fungi—**members of a class of relatively primitive vegetable organisms. They include mushrooms, yeasts, rusts, molds, and smuts.
- **Graft rejection—**an immune response against transplanted tissue.
- **Graft-versus host disease (GVHD)—**a life-threatening reaction in which transplanted cells attack the tissues of the recipient.

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- **Granules**—membrane-bound organelles within cells where proteins are stored before secretion.
- **Granulocytes**—phagocytic white blood cells filled with granules organisms. Neutrophils, eosinophils, basophils, and mast cells are examples of granulocytes.
- **Helper T cells (Th cells)**—a subset of T cells that carry the CD4 surface marker and are essential for turning on antibody production, activating cytotoxic T cells, and initiating many other immune functions.
- **HIV (human immunodeficiency virus)**—the virus that causes AIDS.
- **Hypoplastic anemia** - Progressive non-regenerative anemia resulting from greatly depressed, inadequately functioning bone marrow that may lead to aplastic anemia.
- **Immune response**—reaction of the immune system to foreign substances.
- **Immunoglobulin**—a family of large protein molecules, also known as antibodies, produced by B cells.
- **Immunophenotyping** - is a technique notably used in the diagnosis of leukemia. It involves the labeling of white blood cells with antibodies directed against surface proteins on their membrane. By choosing appropriate antibodies, the origin of leukemic cells can be accurately determined. The labeled cells are processed in a flow cytometer, which a laser based instrument capable of analyzing thousands of cells per second. The whole procedure can be performed on cells from the blood, bone marrow or spinal fluid in a matter of a few hours.
- **Immunosuppressive**—capable of reducing/inhibiting immune responses.
- **Inflammatory response**—redness, warmth, and swelling produced in response to infection, as the result of increased blood flow and an influx of immune cells and secretions.

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- **Interferons—**proteins produced by cells that stimulate anti-virus immune responses or alter the physical properties of immune cells.
- **Interleukin** - The name given to a group of multifunctional cytokines once their amino acid structure is known. They are synthesized by lymphocytes, monocytes, macrophages, and certain other cells.
- **Interleukin-2 (IL-2)** - A cytokine derived from T helper lymphocyte that causes proliferation of T lymphocytes and activated B lymphocytes.
- **Invertebrate -** Any animal that lacks a vertebral column, or backbone. They include the protozoans, annelids, cnidarians, echinoderms, flatworms, nematodes, mollusks, and arthropods. More than 90% of living animals are invertebrates. Worldwide in distribution, they range in size from minute protozoans to giant squids. Apart from the absence of a vertebral column, invertebrates have little in common. They are generally soft-bodied and have an external skeleton for muscle attachment and protection.
- **Ion channel** - ion channel a specific macromolecular protein pathway, with an aqueous "pore," that traverses the lipid bilayer of a cell's plasma membrane and maintains or modulates the electrical potential across this barrier by allowing controlled influx or exit of small inorganic ions such as Na^+, K^+, Cl^-, and Ca^{2+}. It plays an important role in propagation of the action potential in neurons, but also may control transduction of extracellular signals and contraction in muscle cells. In general, ion channels are characterized by their selectivity for certain ions, their specific regulation or gating of these ions, and their specific sensitivity to toxins.
- **Ionophore** - A compound or substance that forms a complex with an ion and transports it across a membrane.

- **Isoform** – A protein isoform is a version of a protein with small differences, usually a splice variant or the product of some posttranslational modification.
- **Leukocytes**—all white blood cells.
- **Lymph nodes—**small bean-shaped organs of the immune system, distributed widely throughout the body and linked by lymphatic vessels. Lymph nodes are garrisons of B, T, and other immune cells.
- **Lymph—**a transparent, slightly yellow fluid that carries lymphocytes, bathes the body tissues, and drains into the lymphatic vessels.
- **Lymphatic vessels—**a body-wide network of channels, similar to the blood vessels, which transport lymph to the immune organs and into the bloodstream.
- **Lymphocytes—**small white blood cells produced in the lymphoid organs and paramount in the immune defenses. B cells and T cells are lymphocytes.
- **Lymphoid organs**—the organs of the immune system, where lymphocytes develop and congregate. They include the bone marrow, thymus, lymph nodes, spleen, and various other clusters of lymphoid tissue. Blood vessels and lymphatic vessels are also lymphoid organs.
- **Lymphokines**—powerful chemical substances secreted by lymphocytes. These molecules help direct and regulate the immune responses.
- **Lymphopenia** - A reduction in the number of lymphocytes in the blood. Also called *lymphocytic leukopenia*, *lymphocytopenia*.
- **Lysozyme** - An enzyme occurring naturally in egg white, human tears, saliva, and other body fluids, capable of destroying the cell walls of certain bacteria and thereby acting as a mild antiseptic.

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- **Macrophage—**a large and versatile immune cell that devours invading pathogens and other intruders. Macrophages stimulate other immune cells by presenting them with small pieces of the invaders.
- **Major histocompatibility complex (MHC)** - a group of linked loci, collectively termed H-2 complex in the mouse and HLA complex in humans, that codes for cell-surface histocompatibility antigens and is the principal determinant of tissue type and transplant compatibility. See Also: human leukocyte antigens.
- **Mast cell**—a granulocyte found in tissue. The contents of mast cells, along with those of basophils, are responsible for the symptoms of allergy.
- **Memory cells—**a subset of T cells and B cells that have been exposed to antigens and can then respond more readily when the immune system encounters those same antigens again.
- **Microbes—**microscopic living organisms, including bacteria, viruses, fungi, and protozoa.
- **Microorganisms—**microscopic organisms, including bacteria, virus, fungi, plants, and parasites.
- **Molecule—**the smallest amount of a specific chemical substance. Large molecules such as proteins, fats, carbohydrates, and nucleic acids are the building blocks of a cell, and a gene determines how each molecule is produced.
- **Monoclonal antibodies** - Any of the highly specific antibodies produced in large quantity by the clones of a single hybrid cell formed in the laboratory by the fusion of a B cell with a tumor cell or antibodies produced by a single cell or its identical progeny, specific for a given antigen. As tools for binding to specific protein molecules, they are invaluable in research, medicine, and industry.

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- **Monocytes—**large phagocytic white blood cells which, when entering tissue, develop into macrophages.
- **Monokines—**powerful chemical substances secreted by monocytes and macrophages. These molecules help direct and regulate the immune responses.
- **Natural killer (NK) cells**—large granule-containing lymphocytes that recognize and kill cells lacking self antigens. Their target recognition molecules are different from T cells.
- **Neutrophil—**white blood cell that is an abundant and important phagocyte.
- **Opportunistic infections** - An infection by a microorganism that normally does not cause disease but becomes pathogenic when the body's immune system is impaired and unable to fight off infection.
- **Parasites—**plants or animals that live, grow, and feed on or within another living organism.
- **Passive immunity—**immunity resulting from the transfer of antibodies or antiserum produced by another individual.
- **Pathogen—**a disease-causing organism.
- **Phagocytes—**large white blood cells that contribute to the immune defenses by ingesting microbes or other cells and foreign particles.
- **Phagocytosis**—process by which one cell engulfs another cell or large particle.
- **Phytohemagglutinin (PHA)** -A phytomitogen from plants that agglutinates red blood cells. The term is commonly used specifically to refer to the lectin obtained from the red kidney bean (*Phaseolus vulgaris*), which is also a mitogen that stimulates T lymphocytes more vigorously than B lymphocytes. Syn: phytolectin.
- **Plasma cells—**large antibody-producing cells that develop from B cells.

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- **Platelet—**cellular fragment critical for blood clotting and sealing off wounds.
- **Progeny** - One born of, begotten by, or derived from another; an offspring or a descendant. Offspring or descendants considered as a group.
- **Senescent** - Growing old; aging.
- **Serum—**the clear liquid that separates from the blood when it is allowed to clot. This fluid contains the antibodies that were present in the whole blood.
- **Spleen—**a lymphoid organ in the abdominal cavity that is an important center for immune system activities.
- **Stem cells—**immature cells from which all cells derive. The bone marrow is rich in stem cells, which become specialized blood cells.
- **T cells—**small white blood cells (also known as T lymphocytes) that recognize antigen fragments bound to cell surfaces by specialized antibody-like receptors. "T" stands for thymus, where T cells acquire their receptors.
- **T cytotoxic cells (Tc)** - a subset of CD8 T lymphocytes that bind to other cells via class I MHC and are involved in their destruction.
- **T lymphocytes**—see T cells.
- **Thymus—**a primary lymphoid organ, high in the chest, where T lymphocytes proliferate and mature.
- **Toxins—**agents produced in plants and bacteria, normally very damaging to cells.
- **Vaccines**—preparations that stimulate an immune response that can prevent an infection or create resistance to an infection. They do not cause disease.

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- **Vertebrate -** Any animal of the chordate subphylum Vertebrata, which includes the fishes, amphibians, reptiles, birds, and mammals. Vertebrates have an internal skeleton formed of cartilage, bone, or both. The skeleton consists of a backbone (vertebral column), which partly encloses a spinal cord; a skull, which encloses the brain; and usually two pairs of limbs. Nerves extending from the spinal cord and brain permeate the skin, muscles, and internal organs. The muscular system consists primarily of bilaterally paired masses attached to bones or cartilage. Skin and scales, feathers, fur, or hair cover the outer surface.
- **Viruses**—microorganisms composed of a piece of genetic material—RNA or DNA— surrounded by a protein coat. Viruses can reproduce only in living cell.