An *in vitro* study on the immunotoxicity of sewage effluents discharged into the Eerste River-Kuils River water catchment system

Tandeka Magcwebeba

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Key words

Cell-mediated immunity
Cytotoxicity
ELISA
IL-6
IFN-γ
IL-10
Inflammatory activity
Humoral immunity
Human whole blood cultures
LDH assay

Abbreviations

AIDS-	Acquired immunodeficiency syndrome
C1-INH-	C1 inhibitor
CTLA-4	Cytotoxic T-lymphocyte antigen-4
ELISA-	Enzyme-linked immunosorbent assay
HIV-	Human immunodeficiency virus
ikB-	Inhibitor of kappa Beta
IL-2-	Interleukin-2
IL-4 -	Interleukin -4
IL-5-	Interleukin-5
IL-6-	Interleukin-6
IL-8-	Interleukin-8
IL-10-	Interleukin-10
IL-12-	Interleukin-12
IL-12R-	Interleukin-12 receptor
IL-12β-	Interleukin-12 Beta
IL-13-	Interleukin-13
IL-18-	Interleukin-18
IL-27 –	Interleukin-27
IFN-γ-	Interferon-gamma
IFN-γR-	Interferon-gamma receptor
TNF-α-	Tumor necrosis factor –alpha
Jak –	Janus kinase
STAT-	Signal transducer and activator of transcription
LDH-	Lactate dehydrogenase
TCR-	T-cell receptor
Tyk-	Tyrosine kinase
MASPS –	MBL associated serine proteases
MHC-	Major histocompatability complex
NFAT-	Nuclear factor of activated cells
NF-kB-	Nuclear factor kappa beta
TB-	Tuberculosis

Abstract

The immune system is comprised of the innate and adaptive immunity which have mechanisms that protect the host against invading foreign pathogenic substances. Cytokines, with the aid of other molecular components play a pivotal role in the induction and regulation of these mechanisms thus maintain homeostasis. Surface water which is used in the irrigation of crops, for domestic purposes and recreation can be polluted via point source and non-point source pollution with environmental contaminants emanating from anthropogenic activities. Exposure to these contaminants via consumption of, or direct contact with affected surface water can cause diarrheal disease and they can also modulate the functions of cytokines and other mediators of the immune system thus resulting in immune dysfunction and the development of disease in humans and animals.

The Eerste River water catchment system which runs through Stellenbosch is used in the irrigation of crops and recreation. Studies have reported that the quality of the river is at its cleanest near its origin in Jonkershoek and in the central business area of the town. However upon confluence with the heavily polluted Plankenbrug River which runs through an agricultural area and an informal settlement the water quality of the Eerste River deteriorates rapidly. The degradation in the water quality of the Eerste/Plankenbrug river system is often attributed to the inadequately serviced informal settlement of Kayamandi. There is no literature on the immunotoxicity of the Eerste/Plankenbrug River system and there are no studies that have compared the contamination levels between the agricultural area and informal settlement. Most techniques only measure the physico-chemical properties of water which do not give insight on the potential adverse health effects that can be imposed in humans upon exposure to polluted water. Hence an in *vitro* human whole blood culture assay has been developed where IL-6 is the 'gold standard' biomarker used to measure the inflammatory activity in water as an indicator of water quality.

The aim of this study was to use *in vitro* human whole blood cultures to screen the water samples collected from the Eerste/Plankenbrug river system for cytotoxicity and inflammatory activity and for the first time investigate the impact on the cell-mediated and humoral immune pathways.

Water samples were collected from the sites during the dry summer season and rainy winter season. Blood was collected from the healthy male volunteers and diluted with RPMI 1640. For cytotoxicity and inflammatory activity 2.5µl of water extracts or DMSO were incubated with 250µl of blood for 18-20hrs at 37˚C. For the cell-mediated and humoral immune pathways blood was stimulated with PHA and then 2.5µl of water extracts or DMSO were incubated with 250µl of

blood for 48hrs at 37˚C. The LDH assay was used to analyse the blood samples for cytotoxicity and the ELISA was used for cytokine analysis. IL-6 was used as a biomarker for inflammatory activity, IFN-γ was used as a biomarker for the cell-mediated immune pathway and IL-10 was the biomarker for the humoral immune pathway. The results were analysed statistically using the ANOVA test.

Results showed that water samples were not cytotoxic. Water extracts collected from the confluence point of the Eerste River and Plankenbrug River (Plankenbrug) induced significantly ($p<0.001$) the highest IL-6 production and hence had the highest inflammatory activity. This was followed by the site after the confluence (Post-Plankenbrug) which had a significantly higher ($p<0.001$) IL-6 production than the control site (Jonkershoek) and the central business area of the town (Pre-Plankenbrug). Jonkershoek had significantly ($p<0.001$) the lowest IL-6 production compared to the other sites. Water extracts from the other sites had no effects on the immune pathways of the adaptive immunity except for the Plankenbrug site which significantly ($p<0.001$) suppressed both IFN-γ and IL-10 production in the blood samples. All these effects were only observed during the dry summer season. Further investigation in the Plankenbrug River during the summer season showed that the agricultural area (Pre-Kayamandi) and the informal settlement (Post-Kayamandi) equally contribute to contamination of the Plankenbrug River because these two sites had significantly a higher ($p<0.001$) IL-6 production than Jonkershoek but there was no significant difference in IL-6 production in these two sites. Only the water extract from Post-Kayamandi significantly suppressed both IL-10 and IFN-γ ($p<0.001$).

This study shows that water from the Plankenbrug River is heavily polluted by contaminants coming from both the agricultural area and informal settlement of Kayamandi. These contaminants can be potentially immunotoxic during the summer season and they can result in inflammatory diarrheal disease and immunosuppression in exposed individuals. To prevent an increase in the morbidity rate in this area environmental health officials and municipality workers should put in place monitoring programmes that will involve the community. They should also improve on the services provided to Kayamandi thus to limit pollution. Lastly, the community should be educated on the importance of keeping the river clean

Declaration

I declare that this is work and that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Tandeka Magcwebeba January 2008

Signed …….

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Chapter 1: The immune system

Introduction

The immune system is designed to protect the body from the invasion of any foreign matter that might be pathogenic. It is comprised of the innate and adaptive immunity. These two branches have complementary and co-operative mechanisms that are interlinked in the quest to eradicate foreign substances. Cytokines, with the aid of other molecular components, play a pivotal role in the induction and the regulation of the immune system.

1.1 Innate immunity

The innate immunity provides the first line of defense against invasion by pathogens using physical, mechanical and chemical barriers. These barriers are located in all the areas of the body that are directly exposed to the environment. They include the keratinised cells of the skin and mucous membranes of mouth, respiratory tract, gastro intestinal tract and genitourinary tract. Characteristics of these barriers are a slightly acidic pH, the presence of normal commensals and a cell lining with an epithelial layer on the surface. The cell structures of barriers are organised in tight cell-cell junctions that make it difficult for microbes to penetrate and invade the underlying tissue.

Contact of epithelial cells of the mucous membranes and skin with pathogen's molecular patterns induces the synthesis and secretion of various molecules. The synthesised molecules display antimicrobial properties that are associated with defence mechanisms. The secreted molecules include the β-defensins and lysozymes. The expression and release of these antimicrobial enzymes is activated by the presence of cytokines such as TNF-α, IL-1β and IL-6 (King and Willis. 2005, Tosi. 2005, Gennery et al. 2006).

The other first line defense mechanisms provided by the barriers involve the secretion of viscous mucus by mucous membranes, which can trap foreign particles. The epithelial hair and cilia lining the membranes assist in sweeping away the trapped foreign particles. Furthermore, to prevent microbial invasion the barriers have flushing mechanisms, these include the washing effects of tears and saliva. Other mechanisms involved in ensuring expulsion of foreign matter are through urination, defacation and vomiting (Tortora and Grabowski. 1996, Gennery et al. 2006).

The combined effects of these mechanisms make it difficult for microbes to attach, penetrate and invade the tissue material underneath the epithelial surface.

However when these barriers are breached and the pathogen invades the tissue, cellular components and complement proteins in the innate system provide the second line of defense. This is mediated through phagocytosis coupled with inflammation and cell lysis.

In phagocytosis the most prominent cells are neutrophils, macrophages and dendritic cells. Eosinophils have also been reported to partake in phagocytosis but their primary role is to release substance that will kill microbes and helminths extracellularly (King and Willis. 2005).

1.1.1 Phagocytosis

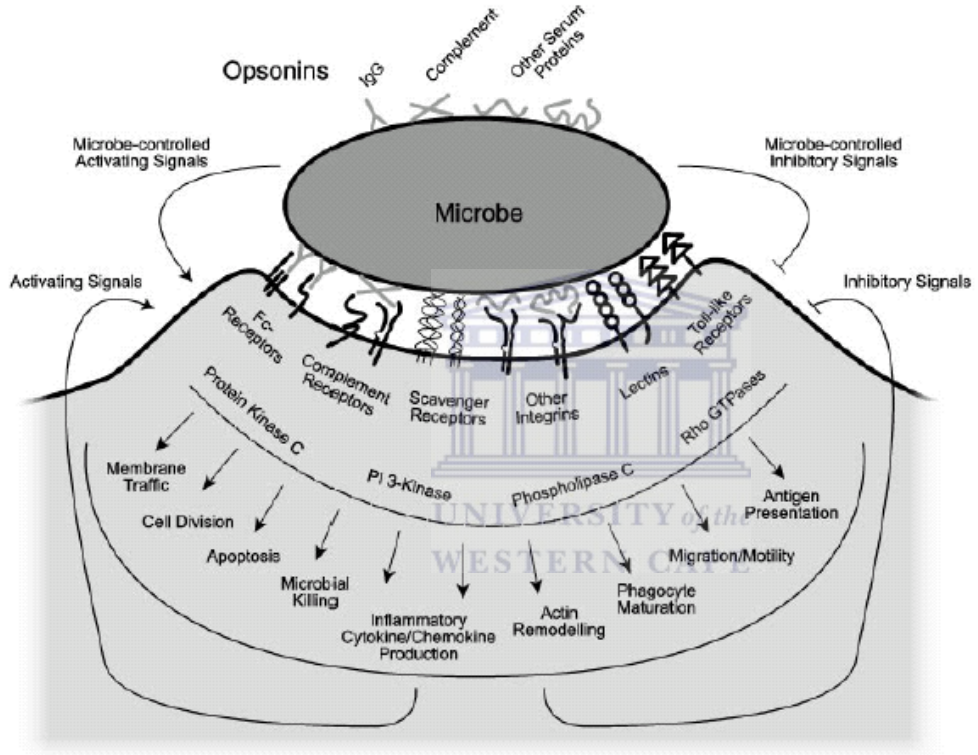

Figure 1.1: This diagram shows all the pattern recognition and opsonic receptors expressed on a cell surface membrane upon an encounter with a pathogen. Beneath the membrane there is also signaling molecules .The receptors together with the signaling molecules function in pathogen identification, binding and internalization. Diagram from Underhill and Ozinsky. 2002

Phagocytes and epithelial cells express a vast array of receptors and signalling molecules on their cell surface membranes that facilitate the process of phagocytosis (See Fig 1.1).

Receptors are grouped into pattern recognition receptors and opsonin receptors. Pattern recognition receptors include Toll-like receptors, lectin and scavenger receptors, whereas opsonin receptors include complement receptors and Fc receptors. Briefly looking at the receptors, pattern recognition receptors recognise and bind to the pathogen's patterns such as lipopolysaccharides

and furthermore they are able to bind to the whole pathogen. On the other hand opsonin receptors either bind to the Fc portion of immunoglobulins that have formed immune complexes with an antigen (Fc receptors) or to immune complexes that have been coated by complement molecules (complement receptors). Since there are different types of immunoglobulins, cells express various Fc receptors designated for each immunoglobulin complex i.e. FcRγ for IgG, FcRα for IgA, FcRε for IgE, FcRδ for IgD and FcRμ for IgM (Peiser et al. 2001, Underhill and Oznsky. 2002, Underhill and Gantner. 2004, Batjay et al. 2006, Ivan et al. 2006). Receptors are also divided into Trans and Cis categories according to their ability to internalise microbes during phagocytosis (Underhill and Gantner. 2004). Trans receptors have a high detecting range thus they can 'sense' the presence of an infection by recognising the released ligands of a pathogen from a distance. In response they stimulate a generalised inflammatory reaction but they are not able to facilitate the internalisation and killing of the pathogen. Trans receptors are expressed in non-phagocytic and phagocytic cells and they include Toll-like receptors and C5a receptors. On the other hand, cis receptors have a low detecting range thus they can only recognise the physical presence of a pathogen. Direct contact of receptors with a pathogen stimulates internalisation and killing activity of the phagocytes hence cis receptors are also called phagocytic receptors. According to Underhill and Ozinsky. 2002, the most thoroughly characterised phagocytic receptors are the Fcγ receptors but other cis receptors include the lectin receptor: Dectin-1, and the complement receptors: CR1, CR3 and CR4 (Underhill and Oznsky. 2002, Underhill and Gantner. 2004). Pathogen internalisation involves cytoskeletal rearrangement and alteration in membrane trafficking and morphology. This process is mediated by signalling molecules expressed beneath the surface membranes of phagocytes. Signalling molecules include Protein Kinase C, Phospholipase C, Phosphoinositide 3-Kinase and RhoGTPases (Underhill and Oznsky. 2002).

The mechanisms leading to the elimination of a pathogen from a system involves leukocyte activation and recruitment of these cells to the site of an infection where they initiate phagocytosis. The influx of cells to the affected area and the release of bactericidal substances results in inflammation. The detailed mechanism of the inflammatory response is beyond the scope of this thesis however the next section will briefly describe the molecular event responsible for leukocyte activation and recruitment during an infection. It will also briefly discuss the bactericidal activity during phagocytosis.

An encounter with a pathogen results in the induction of molecular structural changes that prepares the cellular components of the innate immunity to fight against the invading pathogens. For instance upon the entry of a foreign antigen, Toll-like receptors recognise and bind to the pathogen's molecular patterns. Contact of these Toll-like receptors with pathogen-associated

molecular pattern triggers intracellular signalling cascade leading to the nuclear translocation and liberation of the transcriptional factor nuclear factor-kappa Beta (NF-kB) from ikB. Nf-kB promotes the gene expression and release of a wide variety of pro-inflammatory cytokines such as, TNF-α, IL-1, IL-6, IL-12, IFN-γ etc (Liang et al. 2004, Tosi. 2005)

Production of TNF-alpha, IL-1, IL-6 and the presence of the infecting agent further activate endothelial cells and the leukocytes to enhance the expression of cellular adhesion molecules. These cellular adhesion molecules consist of selectins, integrins and glycoproteins, and they belong to the immunoglobulin superfamily. The glycoproteins include intercellular adhesion molecules (ICAMs) and together with selectins are expressed by endothelial cells. ICAMs serve as receptors for the integrins molecules expressed on the leukocytes. Adhesion molecules assist in the transmigration of leukocytes from the inside the blood vessel to the affected area (Meager.1999, Kaplanski et al. 2003). To aid in the migration of leukocytes to the infected site, the adhesion molecules cause circulating cells to 'slow down'' and attach to the endothelial surface. Once attached leukocytes roll and squeeze through the interstitial spaces of the endothelial cells and move to the site of infection to initiate phagocytosis (Meager. 1999, Kaplanski et al. 2003, Tosi. 2005). Neutrophils are the first leukocytes to accumulate at the site of injury after an infection. They are recruited to the site of injury by chemoattractants and IL-8. IL-8 is a chemokine usually produced early in the first 24hrs of infection in endothelial cells and other relevant cells under the stimulation of inflammatory cytokines. Neutrophils only accumulate in the early stages of inflammation, where they are responsible for the killing of the pathogen through the release of microbicidal enzymes.

Interleukin-6 and IL-8 aid in the transition from neutrophil to monocytes dominance at the site of injury. IL-6 is reported to induce production of the monocyte chemotactic protein-1 by neutrophils and endothelial cells through binding to the soluble IL-6Rα expressed on the relevant cells. On the other hand, a high concentration of IL-8 in the vessels, due to a prolonged production inhibits neutrophil adhesion and extravasation (Kaplanski et al. 2003).

Macrophages and dendritic cells with phagocytic properties engulf the pathogen into a sealed phagosome. The formed phagosome fuses with lysosomal compartments containing oxygen dependent and oxygen independent microbicidal enzymes. Formation of a phagolysosome results in the release of nitric oxides, superoxides, lysozymes and α-defensins. These substances, which are responsible for the intracellular killing of the pathogens, are released from the primary and secondary granules of the polymorphonuclear leukocytes (Underhill and Ozinsky. 2002, Tosi. 2005, King and Willis. 2005, Gennery et al. 2006).

1.1.2 The role of the complement system in the innate immunity

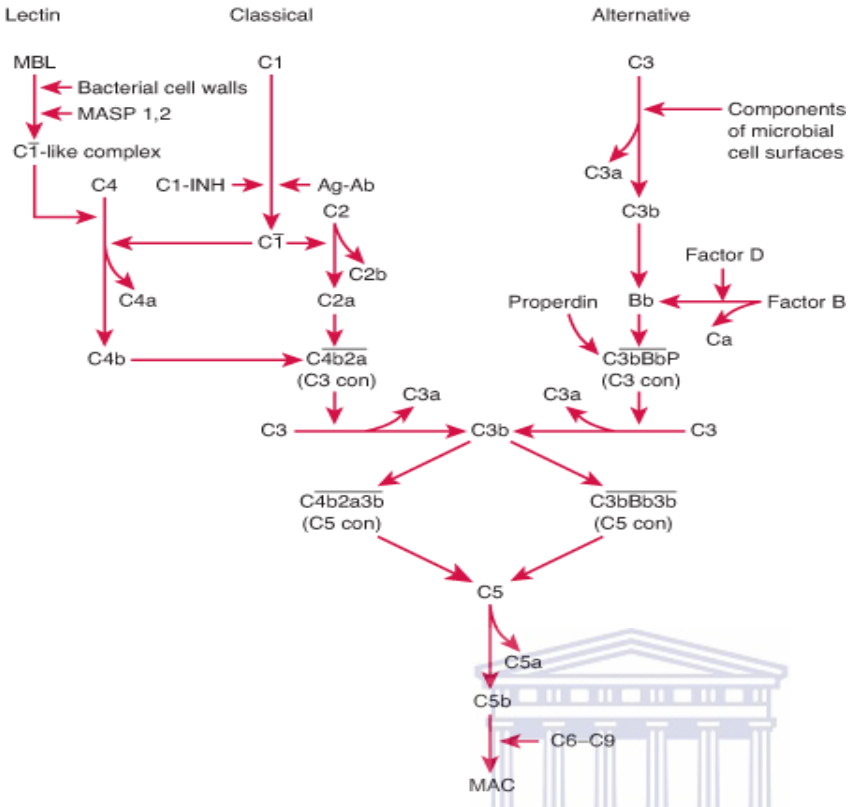

Figure 1.2: This diagram shows the three pathways involved in the complement system. The alternative pathway, mannan-binding lectin pathway and the classical pathway are involved in the humoral immunity. Diagram from Porter and Kaplan. 2005.

The complement system is comprised of approximately 30 serum proteins. These protein molecules are synthesised in the liver and released into the serum for immune survellaince. Upon exposure to a foreign pathogen the protein molecules in the complement system can directly or indirectly kill a pathogen via the alternative, mannan-binding lectin and classical pathway (King and Willis. 2005, Tosi. 2005).

1.1.2.1 The alternative pathway

The alternative pathway is mediated by the C3 molecule (See Fig 1.2). C3 is found in the serum where it is cleaved at low levels into C3a and the highly reactive thioester C3b. C3b is only activated when it binds to the membrane or cell surface of a pathogen. Once bound to a pathogen, C3b undergoes conformational changes that recruit binding of Factor B. When bound to the C3b molecule, Factor B is cleaved by factor D into Bb and Ba, the latter diffuses away and Bb binds to C3b to form the C3 convertase C3bBb. The C3 convertase, which is stabilised by Properdin, is

responsible for the positive feedback loop that amplifies the alternative pathway through the generation of C3b. C3 convertase cleaves the C3 molecule available in serum into C3a and into C3b in a continuous process. C3a acts as an anaphylotoxin whereas the newly formed C3b molecules have various functions. Firstly they opsonise the pathogen for phagocytosis, they serve as precursors in the positive feedback loop and lastly they bind to the C3 convertase to form C3bBbC3b a C5 convertase. This enzyme cleaves C5 into C5a and C5b. C5a is potent chemoattractant that also serves as an anaphylotoxin. C5b is the first component in the membrane attacking complex. To prevent chronic activation, Factor H and Factor I and the Decay accelerating Factor regulate the alternative pathway. These alternative pathway regulators prevent the binding of factor B to C3b by competing for the binding site (Factor H), prevent the formation of the C3 convertase by accelerating the dissociation of Bb from C3b (DAF) and lastly they degrade the C3b molecule (Factor I). The highly reactive thioester cannot bind to the membrane of the host due to the presence of sialic acid but this can also be the defense mechanism of other pathogens that try to escape killing (Chaplin. 2003, Tosi. 2005, Duncan. 2007,)

1.1.2.2 The mannan-binding lectin pathway

The mannan-binding lectin (MBL) pathway is facilitated by the MBL protein which is a plasma protein that belongs to the family of collectins (See Fig 1.2). MBL binds to the cell surface of a wide variety of clinically relevant micro-organisms including viruses, bacteria, fungi and parasites. It recognises the repetitive carbohydrate structures displayed on the pathogen's surface membrane (Petersen et al. 2001). Once bound to the cell surface of its target the activated MBL protein undergoes conformational changes that allow its association with MBL-associated serine proteases often referred to as MASPs. The currently known proteases are MASP-1, MASP-2 and the non-protease sMASP. The molecules of the lectin pathway, MBL and the MASPs, are often compared to the C1 macromolecule with its components due to structural and functional similarities (Degn et al. 2001, Tosi. 2005).

The formed complex of the MBL protein and the MASPS activates C4, which is cleaved into C4a and C4b, C4a floats away and it acts as anaphylotoxin whereas C4b binds to the C2a molecule, a by-product of the cleaved C2 molecule. It has been reported that MASP-2 is responsible for the cleavage of both the C4 and C2 molecules whereas MASP-1 is responsible for the cleavage of C2 (Krarup et al. 2007). The formed complex of C4bC2a acts as C3 convertase that is responsible for generating C3b that falls in the alternate pathway and also acts as an opsonin. C3b also binds to the C4bC2a complex to form the C5 convertase C3bC4bC2a which is responsible for the cleavage of the C5 molecule, the initiator of the membrane attack complex. The inhibitory effects that regulate

the MBL pathway are exerted by the α2-macroglobulin and C1-INH molecules (Tosi. 2005, Degn et al. 2007, Duncan. 2007).

1.1.2.3 The classical pathway

The classical pathway of the complement system is a bridge between the innate and adaptive immunity. Its activation is dependent on antibodies, more specifically IgM and IgG antibodies, which have formed complexes with pathogenic antigens. This pathway is also facilitated by the C1 macromolecule, which is comprised of C1s, C1q and C1r (See Fig 1.2). Upon an encounter with an antigen-antibody complex, the C1q molecule binds to the Fc portion, which activates C1r then C1s. The activated C1 macromolecule proteolytically cleaves C4 into C4a and C4b and C2 into C2a and C2b.

The C4b and C2a molecules bind to form the C3 convertase that will generate C3b for the alternative pathway, C4a acts as anaphylotoxin and C2b is a prokinin that is involved in edema. In addition, C4b can also act as an opsonin that recruits macrophages to phagocytose immune complexes. The generated C3b forms part of the alternative pathway but it also binds to the C3 convertase to form the C5 convertase responsible for activating C5 which enters the in the formation of the membrane attack complex. The classical pathway is negatively regulated by the C1-INH molecules also involved in the MBL pathway (Tosi. 2005, Duncan. 2007).

1.1.2.4 The membrane attacking complex

The cleavage of the C5 molecule by the C5 convertase causes the dissociation of this molecule into C5a and C5b. C5a is a potent chemoattractant that is involved in the recruitment of leukocytes into the site of infection. Secondly together with C3a and C4a, C5a acts as anaphylotoxin that can induce the release of histamine from mast cells and basophils. The release of histamine from these cells increases vasodilation and permeability (Tosi. 2005).

When C5a splits from C5b, it leaves a binding site for C6. The attachment of C6 to C5b forms a stable and soluble complex C5b.6, which recruits the binding of C7. The formed C5b.6.7 complex has a high affinity to attach to the pathogen's cell membrane surface without disturbing the membrane integrity. It is only when C8 is bound that the C5b.6.7.8 complex starts to partially penetrate the membrane by forming pores. Secondly the C5-C8 complex serves as a receptor for the binding of the C9 molecules. The binding of the initial C9 molecule on the exposed site of C8 causes structural changes in C9. It unfolds its globular hydrophilic structure into an elongated amphipathic structure that inserts its pores into the membrane inducing osmotic lysis in the

pathogen. In addition the elongated structure serves as a binding site for more C9 molecules to attach and induce cell lysis (Sims and Wiedmer. 1995, Xiong et al. 2003, Tosi. 2005)

1.1.3. Natural Killer cells

Natural killer (NK) cells are lymphocytes found in the innate immunity and are responsible for inducing cytotoxicity in virus infected cells and malignant tumours before the development of the adaptive immunity. These cells do not express specific receptor for antigens i.e. T-cell receptors or surface immunoglobulins. Instead they display cell-surface receptors that are used to distinguish healthy cells from non-healthy cells. Non-healthy cells lose the normal expression of MHC class I which is a surface marker for self antigens. NK cells are activated and recruited to the site of infection by chemokines and cytokines. Activation of these NK cells results in the cytotoxic killing of virus infected cells and tumours through the release of perforin and granzyme B from the granular storage compartments of these cells. Perforin causes cell lysis by producing pores in the target membrane thus resulting in cell leakage. Secondly, NK cells induce apoptosis by binding with its ligands to the death receptors Fas and Trail-R expressed on the target cells. Granzyme B is associated with facilitating apoptosis by entering the target cell via the pores formed by perforin where it then leads the caspase-induced apoptosis (King and Willis. 2005, Tosi. 2005).

In addition, NK cell express FcγIII receptors that play a role in the killing activity of these cells against pathogens through mechanisms that involve antibody-dependent cell cytotoxiciy. Most importantly, NK cells participate in the cell-mediated cytotoxicity against intracellular pathogens, as they are the main source of IFN-γ (Tosi. 2005).

1.1.4 Antigen processing and presentation

The innate immunity is there to provide a quick and initial response against pathogen invasion. One of its characteristics is that it does not have the ability to program memory cells to provide a vigorous secondary immune response upon subsequent invasion by the same pathogen. Furthermore it is not designed to mount a prolonged immune response. So when the innate immunity cannot eradicate the pathogen, macrophages and dendritic cells become antigen-presenting cells (APC). APCs process intracellular and extracellular antigens and present them to the adaptive immunity.

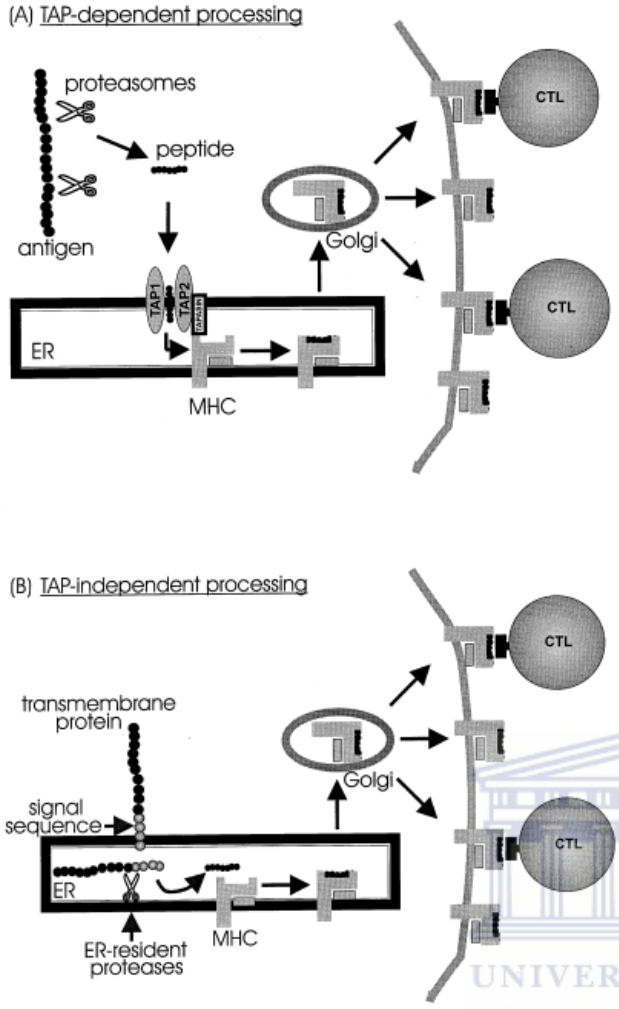

Figure 1.3: This is diagram depicts antigen processing and presentation of intracellular antigens. Diagram from Khanna 1998.

1.1.4.1 Antigen processing for intracellular pathogens

Intracellular pathogens such viruses and mycobacterium TB enter the cell and replicate inside the host's cells. Newly synthesised pathogen proteins accumulate in the cytosol of the host cell where they are broken down into small antigenic peptides by the proteoasome. The peptides are then transported to the endoplasmic reticulum (ER) by the TAP transporter where they bind to the antigenic groove and form a complex with class I MHC molecules (See Fig 1.3). Peptides may also also be transported to the ER independent of TAP. In this pathway peptides can be translocated to the ER lumen by hydrophobic signal sequences (See Fig 1.3). Class I MHC molecules consist of an α-heavy chain associated with β2-microglobulin sheets. They are synthesised in the rough endoplasmic reticulum and then they are transported to the ER for binding with the antigenic peptides. The bound MHC complex is then transported to the cell's surface for antigen presentation to the T-cell receptor of CD8 cytotoxic T-cells and naïve CD4 T-helper cells (York et al. 1996, Khanna 1998, Chaplin 2003, Trombetta et al. 2005).

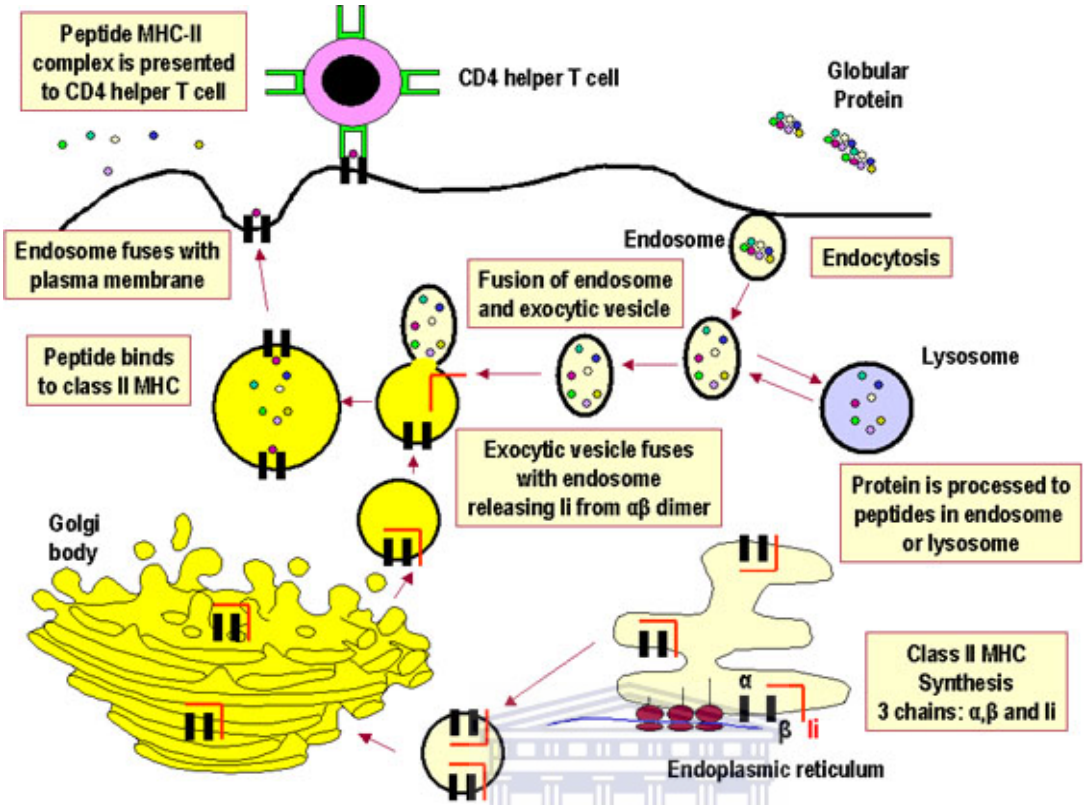

Figure 1.4 This diagram shows antigen processing and presentation of extracellular antigens. Diagram from Bowers. 2004.

1.1.4.2 Antigen processing for Extracellular pathogens

The antigen presenting cells engulf extracellular pathogens such as bacteria and parasites. These pathogens can either be taken up by B-cells where they are broken down and their peptides displayed on the B-cell receptor to attract T-cells or they can be engulfed by monocytes. Pathogens that have been internalised by monocytes are broken down by the proteolytic activity of the lysosomes. The resulting antigenic peptides are then packaged into an endosome and taken to the endoplasmic reticulum (ER). In the ER, class II MHC molecules that are synthesised also consist of the α-heavy chain associated with β2-microglobulin sheets. However an invariant chain (Ii) occupies the antigenic groove. So when the endosome carrying the antigenic peptides is brought to the complex, the invariant chain is degraded. The antigenic peptides then bind to the vacant antigenic groove (See Fig 1.4). The class II MHC complex is also transported to the cell surface of the plasma membrane for antigen presentation to the naïve CD4 T-helper cells (Chaplin. 2003)

1.2 Adaptive immunity

1.2.1 Antigen presentation to the naïve CD4 T-helper cells

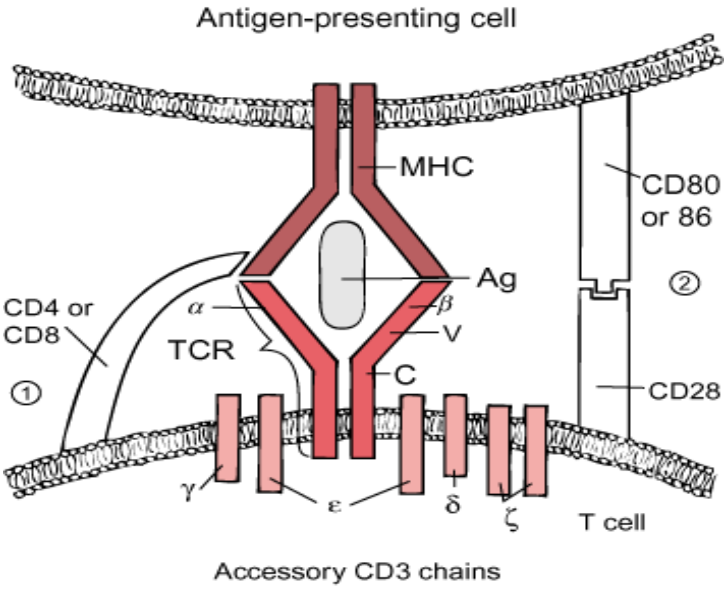

Figure 1.5: This diagram shows all the molecular structures involved in the full activation of naïve CD4 T-cells and CD8 cytotoxic T-cells during antigen presentation. Diagram from Merk Manual 2005.

When the antigenic peptides are presented to the adaptive immunity they bind to the άβ T-cell receptors and CD3 complex of the naïve CD4 T-cells (See Fig 1.5). This interaction partially activates the T-cells; full activation is achieved when the co-stimulatory CD28 ligand on the T-cells interacts with either CD80 or CD86 expressed on the APC. Activation of the CD4 T-cell triggers the adaptive immune response and this initiates the polarization of T-cells (Dong et al. 2000, Chaplin. 2003).

1.2.2 T-cell polarisation

Polarization of the naïve CD4 T-cells restricts them into producing cytokines that influences their differentiation into effector cells. The effector cells are confined to either cell-mediated immunity or antibody mediated immunity. There are several factors, which determine the fate of the T-cells and the immune response that will be mounted. Firstly, the antigen and the type of MHC molecules bound to it, play a big deciding role. Secondly for T-cells to be fully activated CD80 and CD86 receptors on APCs must bind to the CD28 ligand on the CD4 T-cells. The absence of these co-stimulatory molecules can cause anergy, which means that even if the antigen has been presented to the T-cell receptor, the T-cells will remain inactivated. Anergy can happen even if the co-stimulatory molecules are present. This can be caused by the presence of the regulatory molecule, CTLA-4 (Dong et al. 2000, Szabo et al. 2003)

CTLA-4 has high affinity for the CD28 receptor expressed on the T-cells. Its activity suppresses the effects of the CD80/86 by blocking their binding and thus preventing the activation of T-cells (Dong et al. 2000).

The most significant factor influencing T-cell fate is the cytokine environment. Cytokines are the main mediators that drive polarization of the effector cells into their respective immune response domains. Cells that produce cytokines include macrophages, dendritic cells, activated CD4 T-cells, NK cells and also B-cells. The main cytokines that drive the Th1 immune response include IL-2, IL-12, IL-18, IL-23, IL-27 and IFN-γ. The cytokines that are the main mediators of the Th2 response include IL-4, IL-5, IL-6, IL-10 and IL-13 (Reiner 2001, Szabo et al. 2003, de Jong et al. 2004).

Each one of the above mentioned influencing factors is important in mounting a proper immune response against an invading pathogen. Failure to do so can result in the dissemination and progression of an infection. A popular example that is often used to illustrate the importance of polarization and induction of a proper immune response is that of the *Leishmania major* infection in mice. Common inbred strains of mice can mount a Th1 cell-mediated immune response and resolve the lesion. On the other hand Balb/C mice develop a Th2 antibody-mediated immune response that never heals the lesions and renders them susceptible to the infection (Launois et al. 1997).

1.2.2.1 Polarisation into the Th1 response and cell-mediated immunity

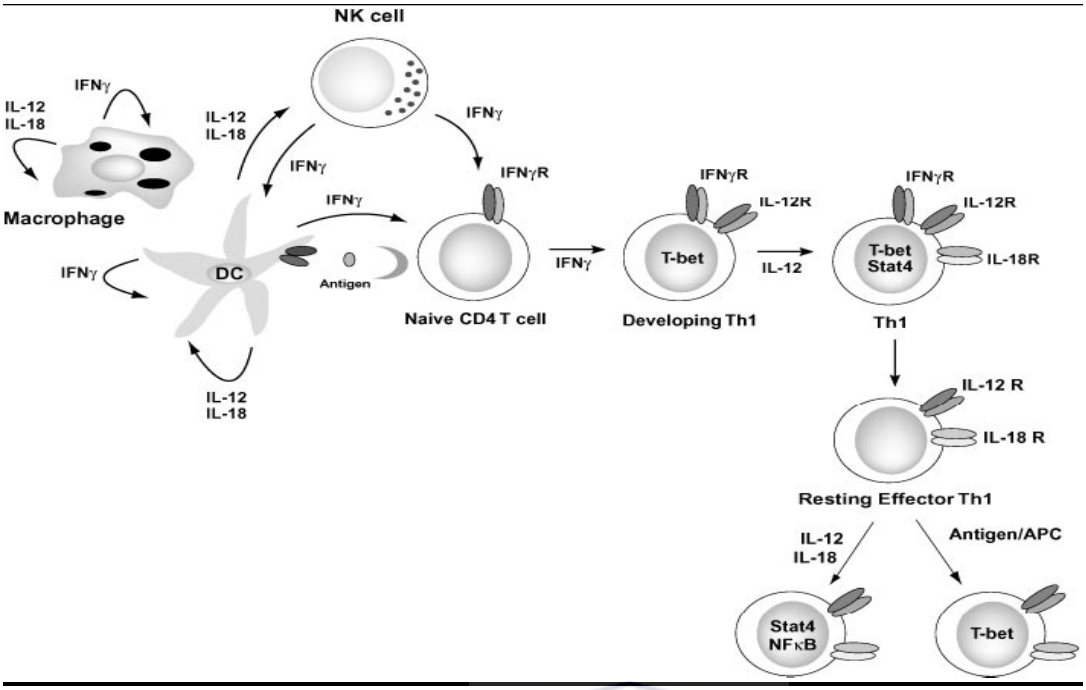

Figure 1.6: This diagram depicts the cellular elements and molecular mechanisms involved in the path to the Th1 immune response that will then give rise to the cell-mediated immunity. Diagram from Szabo et al. 2003.

1.2.2.1a Molecular mechanisms of Th1 polarisation

Upon direct stimulation with microbial products, cells of the innate immune system such as macrophages and immature dendritic cells produce pro-inflammatory cytokines that include the IL-12 family (IL-18, IL-23, and IL-27) and IFN-γ. To drive polarisation into the Th1 immune response, IL-12 signals through the IL-12R complex expressed on NK cells. The IL-12R complex consists of the IL-12β1 and IL-12β2 chains and ligation of IL-12 to the IL-12β2 receptor activates Jak2/Tyk2 underneath the cell membrane (Ortmann et al. 2000, Szabo et al. 2003). Activation of these kinases results in a phosphorylation cascade that causes dimerisation and nuclear translocation of Stat 4 (See Fig 1.6). This transcription factor then causes an increase IFN-γ gene transcription and translation. IL-18, IL-23 and IL-27 all assists the activity of IL-12, which is to enhance production of IFN–γ. Production of IFN-γ from activated CD4 T-helper cells is also induced by IL-12 through receptor signalling, activation of Jak2/Tyk2 and phosphorylation of STAT4 (Szabo et.al. 2003, de Jong et al. 2005).

The IFN-γ that is released by the NK cells and APCs, signals through IFN-γ receptor which is comprised of IFN-γR1 and IFN-γR2 chain on the naïve CD4 T-cell. Signalling activates the

JAK1/JAK2 kinases and induces a phosphorylation cascade that results in nuclear translocation of STAT1. This transcription factor then induces the expression of the main mediating transcription factor of the Th1 response T-bet. T-bet acts in a positive loop mechanism to amplify production of IFN-γ (See Fig 1.6). It does this by two mechanisms. Firstly, it causes chromatin remodelling of the IFN-γ locus and transactivates the IFN-γ gene, which results in more IFN-γ -production. Secondly, T-bet up-regulates expression of the IL-12β2 chain on the T-cell. This enhances the production of IFN-γ through the STAT4 pathway (Szabo et al. 2003, de Jong et al. 2005).

1.2.2.1b <u>Cell-mediated immunity</u>

The presence of IFN-γ, IL-12 and the antigenic peptides displayed on MHC I molecules activates the T-cells to proliferate and divide into memory and effector cells. The memory T-cells stay in quiescent stage and they are activated into effector cells when there is a second invasion by the antigen. The effector cells assist in the binding of the antigen displayed on APCs to the CD8 cytotoxic T-cells. However CD8 cytotoxic T-cells are reported to have the ability to bind to the antigen without assistance from T-helper cells (King and Willis. 2005). Binding of the antigen to the CD8 cytotoxic cells activates them to release granules of perforin and granzyme B that kills the infected cells through perforation of the membrane and induction of caspase dependent apoptosis (King and Willis. 2005). Furthermore activated cytotoxic cells produce IFN-γ. A question that might arise is why all forces focus on the production of IFN-γ. This is because it is the main clear-cut mediator of the Th1 immune response. Functions of the high levels of IFN-γ include delaying viral replication in the affected cells, inducing the expression of the MHC molecules on APCs so as to enhance the presentation of antigenic peptides to the T-cells. This cytokine also activates macrophages resulting in increased phagocytosis of the intracellular pathogens. Further more IFN-γ activates the killing mechanism of NK cells and it is also involved in facilitating antibody–dependent cell cytotoxicity through immunoglobulins from the humoral immunity. Lastly it also induces the production of nitric oxide and super oxide (Szabo et al. 2003, King and Willis. 2005). All of these products aid in the eradication of intracellular pathogens such as viruses and *Mycobacterium tuberculosis.* The presence of the pro-inflammatory cytokines involved in the Th1 immune response together with the transcription factor T-bet down regulates IL-4 and IL-10 production and all the mechanisms related to the Th2 immune response (Szabo et al. 2003).

1.2.2.2 Polarisation into the Th2 response and humoral immunity

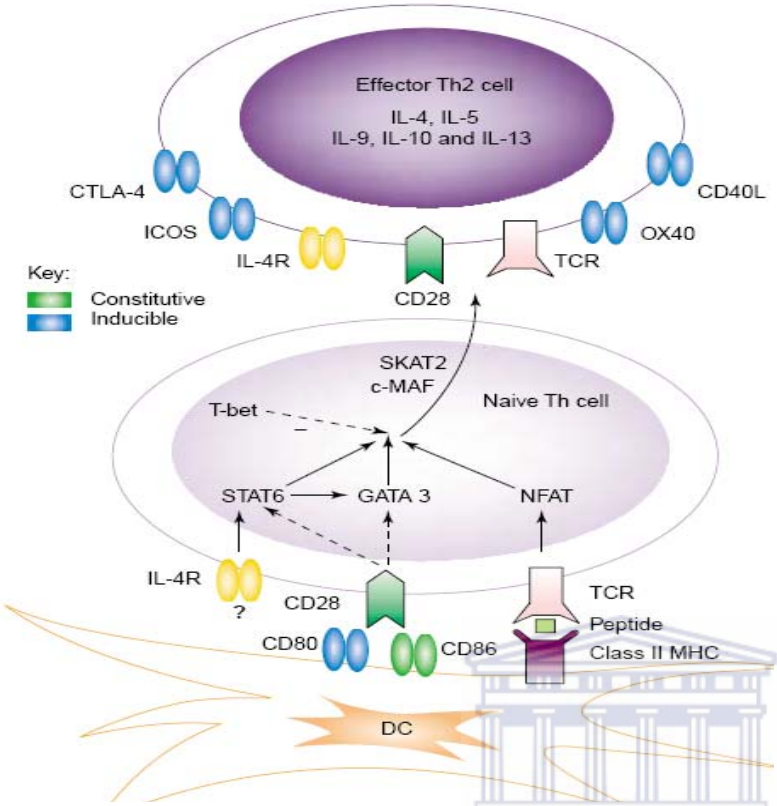

Figure 1.7: This diagram shows the molecular mechanisms involved in the priming of a naïve CD4 T-cell into the Th2 immune response that will give rise to the humoral immunity. Diagram fro Jankovich et al. 2001.

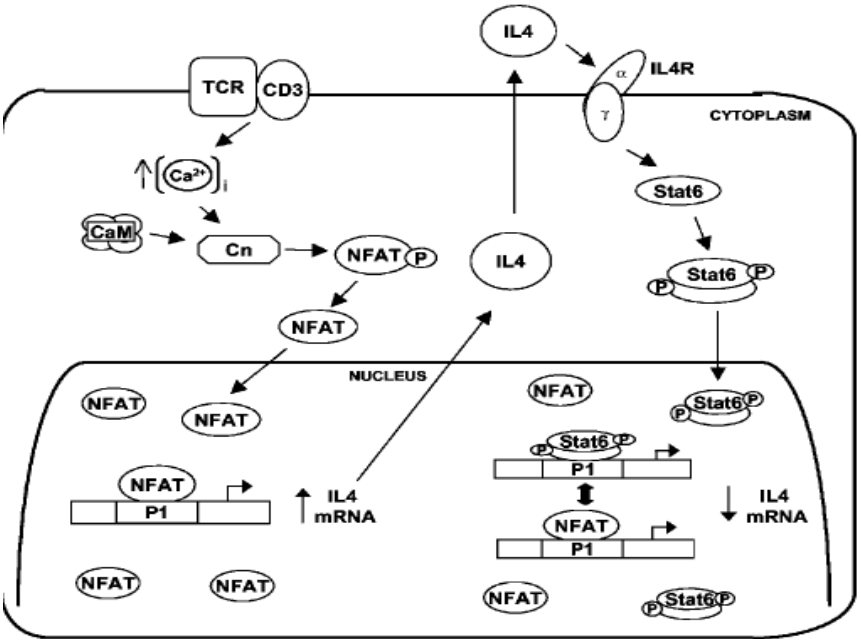

Figure 1.8: This diagram shows the two pathways in which IL-4 production and the polarisation of CD4 T-cells into the Th2 immune response is activated. Diagram from Dorado et al. 2002.

1.2.2.2a Molecular mechanisms of Th2 polarisation

Extracellular antigenic peptides presented to the T-cell receptor by APC and the presence of co-stimulatory molecules polarises naïve CD4 T-cells into producing IL-4. This cytokine is responsible for initiating the proliferation of effector T-cells into the Th2 domain. The exact origin of the exogenous source of this cytokine is not known. It has been speculated that basophils, mast cells and NK cells are the initial source of IL-4 (Dong et al. 2000, Dorado et al. 2002). On the other hand it is widely accepted that the T-cells are the main source of IL-4 whereby its production from the T-cells is activated by signalling through the T-cell receptor. Then it has the ability to enhance its own production from the T-cells through an autocrine fashion and with the help of the transcription factor, GATA-3 (de Jong et al. 2005, Zhu et al. 2006).

Production of IL-4 from T-cells is reported to be activated via two pathways which are the Stat-6 dependent pathway and the Stat-6 independent pathway (See Fig 1.7 and Fig 1.8).

In the Stat-6 independent pathway, presentation of antigenic peptides displayed on MHC II molecules to the T-cell receptor and the presence of co-stimulatory molecules activates NFAT. This transcription factor is located in the cytoplasm; TCR signalling activates the dephosphorylation of NFAT by the calcineurin phosphatase via the calcium/calmodulin pathway. This results in the nuclear translocation of NFAT, where it binds to the AP1 site in the IL-4 promoter and activates the gene expression and production of this cytokine (Dong et al. 2000,

Dorado et al. 2002). It is then postulated that the secreted IL-4 exits the cell to amplify its production (See Fig 1.7) from the T-cell through the Stat-6 dependent pathway (Dorado et al. 2002).

In the Stat-6 dependent pathway, IL-4 from an exogenous source binds to its surface receptor IL-4άR that is expressed on the surface of the CD4 T-cells and it signals through Jak1/Jak3. Phosphorylation of these kinases activates and results in the nuclear tranlocation of Stat-6 to the IL-4 responsive gene promoter where it up-regulate the transcription of IL-4 (Ortmann et al. 2000).

In the literature reviewed it is not yet clear how the production of Gata-3, the master regulator of Th2 polarisation, is activated. It has been speculated that naïve CD4 T-cells express Gata-3 independently of IL-4 before entering the cell cycle and this transcription factor is capable of causing it own auto-induction (Reiner. 2001). Gata-3 is responsible for initiating the remodelling of local chromatin in cytokine loci thereby promoting the transcription of Th2 cytokines such as IL-4, IL-5 and IL-13 in effector cells. In addition, GATA-3 up regulates the gene expression of c-Maf, in activated CD4 T-cells, which is also responsible for the exclusive production of the IL-4 (Dong et al. 2000, Jankovich et al. 2001, Szabo et al. 2003, Kosaka et al. 2006,). Literature states that c-Maf can co-ordinate its function of inducing IL-4 production with NFAT and SKAT2 (Dorado et al. 2002).

Gata-3 also has down-regulatory effects that act on STAT-4 and IL-12β chain thus inhibiting the production of IFN-γ and up-regulating Th2 polarization (Dong et al. 2000, Reiner. 2001, Grogan et al. 2002, Szabo et al. 2003, Zhu et al. 2006). Production of IL-4 and IL-13 is associated with the activation of B-cell to plasma cells that produce antibodies, whereas IL-5 is implicated in eosinophil activation (King and Willis. 2005).

Other cytokines involved in the Th2 immune response include IL-10 and IL-6 produced by regulatory T-cells and macrophages respectively. IL-10 is an anti-inflammatory cytokine that is reported to function in the activation of B-cell proliferation and the suppression Th1 related cytokines and mechanisms (de Jong et al. 2004, Oral et al. 2006). On the other hand, the pro-inflammatory cytokine IL-6 is also associated with the up-regulation of the Th2 response and suppression of the Th1 immunity (King and Willis. 2005).

1.2.2.2b Immunoglobulins and their function in humoral immunity

The combined effects of the Th2 cytokines are responsible for B-cell proliferation and the differentiation of these B-cells into memory cells and effector cells. The effector cells switch into plasma cells that produce Immunoglobulins (Ig), also known as antibodies (Ab) under the influence of Th2 cytokines. There are different types of Ig these include IgA, IgM, IgE, IgD and IgG (King and Willis. 2005).

The function of these immunoglobulins is to eradicate invading foreign antigens and this is achieved in several ways. Binding of antibodies to the antigen immobilises the invading microbe and neutralises its product. Antibodies can also cause agglutination by binding to multiple microbes thus resulting in the precipitation of bacterial products. Agglutination, neutralisation and immobilisation of antigens attract macrophages and cause enhanced phagocytosis (King and Willis. 2005). The formation of the immune complex between the antigen and the antibody activates the C1 molecules. This then triggers the classic pathway in the complement system resulting in MAC formation and microbial lysis. In addition, the C1 molecules coat the immune complex for identification by macrophages and hence phagocytosis of the Ig bound antigen (Terheyden et al. 2005, Tosi. 2005, Duncan. 2007).

The antibodies, specifically IgE bind and activate eosinophils, basophils and mast cells. Binding of IgE to these leukocytes degranulates them and they release inflammatory mediators, cytokines and toxic proteins. These products are responsible for vasochanges and influx of these leukocytes to the relevant affected sites and toxic killing of pathogens and helminths. In as much as the inflammatory response induced by these cells is protective to the host it can also have detrimental effects when there is pathological development of allergic reaction. These allergic reactions include asthma, hay fever and eczema (Abraham et al. 1998, Szabo et al. 2003) and if left untreated they can be fatal.

Immunoglobulins can also clear infections through the antibody–dependent cell cytotoxicity (ADCC). In this case, immune complexes are bound by NK cells through the Fc receptor on the antibody resulting in the release of perforin from NK cells. The perforin is directed to lyse the membrane of the pathogen (Tosi. 2005).

The ADCC pathway is employed in the attack against large microbes such as helminths, which cannot be phagocytosed by macrophages. Helminthic infections are usually accompanied by an increase of the IgE titre (van Riet et al. 2007). This immunoglobulin, which is usually low in the plasma, binds to its receptors expressed on eosinophils. The eosinophils are activated to release toxic substance that will degrade the parasite (Prussin et al. 2003).

1.3 Immune balance

In order to maintain homeostasis the immune system must be intact by avoiding a chronic inflammatory response in the innate immunity and retain the balance between the Th1 and Th2 immune response in the adaptive immunity. Even though the two domains are designed to protect the body from invading foreign pathogenic material, an overactive immunity that is not regulated can be pathophysiological. Chronic inflammatory response that is not regulated can result in necrosis of surrounding tissue due to prolonged release of reactive oxygen species such as nitric oxides and superoxides. Secondly a dominant Th1 immune response suppresses the humoral immunity designed to fight against bacteria and parasitic infections. Suppression of Th2 predisposes the host to extracellular infections. An example of which is the development of opportunistic infections in HIV-positive individuals who develop AIDS. On the other hand, Th2 dominance suppresses the cell-mediated immunity, which is responsible for containing viral infections, *Mycobacterium tuberculosis* and tumors. The suppression of Th1 makes the host susceptible to viral infections, TB and progression of malignant tumors to cancer. Literature has reported a high incidence rate of HIV and TB in individuals with a helminthic infection and hence a dominant Th2 immune response (Lawn 2004, Girard et al. 2005, Whiteside 2006, Tan and Coussens. 2007, van Riet et al. 2007)

The immune system has regulatory molecular mechanisms that ensure stability. However defects in the immune system that by-pass the regulatory mechanisms can result in overactive pathogenic immunity that can progress to autoimmune disease or immunosuppresion that can render the host susceptible to infections. The defects can either be genetic, from acquired infections or exposure to environmental contaminants. Exposure to environmental contaminants can be through direct contact in a working environment or via consumption of contaminated food and water. Environmental contaminants can cause adverse health effects in both humans and animals by altering homeostasis, which may result in the dysfunction of the body's endocrine system. The immune system is amongst the physiological systems of the body that is severely affected by contaminants polluting surface water (Tryphonas. 2001).

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Chapter 2: Surface water quality and immunotoxicity

2.1 Surface water and contamination

Surface water includes rivers, lakes, streams, oceans, reservoirs, estuaries and ponds (Ohe et al. 2004, Taylor 2003). These water bodies are an essential water source world wide. The importance of surface water is seen in its use as a drinking water source in disadvantaged domestic areas where there is a lack of proper water treatment and facilities. It is also used in industries as well as for the irrigation of crops in agriculture, for recreational purposes and religious activities (Taylor. 2003, Ohe et al. 2004).

The global concern lies with the deterioration of surface water quality which affects both developing and developed countries (da Silva and Sacomani. 2001, Massoud et al. 2006). The deterioration of water quality is due to the entry of environmental contaminants from agriculture, industries and domestic areas. These contaminants are in the form of fecal matter, pesticides, nutrients, heavy metals and estrogenic compounds. The introduction of these contaminants to surface water is primarily attributed to anthropogenic activity resulting from urbanization. Anthropogenic activity is responsible for the emission of pathogenic micro-organisms (Taylor 2003) and toxic chemical compounds into wastewaters. The inadequate or lack of treatment of these released effluents results in the accumulation of contaminating compounds in surface water systems (Gassana et al. 2002, Carroll et al. 2005, Evans et al. 2005, Massoud et al. 2006, Xian et al. 2007).

The different ways in which contaminants can be introduced into surface water are through point source pollution, non-point source pollution and atmospheric deposition. In point source pollution industrial effluents, municipal sewage treatment plants and combined sewage storm water are discharge into surface water. These discharges often arise from a single defined location. In non-point source pollution contaminants originate from poorly defined, diffuse sources that typically occur over a broad geographical scale. Examples of how contaminants can enter water ways in non-point source pollution include agricultural run-off following pesticide application, storm water and urban run-off (Ritter et al. 2002). Atmospheric deposition also falls under non-point source pollution; it results in airborne chemical compounds settling in surface water. This can occur through wet deposition where chemical compounds suspended in the air are absorbed through rain fall and deposited into surface water or it can occur through dry deposition where chemical compounds are directly adsorbed to water (Ritter et al. 2002).

Non-point source (NPS) pollution is a growing threat to natural waters and the major problem is that unlike point source pollution, NPS is difficult to identify as the entry point to surface water resources is diffuse and not limited to a single location (Van Zyl and Hearth. 2007).

2.2 Surface water contaminants and their impact on health

2.2.1 Fecal contamination

Fecal contamination of surface water can be from human and animal origin. The sources of fecal pollution can be point source coming from wastewater treatment plants or non-point source coming from domestic areas which lack latrines, and in agricultural areas where there is grazing live stock faeces and also cattle manure spread on cultivated areas. Fecal contamination of surface water can result in the accumulation of pathogenic micro-organisms such as viruses, bacteria and protozoa (Servais et al. 2007). The universally used indicator of water fecal contamination is the gram negative bacteria *Eschericha coli*; its presence in water reflects the potential prevalence of other pathogenic micro-organisms of fecal origin (An et al. 2002).

Exposure to water contaminated with fecal matter through consumption is associated with diarrhoeal diseases which can either be from viral, bacterial or protozoan agents. Examples of causative agents involved in diarrheal disease are *E.coli*, *Giardia lambia*, *Salmonella typhi, Vibrio cholera, Cryptosporiduium parvum* and *hepatitis A* virus. Other clinical manifestation associated with coming into contact with contaminated water includes the skin disease from schistosoma infection (Ashbolt 2004, Water and ethics 2004).

Diarrhoeal diseases resulting from contaminated water are the most dangerous. According to a WHO report given in 2004, 1.8 million people die every year from diarrhoeal disease (including cholera) and out of these death 90% are children under the age of 5 years. This mostly happens in developing countries where 88% of the diarrhoeal diseases are attributed to unsafe water supply, inadequate sanitation and hygiene (WHO 2004).

2.2.2 Nutrient contamination in surface water

The increase in anthropogenic activity, intensive agriculture, and industrial activities along with deficient water management has led to the enhanced eutrophication of surface water used for recreational purposes and as a drinking water source (de Figueiredo et al. 2004).

The use of fertilizers in agricultural areas for the maintenance of crops results in the accumulation of nutritious compounds such as nitrogen and phosphorus in the soil. Through non-point source pollution these nutrients are washed off into surface water where they retain their nutritional activity causing excessive growth of phytoplankton blooms. One of the pathogenic phytoplankton is the toxic blue-green algal blooms or other wise known as cyanobacteria (Paerl et al. 2001). The development of cyanobacterial blooms is associated with the production and accumulation of secondary metabolites toxic to many organisms, including humans (de Figueiredo et al. 2004). Microcystins are amongst the toxic metabolites produced by cyanobacteria. These toxins induce hepatotoxicity and neurotoxicity and they have been reported to have carcinogenic properties over long-term exposure in humans and animals. Due to the acute toxicity of cyanobacteria toxins, death of animals upon exposure to contaminated water has been reported (Harding et al 1995, van Halderen et al.1995, de Figueiredo et al. 2004,). Nitrogen exposure is also implicated in other adverse health effects. The naturally occurring ion of nitrogen, nitrate, which forms part of the nitrogen cycle, can combine with haemoglobin resulting in methemoglobinemia (Fewtrell. 2004). This illness affects babies who are still being bottle fed and it results from the reduction of hemoglobin to methemoglobin. Methemoglobin does not bind oxygen as a result it causes lack of oxygen in babies characterized by formation of the bluish skin colour and chocolate brown colour of blood (Fewtrell. 2004). Microcystins have also been reported to have immunomudulatory effects indicative of immunotixicity (Yea et al. 2001).

The ecological impacts associated with eutrophication include a reduction in dissolved oxygen in the water system resulting in hypoxia and anoxia of the water system making it an unfavourable environment for the invertebrates and vertebrates that inhabit the water body. Also accumulation of toxins can kill invertebrates and vertebrates in the water (Paerl et al. 2001).

2.2.3 Heavy metal contamination in surface water

The introduction of metals into surface water can be from natural sources or from anthropogenic sources. Metal contamination of surface water resulting from anthropogenic sources includes long-term discharges of untreated domestic and industrial wastewater, accidental spills, direct soil waste dumping and urban storm run-off. (Cheung et al. 2003, Turgut 2003, Gowd and Govil. 2008)

Some metals found in trace amounts in the water system are essential for the biological mechanisms involved in the proper functioning of organisms and they can be tolerated. However, if found in high concentrations these metals can become toxic. Heavy metals such as cadmium, mercury, lead and chromium are known to be toxic at very low levels and they can become major environmental and occupational hazards. Heavy metals are non-biodegradable with a very long biological half-life (Barbier et al. 2005) and thus can become persistent in the water environment for long periods. Exposure to water contaminated with heavy metals can induce adverse health effects associated with carcinogenesis, genotoxicity, hepatoxicity, immunotoxicity and since metals are processed by the kidneys they can cause renal dysfunction (Ohe et al. 2004, Barbier et al. 2005, Hemdan et al. 2006).

Heavy metals are also associated with cell damage by different mechanisms including direct damage of cell membranes and certain organelles, altered signaling transduction pathways, or they can affect the intracellular enzymatic system (Tsangaris et al. 1998), which may be the underlying cause of their carcinogenic properties. Excessive exposure to some of the heavy metals particularly Cadmium can result in death (Hemdan et al. 2006).

2.2.4 Pesticide contamination in surface water

Pesticides are widely used in agricultural areas for the maintenance of crops. They are the only toxic chemicals intentionally released into the environment because of their ability to kill off pests (Ongley. 1996).

The contamination of surface water by pesticides is mainly through non-point source pollution. During rainy seasons, storms wash off pesticide residues that have accumulated in agricultural fields into the water system. Atmospheric dry or wet deposition also plays a role in transporting pesticides into the river system. Point source pollution also contributes where industrial wastewater contaminated with pesticides is discharged into surface water (Konstantinou et al. 2006, Zhou et al. 2008).

The different types of pesticides found in contaminated surface water include organochlorine pesticides, organophosphate pesticides and carbamate pesticides. Organochlorine pesticides were used several decades ago, mainly in agriculture and in industries. Their use was banned because

they accumulate as persistent contaminants in water. Their persistence as water contaminants can be attributed to their characteristics of low polarity, low aqueous solubility, ability to resist biodegradation and bioaccumulation in fatty tissue (Kasozi et al. 2006). With regards to the latter, due to their highly lipophilic nature, organochlorine pesticides can be concentrated in fish muscle or tissue thus enabling these compounds to move through the food chain and affect humans and other animals in the higher trophic levels of the food chain (Hall et al. 1999, Barlas et al. 2006, Kasozi et al. 2006, Wang et al. 2007, Zhou et al. 2008). The major problem with organochlorine pesticides is that even long after they were banned they still persist as environmental contaminants and are found also in surface water (Wang et al. 2007).

Organophosphate pesticides were introduced to substitute the non-biodegradable organochlorines. This class of pesticide degrade rapidly in the environment and they do not accumulate in the tissue or muscle of invertebrates. The primary concern surrounding these pesticides is that they are highly toxic to vertebrates compared to the other classes of pesticides (Leong et al. 2007). Carbamate pesticides are degraded rapidly in the environment therefore have low persistence and are less toxic.

The adverse health effects associated with exposure to pesticides include neurotoxicity, birth defects, reproductive system dysfunction, endocrine disruption, cancer and immunotoxicity (Kasozi et al. 2006, Wang et al. 2007).

2.2.5 Estrogenic compounds in surface water

Estrogenic compounds are chemical substances that can interact with the estrogen receptor and induce estrogenic or anti-estrogenic activity (Garritano et al. 2006). These compounds include natural, synthetic and industrial estrogenic compounds. Synthetic estrogens, which include 17ά ethinylestradiol, mestranol or estradiol valerate, are widely used by women for birth control and hormonal replacement therapy. In addition to these compounds women and animals produce natural estrogens as part of the hormonal cycle. These include 17β-estradiol, estrone and estriol (Cargouët et al. 2004). Natural and synthetic hormones are voided from the body of women and animals through urine and can be found as contaminants in wastewater treatment plants, grazing fields and ultimately surface water.

Apart form natural and synthetic estrogens excreted by humans and animals, many chemicals of commercial and household use, such as Bisphenol A, alkylphenolic compounds, phthalates, PCBs, certain pesticides and metals, exhibit estrogenic activity (Cargouët et al. 2004, Stoker et al. 2003, Beck et al. 2006, Julius et al. 2007).

The main source of estrogenic contamination into the surface water system is the inadequate wastewater treatment effluents discharged into surface water. These wastewater effluents not only deposit traces of estrogens into surface water but they also give rise to metabolites that have more potent estrogenic activity than their precursors. These metabolites result from the degradation of alkyphenols to the shorter chain nonylphenols and octylphenols. However it should be noted that natural estrogens are the most potent endocrine disruptors (Julius et al. 2007, Cargouët et al. 2004, Beck et al. 2006).

Male fish, due to low estrogen concentrations under normal conditions, do not synthesize yolk proteins. However male fish exposed to water contaminated with estrogens synthesize yolk proteins such as vitellogenin. The presence of vitellogenin in the plasma of male fish is thus used as a biomarker for estrogenic activity in surface water. Estrogenic activity results in the presence of ovotestes in affected male fish (Cargouët et al. 2004, Vethaak et al. 2005). Other reproductive irregularities which may be associated with exposure of humans to water contaminated estrogenic compounds include development of hormone dependent cancers, disorders of the reproductive tract, a decrease in sperm production and reproductive fitness in males and immunotoxicity often associated with the development of autoimmune disease (Jin et al. 2007).

All of the surface water contaminants discussed above seem to target their toxic effects towards the immune system thus causing immunotoxity. The next section will review studies on the different contaminants and their immunotoxic effects.

2.3 Immunotoxicity

Immunotoxicity results from exposure to immumonomodulating substances that alter immune functions in a manner that will either cause immunosuppression leading to a decrease in host's resistance against infections or enhance immune activity resulting in autoimmune disease that can lead to self destruction of the host's body tissue.

2.3.1 Immunotoxicity by microcystins

Several studies done on the immunotoxic effects of the different microcystins produced by cyanobacteria have shown that these toxins can either be immunostimulatory or immunosuppressive.

Microcystins suppress the immune organs by decreasing the weight of the spleen and thymus. Since these two organs are responsible for the proliferation of T-and B-cells involved in the adaptive immunity, it has been shown that microcystins negatively affect their proliferation, viability and immune response through suppression. The inhibitory effect of microcystins on

lymphoproliferation is mediated by an increase in the apoptosis and necrosis of the cells in these organs. Furthermore microcystins reduce NK activity which maybe the cause of their tumor promoting properties (Yea et al. 2001, Zhang et al. 2001, Shen et al. 2003, Lankoff et al. 2004, Teneva et al. 2005, Zhang et al. 2006).

Microcystins also suppress the innate immunity parameters, by inducing leukocyte apoptosis, reducing phagocytosis and down regulating the production of nitric oxide responsible for the oxidative dependent killing of pathogens during phagocytosis (Shen et al. 2003, Gonçalves et al. 2007). Microcystins have been reported to suppress the production of cytokines involved in both the innate and adaptive immunity. These include the pro-inflammatory TNF-alpha, IL-1β, GM-CSF and

IFN-γ that is involved in Th1 polarization and cell mediated immunity. The other cytokines that are negatively affected by microcystins include IL-2, which is responsible for lymphocyte proliferation, and IL-4, which is a Th2 cytokine that polarises naïve CD4 T-cell into the humoral immunity (Chen et al. 2004, Lankoff et al. 2004, Shi et al. 2004).

Contrary to these suppressive effects, microcystins have also been reported to induce immunostimulatory effects. These toxins enhance inflammation by promoting chemotactic activity through inducing the spontaneous adherence and increasing the migration of neutrophils. In addition, microcystins positively regulate phagocytosis by increasing the intracellular killing activity through the induction of ROS production in polymorphonucleocytes (Hernández et al. 2000, Kujbida 2006).

2.3.2 Immunotoxicity by heavy metals

The immunotoxic effects of heavy metals (cadmium, chromium, arsenic, mercury, lead and manganese) can either be immunosuppressive or immunostimulatory and these effects can be exerted on immune organs, cellular activity, cytokine production and immune responses. Studies support the view that immunomodulatory effects by metals on the various parameters may compromise the host's defense against infectious agents and also aid in the progression of autoimmune disease. The intensity of the adverse effects due to metal toxicity are dependent on the animal species, route and duration of exposure, and the type of metal being tested (Bernier et al. 1995).

Exposure to heavy metals such as cadmium (Cd), lead (Pb) and mercury (Hg) can cause thymic damage and splenomegaly. The latter is associated with an increase in the weight of the spleen. Thymic damage is often associated with induction of apoptosis in thymocytes and a resultant decrease in thymic weight (Tsangaris et al. 1998, Liu et al. 1999, Kim et al. 2000, Dong et al.

2001, Fernandez-Cabezudo et al. 2003, Pathak et al. 2007, Jadhav et al. 2007). Since the spleen and the thymus are responsible for the proliferation of B-cells and T-cell lymphocytes, the apoptosis induced on these two organs reduces lymphocyte proliferation. The decline in lymphocytes also negatively affects the humoral and cell-mediated immune response involved in acquired immunity through a decrease in antibody production and delayed type hypersensitivity. Furthermore, heavy metals induce oxidative stress that results in necrosis associated with unprogrammed cell death and tissue destruction (Kim et al. 2000, Jelovcan et al. 2003, Fernandez-Cabezudo et al. 2003, Kim et al. 2003, Chen et al. 2004, Mondal et al. 2005, Luebke et al. 2006, Pathak et al. 2007).

The immunosuppressive effects of heavy metals are also exerted on the innate immunity where these toxic compounds decrease leukocyte proliferation and affect macrophage cell morphology, reduce phagocytotic activity by inhibiting cell recruitment and chemoctatic migration (Chen et al. 1999), depressing the production of NO, myeloperoxidase and lysosomal enzyme release involved in the intracellular killing of pathogen. In addition, natural killer cell activity associated with eradication of viral infections and tumors is also inhibited (Kim et al. 2000, Bishayi et al. 2006). Hence Cadmium and Manganese increased the susceptibility of mice to a sublethal infection of viruses as observed by the increase in symptoms and mortality associated with these infections (Seth et al. 2003).

In cytokine production, the Th1-related cytokine (IFN-γ) is more suppressed than Th2 cytokines (IL-4 and IL-10) upon metal exposure. Thus metal exposure seems to bias Th2 polarization and this effect is suggested to play a big role in allergic reactions and the development of autoimmune disease.

The stimulatory effects associated with autoimmunity by heavy metals include, contradictory to immunosuppressive effects, T and B-cell lymphocytes proliferation, increased antibody production in B-cells particularly IgE, IgM and IgG, and increased expression of surface MHC II molecules on antigen presenting cells (Guo et al. 1996, Miller et al. 1998,). Furthermore, Mercury has been reported to increase autoantibody production and formation of immune complex deposits (Kim et al 2003, Silbergeld et al 2005).

The immunotoxic effects of heavy metals (i.e. lead) are reported to be more profound in the developing immune system while their impact in adults are more subtle (Chen et al. 1999). Synergistic effects of combined metal mixtures intensify the adverse effects imposed on an exposed immune system (Bishayi et al. 2006, Jadhav et al. 2007). Heavy metals are co-factors in autoimmune disease increasing the risks and severity of clinical disease in the presence of triggering event that can be either genetic or acquired (Silbergeld et al. 2005).

2.3.3 Immunotoxicity by pesticides

Immunotoxicities due to the different types of pesticides are varied and include pathology of immune organs, immunosuppression of the various immune functions that results in decrease host resistance, and immunostimulatory effects that promote development of hypersensitivity reaction and progression of autoimmune disease (Galloway et al. 2003).

Immunotoxic effects by the non-biodegradable organochlorine pesticides, highly toxic organophosphate pesticides and the low toxicity carbamates are imposed on several organisms. These include aquatic organisms, avian species and humans.

Pesticides cause a reduction in spleen and thymus weight which also affects lymphoproliferation by inhibiting T-cell and B-cell proliferation. These effects have a profound suppressive effect on antibody production and delayed type hypersensitivity involved in the humoral immune response and cell-mediated immune responses respectively (Hart et al. 1997, Elsabbagh et al. 2001, Handy et al. 2002, Rooney et al. 2003, Neishabouri et al. 2004, Chen et al. 2005). Although these immunotoxic impacts primarily affect fish, other animals in the higher trophic levels of feeding, including humans, are also affected due to the biomagnification of organochlorine pesticides. For example, seals consuming Baltic Sea herring exhibited suppressesed immune functions (Van Loveren et al. 2000). Similar effects were seen in young Caspian terns from the Great lakes and from the umbilical cord blood of people involve in subsistence fishing (Grasman et al. 2001, Bilrha et al. 2003).

Pesticides also affect the innate immunity by suppressing phagocytosis through the reduction of macrophage proliferation and the production of reactive oxygen intermediates involved in the intracellular killing of pathogens. This immunomodulation results in the decreased host resistance against infection (Beaman et al. 1999, Chen et al. 2004, de Guise et al. 2004, Giroń-Peréz et al. 2007). In fact fish that were exposed to the organophosphate pesticide malathion had a reduced resistance against induced Yersinia infection (Beaman et al. 1999).

The cytotoxic effects of pesticides are caused by the induction of oxidative stress resulting in increased production of free radicals capable of causing cell necrosis and tissue damage (Koner et al. 1998, Galloway et al. 2003). The pro-inflammatory cytokines IL-6 and TNF-α are sometimes increased due to metal toxicity. The increased pro-inflammatory cytokine levels are indicative of cell necrosis and inflammation.

Pesticides can either suppress or stimulate cytokine production, but in both instances there seem to be a bias where one or other of the Th2-related cytokine levels are elevated and Th1 related cytokines are often suppressed. The affected cytokines include the pro-inflammatory cytokines

IL-6, TNF-alpha, IL-2 associated with lymphocyte proliferation, the Th2 cytokines IL-4 and IL-10, and the Th1 cytokine IFN-γ. Suppression of the pro-inflammatory cytokine TNF-α is due the alteration of NF-kB transcription (Corsini et al. 2006).

Pesticides induce allergic reactions by enhancing Th2 development and in addition contribute to chronic inflammation (Seth et al. 2005, Corsini et al. 2006, Alluwaimi 2007, Kim et al. 2007). Immunostimulatory effects of pesticides have been reported, in workers occupationally exposed to Mancozeb. Exposure to this pesticide caused elevated serum IgG, IgE and β2 macroglobulin and increased T-cell proliferation. Sodium diethyldithiocarbamate was shown to influence maturation and activation of T-cells, natural killer cells, IgG secretion and prolonging immunological memory (Corsini et al. 2006).

Pesticides effects are intensified in the developing immune system while in adults impacts are more subtle (Rooney et al. 2003, Luebke et al. 2006).

2.3.4 Immunotoxicity by estrogenic compounds

The presence of estrogen receptors in thymocytes, splenic CD8 cells, monocytes, B-cells and T-cells accounts for the ability of estrogenic compounds to modulate immune cell functions (Kogiso et al. 2006).

In the innate immune system estrogenic compounds inhibit phagocytotic functions by destabilizing lysosomal membranes involved in bacterial internalization and killing. Estrogens also suppress monocyte adhesion via the down regulation of Rac1GTPase and reducing macrophage proliferation and migration to the site of infection (Konishi et al. 2003, Friedrich et al. 2006, Sugita- Gauthier-Clerc et al. 2006). The suppression of inflammation by the natural estrogen 17β-estradiol in mice is through inhibiting the activation of the NF-kB transcription factor responsible for the production of pro-inflammatory cytokines. Hence the suppression of IL-6 induced by Bisphenol A in mice due to the inhibition of NF-kB.

In addition estrogenic compounds increase the migration of neutrophils to infected sites through enhanced MCP-1 production but reduced leukocyte phagocytic activity (Sugita-Konishi et al. 2003). Estrogenic compounds also increase the number of natural killer cells responsible for viral and tumor killing, but reduce their cytotoxic effects (Guo et al. 2002, Karow et al. 2004, Hao et al. 2007).

Estrogenic compounds induce immunotoxic effects in immune organs by decreasing thymic weight, spleen weight and causing thymic atrophy. The negative effects induced on the thymus results in suppressed lymphocyte proliferation of T-cells due to the induction of apoptosis (Hoffman-Goetz et al. 1999, Guo et al. 2002, Sugita-Konishi et al. 2003, Karow et al. 2004).

However, estrogenic compounds enhance the proliferation of B-cells and antibody production, particularly IgE.

The inhibition of T-cells and natural killer cell activity extends to cytokine production, where the Th1 related IFN-gamma produced by these cells is inhibited. Most estrogenic compounds tend to bias Th polarization towards Th2 by enhancing the production IL-4. Th2 polarizations bias is linked to the development of allergic reaction and autoimmune disease. Induction of allergic reactions by Bisphenol A and Alkylphenols are enhanced by increased IL-4 production in CD4 T-cells and antigen specific IgE levels in the sera via the stimulation of Calcium/calcineurin-dependent Nfat transcription factor activation (Lee et al. 2004). In addition the alkylphenols, octylphenol and nonlyphenol, suppress Th1 development and enhance Th2 development (Iwata et al. 2004)

2.4 RESEARCH PROBLEM

Surface water contamination is a global concern because it threatens the quality of the essential water resources and it has adverse health consequences which predispose animals and humans to disease. Therefore there is a great need for detecting methods that will monitor surface water quality and thus help put preventative measures in place that will contribute to maintain sustainable development of this scarce resource.

Currently, the most commonly used techniques to monitor surface water health measure the physico-chemical properties to detect the presence of known hazardous substances or priority micro organic pollutants. The techniques that are used include HPLC, GC-MS and microbiological assays. The problem with the use of these techniques is that they only target certain compounds which have been selected according to their reported acute toxicity in the environment and in aquatic organisms. These reports are based on studies that often use dosages that are environmentally irrelevant. In addition, attention and alarm thresholds are often directed toward the acute toxicity of a single polluting compound or a group of known pollutants and little concern is given to the fact that water pollutants occur in a mixture that also consist of unknown compounds. Most studies that employ the use of the mentioned techniques do not take into consideration the impacts of long-term exposure to the low-level mixture of known and unknown compounds. In addition, these techniques can only assess the current status of surface water. However they do not give information about the health risks that can be imposed on humans and animals associated with exposure to contaminated surface water. Lastly most studies that investigate the impact of water contaminants *in vivo* are performed in experimental animals. Literature states that immunotoxicity studies have accumulated a large database from animal

experiments. This database has been used to extrapolate the potential risk of human exposure. However this has a shortcoming as there are interspecies differences that can lead to the misjudgement on the effects of contaminated water. Thus it would be biologically more meaningful if immunotoxicity data would be based on experiments derived from humans (Tryphonas. 2001)

The use of *in vitro* bioassays combats this problem because they can effectively define the health risks to humans and animals associated with exposure to surface water contaminants (Ohe et al. 2004, Pellacani et al. 2006). In addition, the use of HPLC and GC-MS in the extraction and analysis of water contaminants is a complicated and time consuming process as it requires different procedures for the characterization of known single or group compounds. So the use of the non-selective solid phase extraction (SPE) method is much more advantageous as it eliminates the bias surrounding water contaminants analysis (Galassi et al. 2004).

In immunotoxicity studies, use of isolated leukocytes is very common when detecting immunoregulatory mechanisms such as cytokine production. However isolated leukocytes can be a problem due to pre-activation during the purification process. This can alter the end-result. In addition, when monitoring cellular response, isolated leukocytes do not give an environment resembling the *in vivo* situation hence the use of whole blood culture is much more advantageous. Whole blood cultures require only small amounts of blood, are fast to perform and they are representative of *in vivo* conditions (Fasanmade et al. 1995, Yancy et al. 2001, Poole et al. 2003)

The aim of the current study is to employ SPE to extract hydrophobic contaminants from surface waters. The extracted hydrophobic constituents will then be assesses for potential adverse effects on the immune system using an *in vitro* whole blood culture assay. The immune mechanisms that will be studied include the inflammatory, humoral and cell-mediated pathways.

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Chapter 3: The immunotoxicity of water collected from the Eerste River, Stellenbosch, South Africa

Introduction

Globally there is great concern because of the rapid deterioration of river water quality due to pollution by pathogenic microorganisms and toxic chemical compounds (xenotoxicants). The pollution of the aquatic environment is caused by both point and diffuse source pollution. River water contamination can be attributed to intensive agricultural use, urbanization and industrialization in regions near major water catchment areas. Seasonal variations also contribute to the deterioration of water quality and quantity. Run-off from contaminated areas is concentrated in the aquatic environment when there are low river flows (Ritter et al. 2002, Massoud et al. 2006, da Silva and Sacomani. 2001). Direct contact or consumption of contaminated water is associated with waterborne disease such as diarrhea, reproductive defects, cancer and immunotoxicity (Aravindakshan et al. 2004, Cargouët et al. 2004, Vethaak et al. 2005, Swart and Pool. 2007, Kujbida et al. 2006).

Waterborne diseases, specifically diarrhea, are the leading cause of the death in children and can play a significant role in the progression of AIDS in debilitated HIV positive people who have no access to proper water facilities (Pool et al. 2003, Obi et al. 2006,). Due to the severity of the problems associated with the effects of contaminated water on the immune system it is important to have water quality assessment systems in place that will not only assess the physico-chemical properties of water, but also give early warning signals of potential adverse health effects that contaminated water can pose. In addition, since water contaminants occur in mixtures of varying complexities, it is essential to have biological assays that can monitor these composite mixtures for potential adverse effects (Pellacani et al. 2006). This is specifically important when there are compounds in the pollutant mixture that act antagonistically and/or synergistically with one another.

Cytokines, due to their role in driving immune responses, can be used *in vitro* bio-assays as biomarkers for immunotoxicity. IL-6 has been employed as a biomarker of inflammation and as an indicator of deteriorating river water quality when monitoring water contamination. These studies were done using isolated leukocyte cultures and also with whole blood cultures. The latter system is more advantageous to use because there is no cell purification process that can potentially pre-activate the cells and most importantly, whole blood cultures resemble the *in vivo* situation more closely than isolated cell fractions. Whole blood culture assays using IL-6 as a biomarker have previously been used to determine inflammatory

activity (Fasanmade and Jusko. 1995, Yancy et al. 2001, Poole et al. 2003,) and have also been used to monitor the inflammatory activity and quality of river water (Pool et al. 2000, Pool et al. 2003, Wichmann et al. 2004,). Literature searches have shown no studies on the impacts of water pollutants on the humoral and cell-mediated immune pathways. The aim of the current study is to screen water from the Eerste River, Stellenbosch, South Africa for cytotoxicity, inflammatory activity, and humoral and cell mediated immunotoxicity,

The Eerste River originates in the mountains near Jonkershoek. Figure 1a is a map of the Stellenbosch area indicating specific landmarks that were used for this study. Upstream from Jonkershoek is a nature conservation area and the river water is not impacted on by human activity. There is limited agricultural activity on the banks of the Eerste River upstream from the central business area Stellenbosch. Between Jonkershoek and central Stellenbosch the Eerste River is used for the irrigation of agricultural crops and also for aquaculture. The Plankenbrug River runs into the Eerste River downstream from central Stellenbosch. The Plankenbrug River is heavily polluted by chemicals generated due to various human activities. The pollutants include raw domestic effluents from inadequately serviced formal and informal settlements in Kayamandi, effluents from various industrial factories near Plankenbrug and agricultural run-off from wine, chicken, pig and cattle farming near Koelenhof (Nleya. 2005, Ngwenya. 2006). The Eerste River/Plankenbrug River system thus provides a unique opportunity to study the impacts of both pristine and contaminated aquatic environments on the immune system and also human health in general.

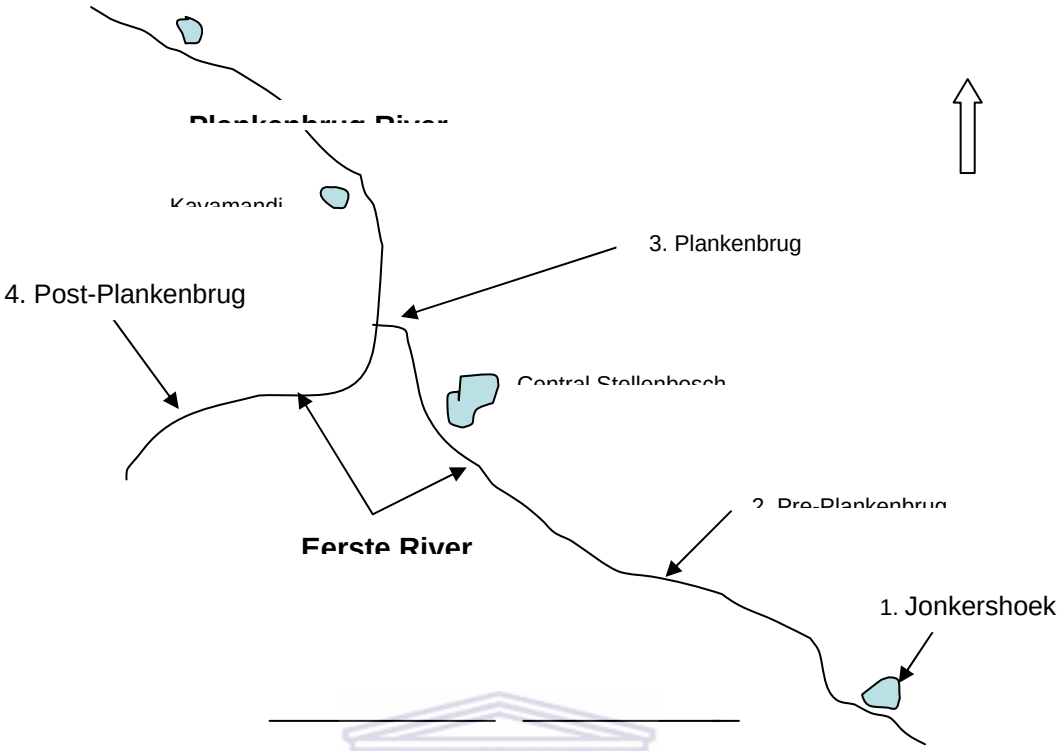

Figure 1a: Map of the Eerste River in Stellenbosch, Western Cape, South Africa.

Methods

Water collections. Water samples were collected in clean 2L glass bottles. The bottles were cleaned by washing with soap, followed by rinsing with tap water. The bottles were then rinsed with reverse osmosis water and finally it was rinsed with HPLC grade ethanol (Merck, Germany). The bottles were then allowed to dry up-side down in a dry rack. Eerste River water samples were collected at Jonkershoek, at 100 m before the confluence of the Eerste and Plankenbrug rivers (pre-Plankenbrug sampling site) and also at a site 100 meters downstream of the confluence of the two rivers (post-Plankenbrug sampling site). Water samples were also collected from the Plankenbrug River 50 m before the confluence with the Eerste River. Samples were collected in January, at the peak of the dry summer season and in the rainy season in June. Water samples were taken to the laboratory and were processed immediately. Neat water samples were stored at -20˚C.

Water Extraction. Water samples were first filtered through Whatman 1 filters (W&R Balston Limited, England) to remove particulates. Filtered water samples were extracted using C18 columns as per our laboratory protocol. Briefly, C18 hydrophobic columns (Anatech) were charged by first washing it with 4 ml solvent mixture (40% v/v hexane, 45% v/v methanol and 15% v/v 2-propanol). This was followed by a wash with 4 ml of ethanol and finally with 4 ml distilled water. Care was taken not to let the columns run dry between the additions of the various liquids. After this the environmental water sample was applied onto the column in a single loading. At the end of the sample application, the column was washed again with 4 ml of distilled water. The column was then dried for at least 1 hour on a vacuum pump. The hydrophobic molecules bound to the column were then eluted with 3 ml of the solvent mixture. The eluate was dried under a stream of air. The dried water extracts were reconstituted in dimethyl sulfoxide (DMSO) to give a final volume 1/1000 times that of the original sample volume.

Blood collection. Blood was obtained from healthy male volunteers by a nurse or doctor at the campus health clinic. Blood samples were collected directly into heparinised vacuum tubes by venipuncture. The blood was stored at ambient temperature and used within 4 hours after collection.

Inflammatory activity. All procedures were done under sterile conditions in a laminar flow cabinet. Four replicates of all samples were assayed. The water samples (2.5 µl/well) were added to wells of a 96 well tissue culture plate (Nunc^{TM}, Denmark). Four replicates of a control containing 2.5 µl/well of DMSO were also included on each plate. Heparinised whole blood was diluted 1:10 with RPMI 1640 (Sigma, Germany). The diluted blood was added to the plate (250 µl/well) and the plate was then incubated at 37˚C for 18-20hrs. At the end of the incubation period the culture supernatants were collected and assayed for IL-6 and LDH.

Th1 and Th2 activity. Heparinised blood was diluted at a ratio of 1: 9 with RPMI-1640 (unstimulated blood) or at a ratio of 1: 9 with RPMI containing 4 µg/ml phytohaemagglutinin (Sigma, Germany) for the stimulated blood. Water extracts (2.5 µl of reconstituted extract per well) or controls (2.5 µl/well DMSO) were added to wells of a 96 well culture tray (Nunc^{TM}, Denmark). Four replicates were done for each sample. Diluted unstimulated or

PHA stimulated blood (250 µl/well) was added to the samples and the mixtures were incubated for 48 hours at 37 °C. At the end of the incubation period the supernatants were collected and assayed for Interferon-gamma (IFN-γ) or Interleukin- 10 (IL-10).

Cytotoxicity. A commercially available chromogenic LDH assay kit was used to analyze the samples (Sigma, Germany). Supernatants collected from the blood samples incubated for inflammatory activity were diluted 1 in 10 with saline. Samples were transferred to wells of a 96 well plate. For the 100 % standard a control blood sample (250 µl) received 2.5 µl of a 10 % v/v Triton X100 solution. The blood lysed immediately. The lysed blood sample was mixed and an aliquot of the lysed blood was diluted in saline at a ratio of 1:10. The diluted blood sample was used as the 100 % cytotoxicity control. Dilutions of this sample were used to construct a standard curve. The standards were also transferred to the 96-well plate. LDH kit substrate was added to the samples and the wells were mixed by tapping the plate. Absorbance readings were taken at 10 minute intervals at 592 nm. Between readings the plate was incubated at room temperature away from the light. The cytotoxicity of the samples were read off a standard curve constructed using the standards.

Cytokine analysis. Double Antibody Sandwich (DAS) ELISAs (e-Bioscience,Germany) were used to quantify cytokine production from the supernatants of whole blood cultures. Nunc maxisorp (Nunc^{TM}, Denmark) plates were used for the assays. All reagents and diluents required for the ELISA are in kit. The assay was performed according to the protocol provided in the kits. In brief: Primary antibody against the respective cytokine was coated onto the plate. The coating was allowed to proceed overnight at 4 ºC. The next morning the plates were washed with phosphate buffered saline (PBS) containing 0.05 % Tween. The plates were then blocked with an assay buffer from the kit for 1 hour, after which the wells received either sample or cytokine standards. The plate was again incubated for 2 hours at ambient temperature on a shaker. After washing the plate the wells received biotinylated secondary antibody against the respective cytokine. The plate was incubated for 1 hour at ambient temperature on a shaker followed by washing as before. The wells then received avidin-peroxidase conjugate. The plate was again incubated for 30 minutes at ambient temperature on a shaker. After the final wash, substrate was added to the wells. The plate was incubated for 20 minutes after which the reaction was stopped by adding 2M H_2SO_4. The plate was then read at 450 nm on a plate reader. A standard curve for each ELISA plate

was generated using Excel and this was used to determine the cytokine concentrations induced by the water extracts and the controls.

Statistical Analysis. Results were analysed for statistical differences between samples using ANOVA test in Sigma Stat

Results

The cytotoxicity of water from the Eerste River. Total LDH in the blood cultures were obtained by lysing diluted whole blood with the detergent Triton X100. The total LDH of the whole blood cultures, when released into the medium, was regarded as 100 % Toxicity. LDH was assayed using a chromogenic substrate for LDH. A standard curve was constructed using dilutions of the 100 % Toxicity sample (Fig 2a). This curve shows that there is a good correlation between the absorbance values and the % Toxicity (R^2=1.00).

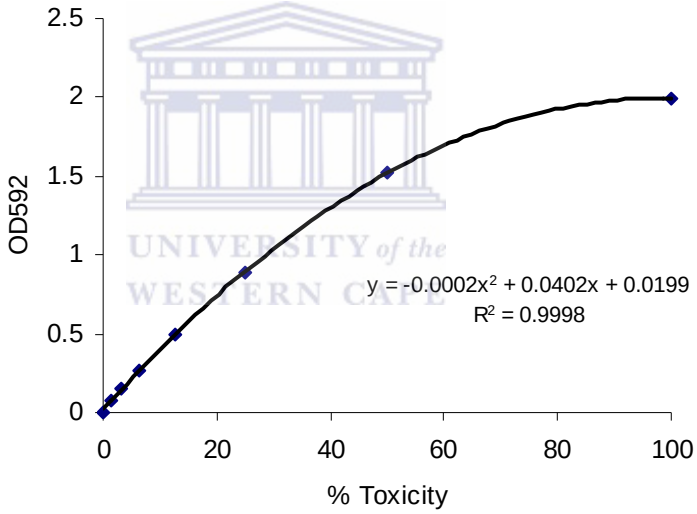

Figure 2a: Standard curve for cytotoxicity. Total LDH of the cell homogenate was used as 100 % Toxicity level.

The data shows that the toxicity of all the samples analyzed were below 10 % (figure 3). Similar % toxicity values were also obtained for samples incubated with the DMSO control. Analysis of the sample data shows that there are no statistically significant differences in cytotoxicity between the various sampling sites (Fig 3a). Follow-up experiments were conducted to determine the origin of the low LDH level found in the medium of sample incubations and the controls. Experiments with freshly collected blood shows that the LDH detected in the culture supernatants originated from the blood plasma of the patient (i.e. background LDH of assay) and that it was not due to cytotoxic reactions occurring during the

incubation period with samples (data not shown). From these results it can be concluded that none of the samples caused cell death and LDH leakage.

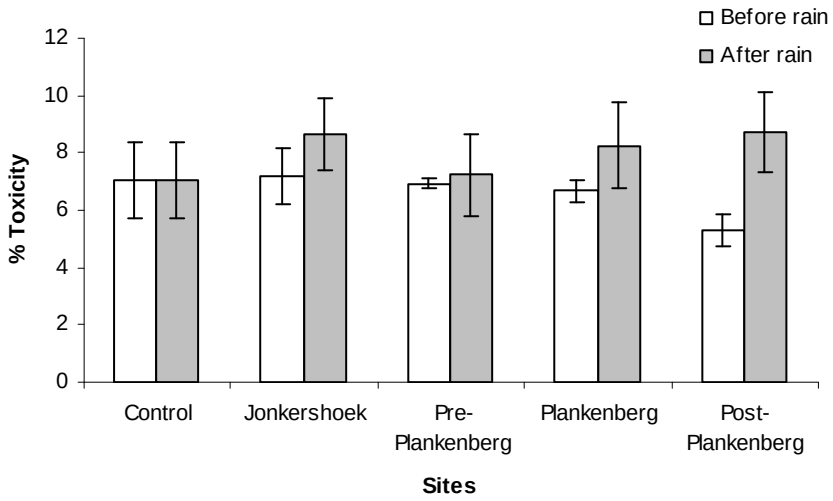

Figure 3a: The cytotoxicity of the river samples expressed as a % toxicity. Total cellular LDH was used as 100 % toxicity. Data are presented as the mean ± SEM (n=8). n is the number of patients used in the experiment

The inflammatory activity of water from the Eerste River. IL-6 synthesis by whole blood cultures was used as a biomarker for inflammatory activity. The standard curve (Fig 4a) obtained using the IL-6 ELISA kit standards showed that there is a good correlation between the absorbance values and IL-6 concentration (R^2=0.98).

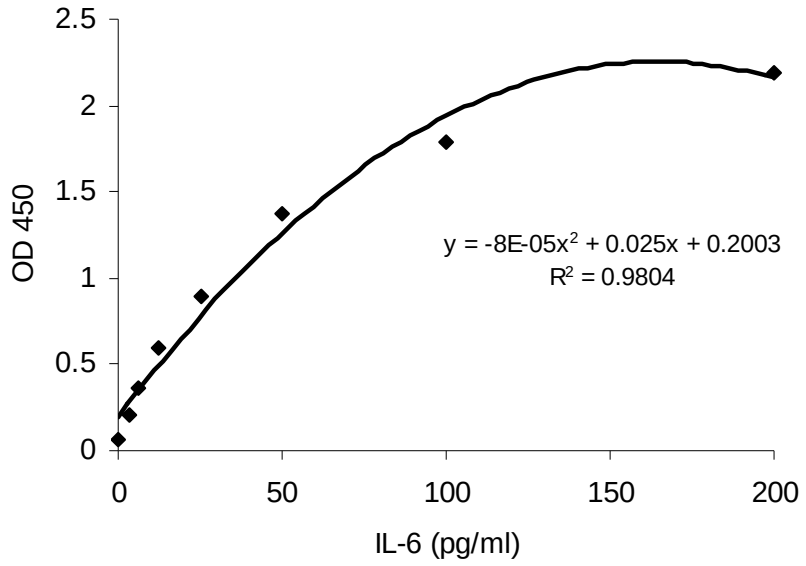

Figure 4a: Standard curve for IL-6. The kit standards were assayed by the DAS ELISA according to the manufacturer's instructions.

All the samples induced statistically higher levels of IL-6 synthesis than the control ($p < 0.001$). There was no difference between the levels of IL-6 induced by water from the different sampling sites after the seasonal rains. However, samples collected at the end of the dry season showed that the levels of IL-6 induced by water extracts from the sampling sites differed significantly from each other. The water from the Plankenbrug site induced the highest level of IL-6 while the lowest IL-6 induction was by the Jonkershoek sample. The sample collected from the Plankenbrug site induced significantly higher ($p < 0.001$) levels of IL-6 when compared to the other samples collected on the Eerste River (Fig 5a). Water from post-Plankenbrug induced higher IL-6 levels than pre-Plankenbrug as well as Jonkershoek ($p < 0.001$). Water from pre-Plankenbrug induced higher IL-6 levels than the Jonkershoek sample ($p < 0.005$). There was no seasonal effect on IL-6 induction for the Jonkershoek site. However, statistically significant differences were found for the IL-6 induction by the pre- and post seasonal rain samples collected from pre-Plankenbrug ($p < 0.005$), Plankenbrug ($p < 0.001$) and Plankenbrug ($p < 0.001$) sampling sites.

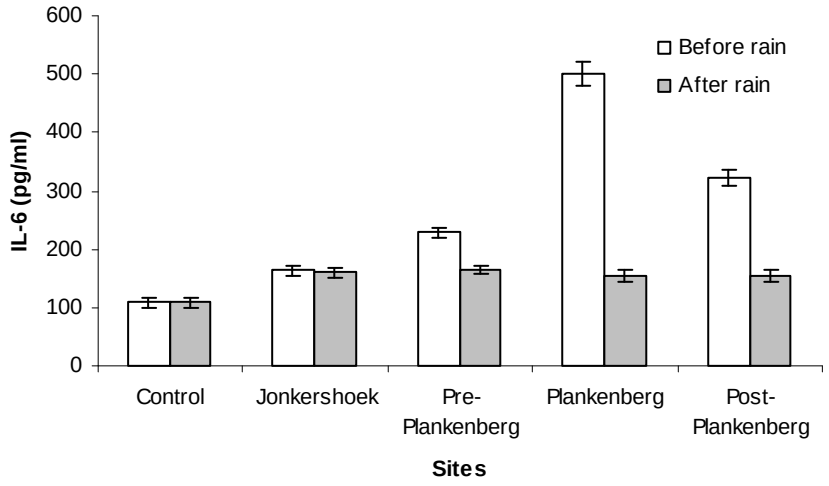

Figure 5a: The inflammatory activity of the river samples. Results are expressed as IL-6 secreted by whole blood cultures in the presence of river water samples. Data are presented as the mean ± SEM (n=16). n is the number of patients used in the experiment

The effects of water from the Eerste River on humoral immunity. IL-10 synthesis by whole blood cultures upon stimulation with PHA was used as a biomarker for the humoral immune pathway. The standard curve (Fig 6a) obtained using the IL-10 ELISA kit standards shows that there is a good correlation with the absorbance values and IL-10 concentration (R^2=1.00).

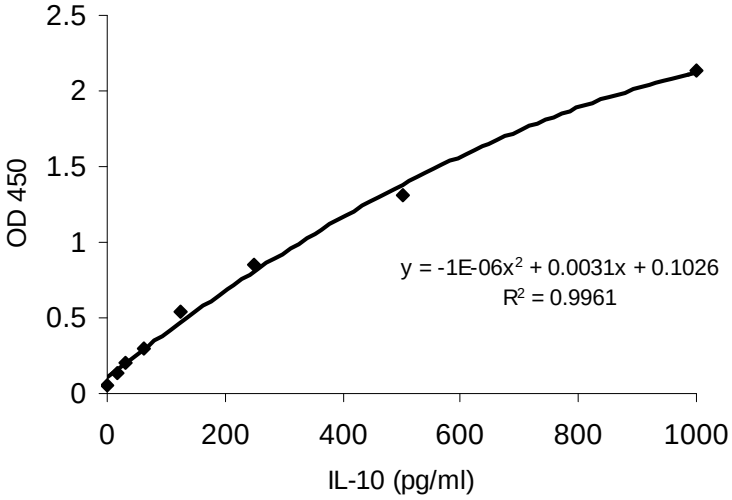

Figure 6a: Standard curve for IL-10. The kit standards were assayed by the DAS ELISA according to the manufacturer's instructions.

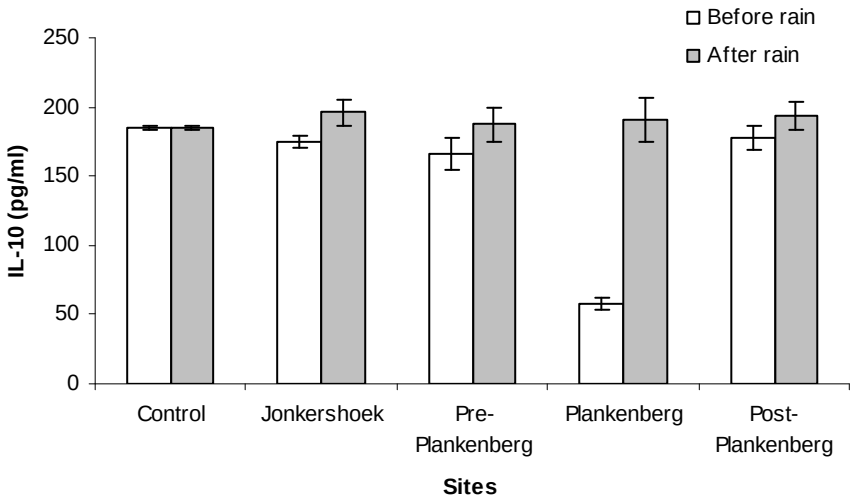

Figure 7a: The effects of the river samples on IL-10 secreted by PHA stimulated whole blood cultures. Data are presented as the mean ± SEM (n=16). n is the number of patients used in the experiment

Initial experiments showed that the unstimulated cultures (absence of PHA) did not induce IL-10. Due to this only data for the PHA stimulated blood cultures are presented (Fig 7a). Samples collected at the end of the dry season showed that the Plankenbrug site caused a significant decrease ($p < 0.001$) in IL-10 secretion when compared to the other collection sites on the Eerste River. There was no difference in the level of IL-10 induced by the control, Jonkershoek, pre-Plankenbrug and post Plankenbrug samples at the end of the dry season. There was no significant difference between IL-10 induced by the different sites after the seasonal rains. The levels of IL-10 induced by the different sites after the seasonal rains were similar to that of the control.

The effect of water from the Eerste River on cell mediated immunity. IFN-γ synthesis by whole blood cultures upon stimulation with PHA was used as a biomarker for cell mediated immunity. The standard curve (Fig 8) obtained using the IFN-γ ELISA kit standards shows that there is a good correlation between the absorbance values and IFN-γ concentration (R^2=0.98).

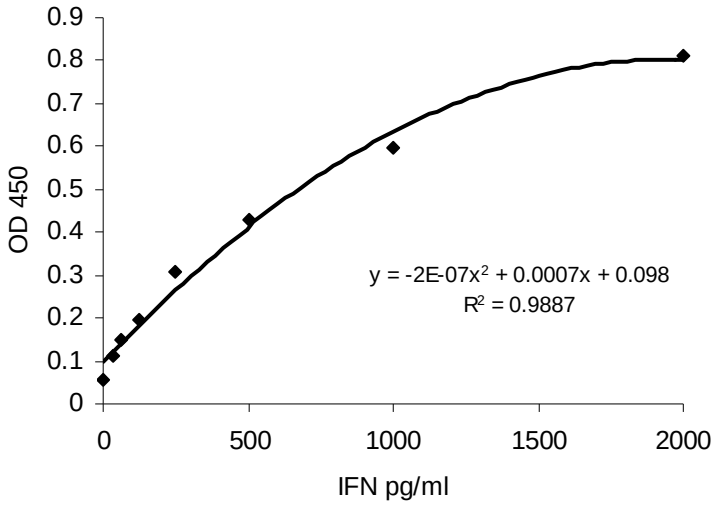

Figure 8 : Standard curve for IFN-γ. The kit standards were assayed by the DAS ELISA according to the manufacturer's instructions.

Initial experiments showed that the unstimulated cultures (absence of PHA) did not induce IFN-γ. Due to this only data for the PHA stimulated blood cultures are presented (Fig 9). Samples collected at the end of the dry season showed that the Plankenbrug site caused a significant decrease ($p \leq 0.001$) in IFN-γ secretion when compared to the other collection sites on the Eerste River (Fig 9). There was no statistical difference in the level of IFN-γ secretion by the control, Jonkershoek, pre-Plankenbrug and post Plankenbrug samples at the end of the dry season. There was no significant difference between IFN-γ induced by the different sites after the seasonal rains. The levels of IFN-γ induced by the different sites after the seasonal rains were similar to that of the control.

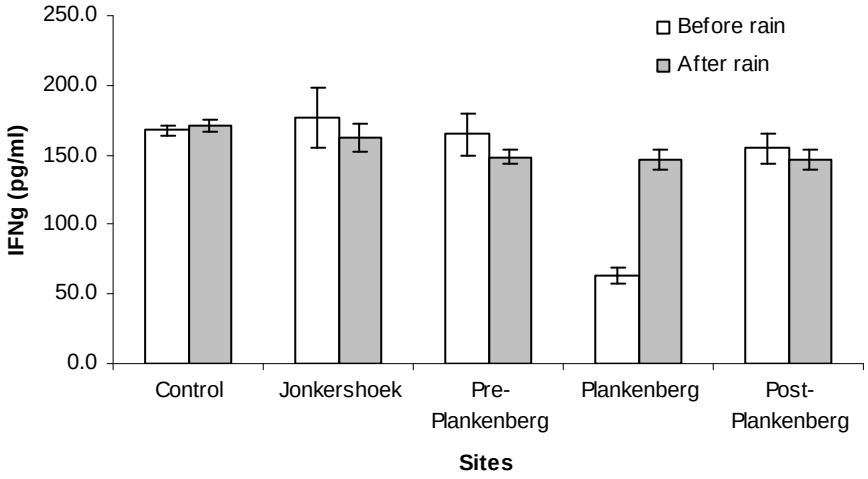

Figure 9: The effect river samples on cell-mediated immunity expressed as IFN-γ secreted by PHA stimulated whole blood cultures. Data are presented as the mean ± SEM (n=17). n is the number of patients used in the experiment

Discussion

The samples used for this study showed no cytotoxicity using the cellular LDH release assay. Although this assay is a very sensitive assay for the determination of cytotoxicity, results obtained from this method does not exclude effects that may affects specific cellular pathways. Previous studies showed that the absence of cytotoxicity (cell death) does not necessarily mean that the samples have no effect on the physiological systems of the cell (Ganey et al. 1993). Studies comparing cytoxicity and immunotoxicity showed that some chemicals have effects on immune pathway biomarkers at concentrations much lower than those required to cause cell death (Ganey et al. 1993, Vandebriel et al 1999, Champeau and Narbonne. 2005).

IL-6 is a sensitive biomarker for inflammatory activity and water quality (Pool et al. 2000). The results obtained for the IL-6 levels in this study are consistent with previous findings of Pool et al. (2000). The data also agrees well with the water quality findings of Ngwenya. (2006) and Nleya. (2005). The results from this study indicate that the water form the Plankenbrug River has very high inflammatory indicing activity in summer. Studies have shown that inflamed intestinal cells have an impaired electrolyte transport mechanism (Seidler. 2006). This results in defective fluid absorption by the intestine and ultimately diarrhea. Residents, specifically children, from an inadequately serviced informal settlement on the bank of the Plankenbrug River near Kayamandi use the water for recreation and also for other domestic purposes. Consumption of water from the Plankenbrug River in summer can pose a health risk to consumers as it can result in increased incidence of diarrhea.

People with congenital or acquired deficiencies of the humoral system are very susceptible to extracellular pathogens such as the encapsulated bacterial pathogens (Buckley. 1986). Consumption of contaminated river water by disadvantaged communities has been associated with faster progression from the time of HIV infection to AIDS and its associated opportunistic infections (Obi et al. 2006). The current study shows that water from the Plankenbrug River collected in the dry season suppresses IL-10. IL-10 is a biomarker for the humoral immunity pathway. IL-10 induces B-lymphocytes to synthesize antibodies. Antibody synthesis is required for immune defenses against extracellular pathogens such as bacteria, fungi and yeast. These microbes are a major cause of disease in humans, specifically people with disorders resulting in suppressed humoral immunity. Thus exposure to water from Plankenbrug might pose a risk to people due to the fact that their immune system will not be able to cope with extracellular pathogens. Of specific concern are pathogens such as *E.coli* and the *staphylococci and streptococci.* These microbes can cause

diarrhea, skin infections such as boils and abscesses and impetigo. A further concern is the fact that a compromised immune system will allow several normally harmless microbes to become opportunistic and cause infections.

The results also indicate that exposure to water from the Plankenbrug River will suppress the cell-mediated immunity as IFN-γ is responsible for driving this immune response against intracellular pathogens and tumors. Suppression of cell-mediated immunity is associated with the development pro-malignant tissue that can not be contained. Secondly *mycobacterium tuberculosis*, which is predominant as an opportunistic infection in HIV affected individuals, is eradicated by the cell-mediated immunity. Therefore, consumption of water from the Plankenbrug can potentially have devastating effects on the community of Kayamandi, who like the rest of the Western Cape, South Africa already are in the grips of a TB epidemic. Of specific concern are that the consumption of this water might increase the TB incidence in immunocompromised individuals, such as AIDS patients, in whom the incidence of TB is extremely high. In addition, literature states that 90% of TB infections do not result in an illness. However consumtion of water from this river can potentially increase the incidence rate of TB illnesses and other intracellular infections even in other wise healthy individuals. (Girard et al. 2005, Lawn and Wood. 2005, Whiteside. 2006)

Conclusion

The Plankenbrug River is polluted by contaminants that cause inflammatory responses and also suppress humoral and cell mediated immunity. To our knowledge this is the first time that it has been shown that surface water can be contaminated by immunotoxicants that can suppress humoral and cell mediated immunity. Use of the contaminated water from this river directly for drinking or recreation, or indirectly by consumption of crops irrigated with the water, can potentially pose a health risks for the consumers. Currently studies are being conducted by us to determine the mode of action of these contaminants on the specific immune cell types and the current study is also being extended to determine the long-term effects of these contaminants on the immune system of mice *in vivo*.

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Chapter 4: The immunotoxicity of water collected from the Plankenbrug River, Stellenbosch

Introduction

The Plankenbrug River is situated in the agricultural town of Stellenbosch in the Western Cape, South Africa. This river flows nearby agricultural areas in Koelenhof and also the densely populated residential of Kayamandi before its confluence with the Eerste River.

The Plankenbrug River is heavily polluted by contaminating substances eminating from anthropogenic activities in the formal and informal settlement of Kayamandi and nearby agricultural areas (Nleya 2005, DWAF 2001). A large part of the river's deteriorating quality is attributed to non-point source pollution in the form of fecal contamination and grey water coming from the poorly serviced informal settlement of Kayamandi. People from this area are reported to rely on bus toilets and showers for sanitation. These sanitation facilities do not meet the demand of the population and as a result they resort to using surrounding fields, the storm-water system and buckets to dispose of their fecal matter. During the rainy season, fecal matter and other pollutants accumulating on the ground are washed into the river system. As result there are often reports on the presence of high counts of *E.coli* (DWAF 2001).

The Plankenbrug River downstream is used for the irrigation of agricultural crops during the summer season when there is little or no rain. These agricultural crops are mainly exported (DWAF 2001). Secondly, even though the river is declared unfit for human use people from the informal settlement may rely on it for domestic purposes and children may use it for swimming. A major problem is the adverse health effects and negative impact on the economy associated with exposure to water from this polluted river. The degradation of water quality in the Plankenbrug River has been associated with a high incidence rate of diarrhea in Stellenbosch (DWAF 2001, Barnes et al. 2004).

The Municipality of Stellenbosch has constructed cut-off drains that prevent storm water from entering the river system and storm water diversion channels that direct storm water into the sewage system. Large amounts of money and resources have been earmarked to improve the sanitation and hygiene in the informal settlement of Kayamandi by upgrading their toilet system (Nleya 2006). However, although large amounts of money and resources are being used to upgrade the infrastructure in Kayamandi, the water quality in the Plankenbrug River continues to deteriorate. This is evident in recent studies that have attributed downstream deterioration of water quality in the Eerste River to its confluence

with the Plankebrug River (Ngwenya 2006, Nleya 2005, Pool et al. 2003). It has further been shown that, amongst other sampled sites in the Eerste River catchment system, the Plankenbrug River induced the highest inflammatory activity in human whole blood cultures, thus reflecting high levels of contamination (Pool et al. 2003). Our recent results, from a study that investigated the immunotoxicity of the Eerste River catchment system, also support the literature where the water samples collected from the Plankenbrug River induced the highest inflammatory activity compared to other sites in the catchment. This study also showed that water from the Plankenbrug River can potentially affect human health due to its suppression of the acquired immune pathways that are required to fight off viral and bacterial infections and also cancerous growths. These effects were only seen in the dry summer season while during the rainy season the suppresants were diluted to levels where no effects were detected.

It has been reported that an estimated amount of R37.5 million is still needed to upgrade the toilet infrastructure in Kayamandi (Nleya 2005). Although literature attributes the non-point source pollution of Plankenbrug River to agricultural activities and human activities in the informal settlement (Nleya 2005, Ngwenya 2006), the intensive planning on managing water pollution in the Plankenbrug River only revolves around the informal settlement Kayamandi. There are currently no literature studies available to substantiate the fact that Kayamandi is the main contributor of pollutants in the Plankenbrug River. Secondly there are no studies that compare the immunotoxicity of water upstream and downstream from Kayamandi.

Human whole blood culture assays have been developed to monitor the inflammatory activity of river water as an indicator of water quality. This assay is now being widely used in water studies and IL-6 is employed as the 'gold standard' biomarker of inflammatory activity (Pool et al. 2000, Pool et al. 2003, Wichmann et al. 2004, Jagals et al. 2006).

The aim of the current study is to compare water samples collected from upstream and downstream from Kayamandi in the Plankenbrug River, Stellenbosch, South Africa for inflammatory activity, humoral and cell mediated immunotoxicity using cytokines as biomarkers. This will be monitored during the dry summer season before the first rains. Figure 1 is a map of the Stellenbosch area indicating the location the Plankenbrug River

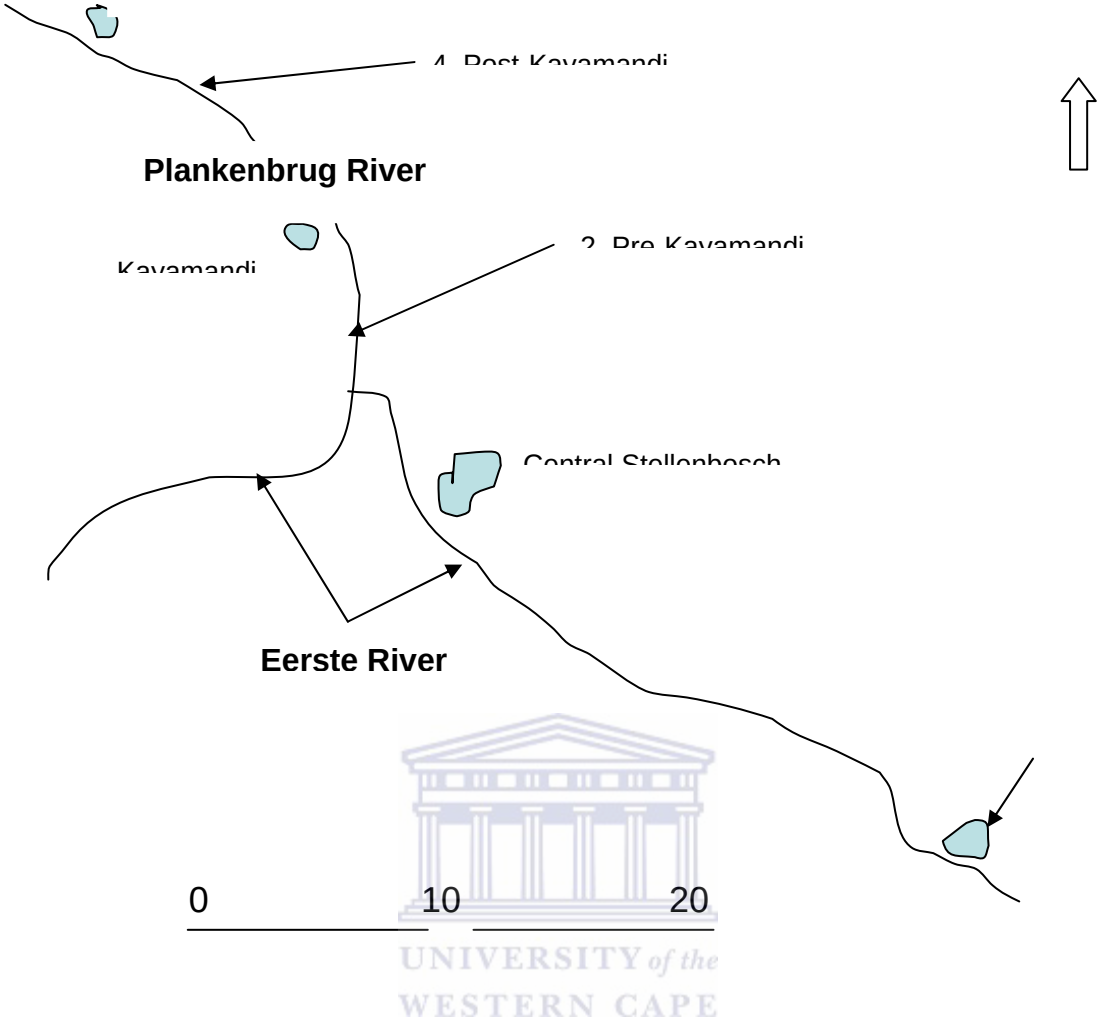

Figure 1b: This map shows the location of the Plankenbrug River in Stellenbosch, Western Cape, South Africa.

Methods

Water collections. Water samples were collected in clean 2L glass bottles. The bottles were cleaned by washing with soap, followed by rinsing with tap water. The bottles were then rinsed with reverse osmosis water and finally it was rinsed with HPLC grade ethanol (Merck, Germany). The bottles were then allowed to dry up-side down in a dry rack. Water samples were collected at Jonkershoek which was used as control site, 100 meters upstream from Kayamandi (Pre-Kayamandi) and 100 meters downstream from Kayamandi (Post-Kayamandi). Samples were collected in January at the peak of the dry summer season and before the first rains. Water samples were taken to the laboratory and were processed immediately. Neat water samples were stored at -4˚C.

Water Extraction. Water samples were first filtered through Whatman1 filters (W&R Balston Limited, England) to remove any particulates Filtered water samples were extracted using C18 columns as per our laboratory protocol. Briefly, C18 hydrophobic columns (from Anatech) were pre-washed with 4ml solvent mixture (40%v/v hexane, 45% v/v methanol and 15%v/v 2-propanol), followed by another wash with 4 ml of ethanol and finally with 4 ml distilled water. Care was taken not to let the columns run dry between the additions of the various liquids. After applying the sample the column was washed again with 4 ml of distilled water. The column was then dried for at least 1 hour on a vacuum pump. The hydrophobic molecules bound to the column were eluted with 3 ml of the solvent mixture. The eluate was dried under a stream of air. The dried water extracts were reconstituted in DMSO to give a volume 1/1000 times that of the original sample volume (Swart and Pool 2007).

Blood collection. Blood was obtained from healthy male volunteers by a nurse or doctor in the campus health clinic. Blood samples were collected directly into heparinised vacuum tubes by venipuncture. The blood was stored at ambient temperature and used within 4 hours after collection.

Inflammatory activity. All procedures were done under sterile conditions in a laminar flow cabinet. Four replicates of all samples were assayed. DMSO or the water samples (2.5 µl/well) were added to wells of a 96 well tissue culture plate (Nunc^{TM}, Denmark). Heparinised whole blood was diluted 1:10 with RPMI 1640 (Sigma, Germany). The diluted blood was added to the plate (250 µl/well) and the plate was then incubated at 37˚C for 18-20hrs. At the end of the incubation period the culture supernatants were collected and assayed for IL-6.

Th1 and Th2 activity. Heparinised blood was diluted at a ratio of 1: 9 with RPMI-1640 (unstimulated blood) or at a ratio of 1: 9 with RPMI containing PHA (Sigma, Germany). Water extracts (2.5 µl of reconstituted extract per well) or controls (2.5 µl/well DMSO) were added to wells of a 96 well culture tray (Nunc^{TM}, Denmark). Four replicates were done for each sample. Diluted unstimulated or PHA stimulated blood (250 µl/well) was added to the samples and the mixtures were incubated for 48 hours at 37 °C. At the end of the incubation

period the supernatants were collected and assayed for Interferon -gamma (IFN-γ) or Interleukin- 10 (IL-10).

Cytokine analysis. Double Antibody Sandwich (DAS) ELISAs (e-Bioscience,Germany) were used to quantify cytokine production from the supernatants of whole blood cultures. Nunc maxisorp (Nunc^{TM}, Denmark) plates were used for the assays. All reagents and diluents required for the ELISA are in kit. The assay was performed according to the protocol provided in the kits. In brief: Primary antibody against the respective cytokine was coated onto the plate. The coating was allowed to proceed overnight at 4 ºC. The next morning the plates were washed with 1X PBS containing 0.05 % Tween. The plates were then blocked with assay buffer from the kit for 1 hour, after which the wells received either sample or cytokine standards. The plate was again incubated for 2 hours at ambient temperature on a shaker. After washing the plate the wells received biotinylated secondary antibody against the respective cytokine. The plate was incubated for 1 hour at ambient temperature on a shaker followed by washing as before. The wells then received avidin-peroxidase conjugate. The plate was again incubated for 30 minutes at ambient temperature on a shaker. After the final wash, substrate was added to the wells. The plate was incubated for 20 minutes after which the reaction was stopped by adding 2M H_2SO_4. The plate was then read at 450 nm on a plate reader. A standard curve for each ELISA plate was generated using Excel and this was used to determine the cytokine concentrations induced by the water extracts and the controls.

Statistical Analysis. Results were analysed for statistical differences between samples using ANOVA test in Sigma Stat.

Results

The inflammatory activity of water from the Plankenbrug River. IL-6 synthesis by whole blood cultures was used as a biomarker for inflammatory activity. The standard curve (Fig 2b) obtained using the IL-6 ELISA kit standards shows that there is a good correlation with the absorbance values and IL-6 concentration (R^2=0.99).

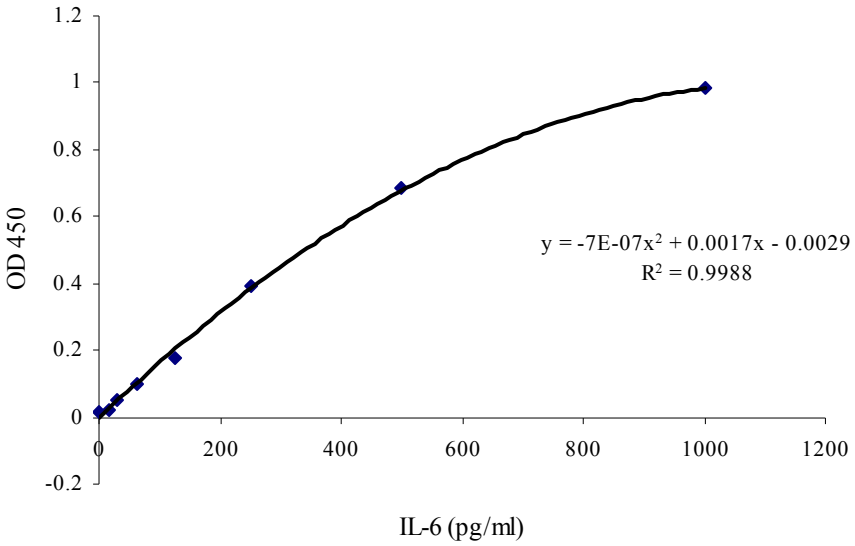

Figure 2b: Standard curve for IL-6. The kit standards were assayed by the DAS ELISA according to the manufacturer's instructions.

Water samples collected at end of the dry season showed that there is significant difference in the inflammatory activity of the Plankenbrug sites compared to the control site at Jonkershoek. Water from both pre- and post-Kayamandi sites induced significantly higher IL-6 than the Jonkershoek site ($p<0.001$). See Fig 3b.

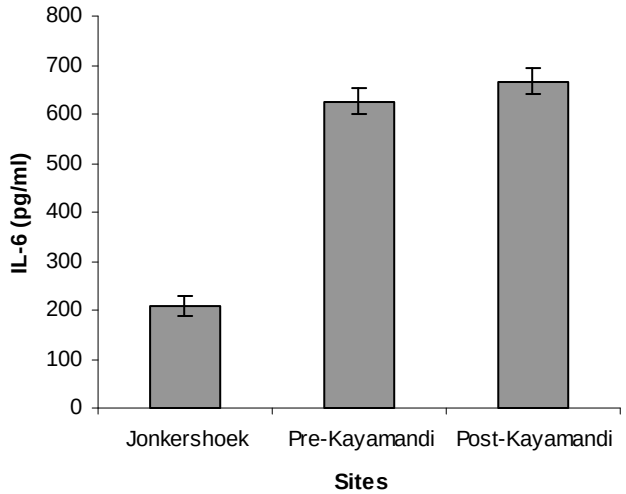

Figure 3b: The inflammatory activity of the river samples expressed as IL-6 secreted by whole blood cultures. The IL-6 secretion by whole blood cultures in the presence of water from the Pre-Kayamandi and Post-Kayamandi was higher than Jonkershoek. Data is presented as the mean ± SEM (n=12). n is the number of patients used in the experiment

The effects of water from the Plankenbrug River on humoral immunity. IL-10 synthesis by whole blood cultures upon stimulation with PHA was used as a biomarker for the humoral immune pathway. The standard curve (Fig 4b) obtained using the IL-10 ELISA kit standards shows that there is a good correlation with the absorbance values and IL-10 concentration (R^2=0.99).

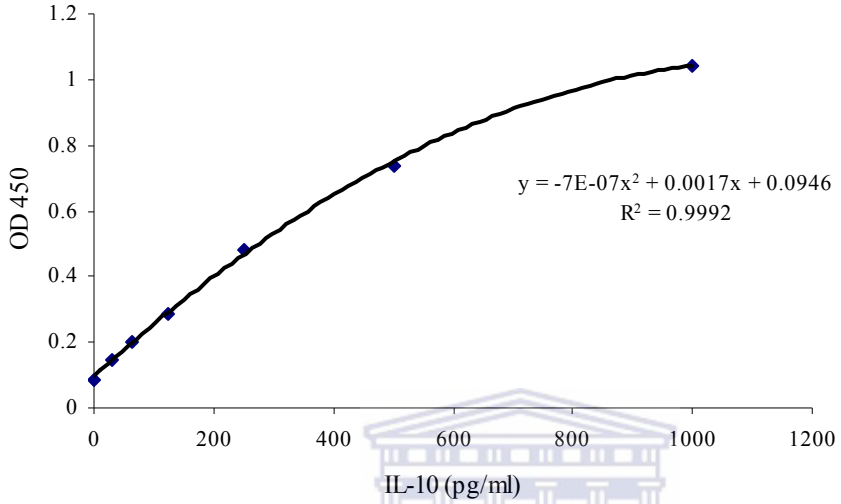

Figure 4b: Standard curve for IL-10. The kit standards were assayed by the DAS ELISA according to the manufacturer's instructions.

Samples collected before the first seasonal rains i.e. at the end of the dry season showed that the Post-Kayamandi site caused a significant decrease in IL-10 secretion when compared to water samples collected from Jonkershoek ($p < 0.001$) and Pre-Kayamandi ($p<0.002$). See Fig 5b.

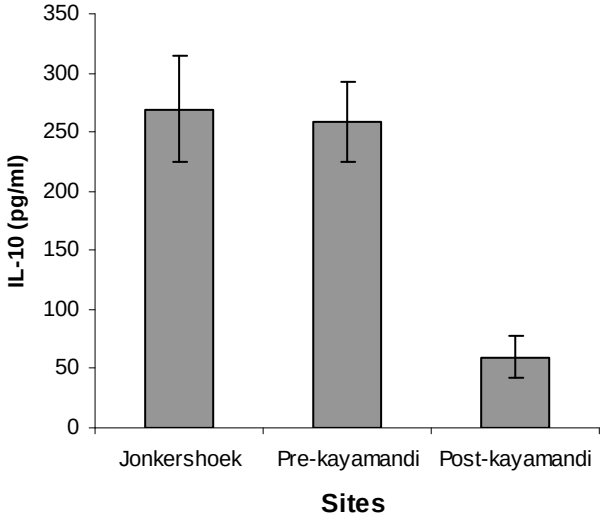

Figure 5b: The effects of the river samples on humoral immunity expressed as IL-10 secreted by whole blood cultures. The IL-10 secretion by whole blood cultures in the presence of water from Post-Kayamandi site in dry season was significantly lower than the IL-10 secretion induced by the other sites. Data is presented as the mean ± SEM (n=12). n is the number of patients used in the experiment

The effect of water from the Plankenbrug River on cell mediated immunity. IFN-γ synthesis by whole blood cultures upon stimulation with PHA was used as a biomarker for cell mediated immunity. The standard curve (Fig 6b) obtained using the IFN-γ ELISA kit standards shows that there is a good correlation with the absorbance values and IFN-γ concentration (R^2=0.99).

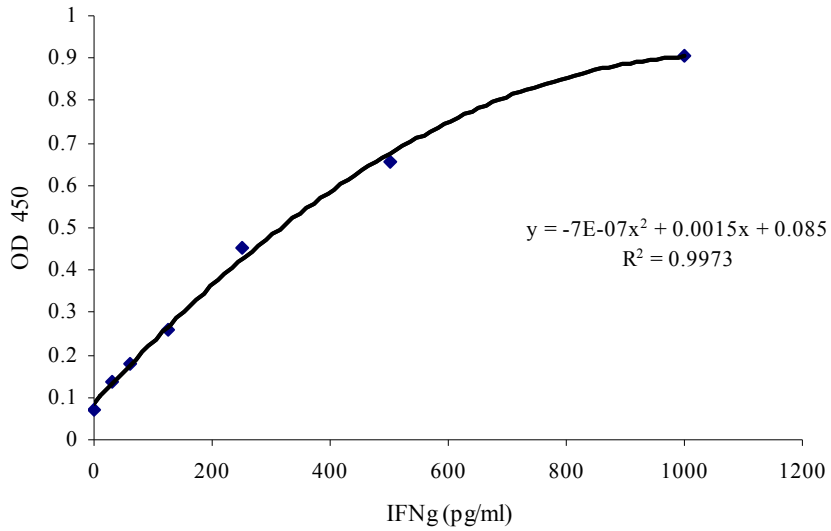

Figure 6b: Standard curve for IFN-γ. The kit standards were assayed by the DAS ELISA according to the manufacturer's instructions.

Samples collected before the first seasonal rains i.e. At the end of the dry season showed that the Post-Kayamandi site caused a significant decrease in IFN-γ secretion when compared to the other water samples collected from Jonkershoek ($p<0.02$) and Pre-Kayamandi sites ($p<0.01$). See Fig 7b.

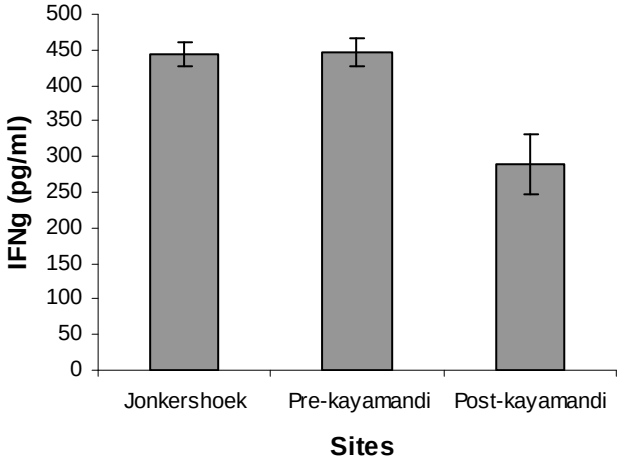

Figure 7b: The effect of river samples on cell-mediated immunity expressed as IFN-γ secreted by whole blood cultures. The IFN-γ secretion by whole blood cultures in the presence of water from the Post-Kayamandi site in the dry season was significantly lower than the IFN-γ secretion induced by the other sites. Data is presented as the mean ± SEM (n=12). n is the number of patients used in the experiment

Discussion

The main aim of this study was to compare the level of contamination in the Plankenbrug River before and after the informal residential area of Kayamandi. Water samples were collected during the dry season upstream and downstream from Kayamandi and Jonkershoek was used as control site. Using *in vitro* human whole blood cultures, the collected water samples were screened for inflammatory activity and for potential immunotoxic effects on cell-mediated and humoral immune pathways. The inflammatory activity induced in whole blood cultures was used as an indicator of water contamination. It was found that water samples collected from Pre-Kayamandi and Post-Kayamandi had a higher inflammatory activity than the samples collected from Jonkershoek. Thus these results indicated high levels of water contamination from the two Plankenbrug sites. However, there was no significant difference in the level of inflammatory activity between Pre-Kayamandi and Post-Kayamandi therefore meaning that these two sites contribute equally to the deterioration of water quality in the Plankenbrug River. These results contradict most reports found in literature, even though contamination of the Plankebrug River by pollutants coming from the agricultural area is acknowledged, a large part of the river's deteriorating water quality is attributed to the informal settlement of Kayamandi (DWAF 2001, Barnes et al. 2004,). This is due to the fact that this residential area lacks proper water facilities and sanitation and as a result they resort to using the surrounding environment for disposing their waste. The presence of skin pathogens and gram-negative bacteria of fecal origin found in the river is also often used as supporting evidence that the informal settlement is the major contributor of contaminants. As a result, to solve the pollution problem large amounts of money have been spent in improving the toilet infrastructure in this area. However this has been in vain and no improvement in river water quality has been observed due to these efforts (DWAF 2001, Nleya. 2005, Barnes et al. 2004, Ngwenya 2006 ,).

Results from this study show that water samples collected from Pre-Kayamandi and Post-Kayamandi sites in the Plankenbrug River have the potential to induce inflammatory diarrhea upon exposure through drinking or recreation use. Exposure of individuals to water contaminated with pathogenic micro-organism triggers an inflammatory response that is mediated by pro-inflammatory cytokines which include

IL-6. The presence of an infectious agent and elevated cytokine levels negatively alters the Na^{+}-K^{+}pump which inhibits the absorption of sodium and thus leading to the excessive

secretion of Na^+ and Cl^- followed by a large release of water therefore resulting in acute or chronic diarrhea (Markossian and Kreydiyyeh. 2005, Payne et al. 2006).

Water extracts collected during the dry summer season from the Post-Kayamandi site significantly suppressed IFN-γ and IL-10 production in human whole blood cultures. The results show that pollution from the informal settlement of Kayamandi introduces immunosuppressive contaminants into the Plankenbrug River and these are more concentrated in the dry season when there is little or no rain thus low river flow. Apart from black water (fecal contamination), poorly serviced residential areas can contribute to water pollution by disposing grey water into the ground that can be washed into river system. They grey water contains detergents that are used in household purposes such as showers, cleaning of cars , washing of clothes and other domestic chores. Some of these detergents may induce immunotoxic effects in exposed individuals (Carden et al. 2007, EPA 2007).

The suppression of IL-10 and IFN-γ can induce adverse health effects associated with a decrease in host's resistances against invading pathogen thus predisposing the host to infection that can become systemic or to the development of cancers. IFN-γ is a cytokine that facilitates polarization of naïve CD4 T-helper cells into the cell-mediated immune response. This immune pathway is responsible for containing intracellular infection such as HIV and *Mycobacterium tuberculosis*, and inducing cytotoxicity in malignant tumors (Lawn. 2004, Girard et al. 2005, Whiteside. 2006, Tan and Coussens. 2007). On the other hand, IL-10 is responsible for B-cell proliferation that isotype switch into antibody-producing plasma cells. The antibodies are involved in the eradication of extracellular pathogen (Oral et al. 2006). A study that was done Barnes et al. (2004) revealed that the river is contaminated with skin pathogens such as *Staphyloccocus spp* and *Streptoccocus spp* which may cause impetigo especially in children who might use the river water for recreational purposes. Our results indicated that individuals who are challenged with *Staphyloccocus spp* and *Streptoccocus spp* will not be able to fight off these skin infections caused by these pathogens due to the suppression of IL-10 which drives humoral immunity against extracellular micro-organisms. Secondly Barnes et al. (2004) reported that there is a high incidence rate of HIV and because of malnutrition the people suffer from TB which can result in death in immunocompromised patients with HIV. Our result then show that exposure from the water in Post-Kayamandi can exacerbate the situation of HIV and TB in Kayamandi and further predispose the people to the development of cancer as water samples from this site suppressed IFN-γ .

Conclusion

The Pre-Kayamandi site and the Post-Kayamandi site equally contribute to the inflammatory activity of the Plankenbrug indicating that both these sites are responsible for the rapid deterioration of water quality in this river. The dry season concentrates immunosuppressive contaminants which may be due to grey water coming from the informal settlement of Kayamandi. Exposure to water from these two sites can be associated with diarrheal disease, skin disease such as impetigo and boils especially in children who use the river for swimming in the summer season. Secondly people may be at risk of developing TB which might accelerate the progression to AIDS in people living with the HIVirus.

To prevent an increase in the morbidity rate in this area environmental health officials and municipality workers should put in place monitoring programmes that will also involve the community in keeping the river and surrounding areas clean. The community should be taught the importance of non-polluting river water and the health consequences associated with pollution of the river. Lastly the municipality should improve on the services provided to the informal settlement so as to limit the rate of potentially harmful pollutants from entering the river.

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Chapter 5: Summary, Conclusion and Recommendations

Summary

Cytokines play a pivotal role in driving immune responses against extracellular and intracellular pathogens, foreign toxins and malignant tumours therefore preventing systemic infections that can be fatal. IL-6 is responsible for inducing the initial acute inflammatory response provided by the innate immunity upon an invasion by pathogenic micro-organisms and other foreign matter. Prolonged infections are relayed to the adaptive immunity by antigen presenting cells where IFN-γ produced by NK cells and macrophages polarises naïve CD4 T-helper cells into the Th1 domain. The T-helper cells proliferate into effector and memory cells that enter into the cell-mediated immune response against viral and mycobacterial infections. On the other hand IL-10 produced by T-regulatory cells induces the proliferation of B-cells that isotype switch into plasma cells that produce antibodies which are responsible for humoral immune response against bacterial and parasitic infection. These antibodies form immune complexes with antigens which results in the perforation of pathogen's membrane through the classical and alternative pathways (Chaplin 2003, Oral et al. 2006)

Even though these cytokines facilitate the protection of the host against insult, prolonged production or suppression can be pathophysiological. It is for this reason that these cytokines have been used as biomarkers of adverse effects in immunotoxicology. Immunotoxicity studies have shown that cytokines can be a target for environmental pathogens and chemicals. The change in cytokine levels induced by these contaminants has been associated with disease in exposed individuals.

Most immunotoxicity studies have been done *in vivo* using laboratory animals and the data extrapolated to assess the immunotoxic adverse health effects that would be posed by environmental contaminants in humans upon exposure. Although this procedure is helpful in indicating potential risks in humans but it is not biologically relevant as there are interspecies differences that cannot be overlooked. As a result of this short-coming, the use of *in vitro* bioassay employing the cell culture model was promoted. However this system also has its disadvantage because it is subjected to pre-activation and it is not reflective of the physiological picture inside the host like whole blood cultures, which are also time-effective. Infact human whole blood cultures are now widely used for the production of cytokines in immunotoxicity studies (Fasanmade and Jusko. 1995, Yancy et al. 2001, Pool et al. 2003)

Surface water in our country is essential as there are some disadvantaged communities i.e. rural areas and informal settlements, which still rely on rivers for domestic purposes and recreation. Secondly, agricultural areas depend on rivers during the dry summer season, especially in the Western Cape, for the irrigation of crops. However anthropogenic activity, improper water facilities and sanitation results in the contamination of river water. Although there are techniques available to monitor water quality, those techniques can only assess the physico-chemical properties of water, which do not give an indication on potential adverse health effects on humans upon exposure. Hence, Pool (2000, 2003) developed an in vitro human whole blood culture assay using IL-6 as biomarker for inflammatory activity and therefore an indicator of water quality and potential immunotoxic adverse health effect, which is now used internationally (Wichmann et al 2004, Jagals et al 2006). This assay, which is quick and cost-effective, provides an additional tool that can screen water quality and the associated human health impacts. It for this reason that IL-6 was used to measure the inflammatory activity of the Eerste River water catchment system in Stellenbosch, South Africa. The water quality of the Eerste River catchment system has been reported to deteriorate rapidly after the confluence with the Plankebrug River.

The aim of our study was to determine, using the inflammatory activity induced in human whole blood cultures, if the Plankenbrug River increased the contamination level in the catchment. This study also wanted to ascertain if the contaminants in the water induce cell damage by detecting cytotoxicity using the LDH assay. For the first time, our study also investigated the relationship between inflammatory activity and the effect on cell-mediated and humoral immune pathways and seasonal variations. The catchment was not cytotoxic at the time when water samples were collected.

Our results were consistent with other studies and reports made on this catchment system (Pool et al. 2003, Nleya. 2005, Ngwenya. 2006). Low inflammatory activity and hence low contamination was found near the origin of the river and in the central business area of Stellenbosch. However after the confluence with the Plankenbrug River the inflammatory activity was high which was indicative of high levels of contamination. Secondly, this study showed that the contaminants in the Plankenbrug River were immunosuppressive to the adaptive immune response as both IL-10 (indicator of humoral immunity) and IFN-γ (indicator of cell-mediated immunity) were suppressed. All of these effects were observed in the dry season when contaminants are concentrated due to low river flow.

These results prompted the second study, which investigated the source of pollution in the Plankenbrug River during the dry season. Most preceding reports have attributed the

pollution to the informal settlement of Kayamandi, which relies on bus toilets for sanitation and communal taps for water. Secondly, a microbiological study of the Plankenbrug River found high prevalence of *E.coli* and other pathogenic micro-organisms and this was used as supporting evidence that Kayamandi is the major contributor in the severe deterioration of water quality in the Plankenbrug River (DWAF 2001, Barnes et al. 2004). Our results contradicted this conclusion because the inflammatory activity of water from the agricultural area and the informal settlement was not different, which showed that these two areas contribute equally to the pollution of this river. However the immunosuppressive effects on the adaptive immune system were only induced by water coming from the informal settlement. This can be result of grey water containing detergents that is just disposed into the environment and is washed off into the river.

Conclusion

Water running through the formal sector of Stellenbosch, which is also the central business district of this town, is kept clean as this area is adequately serviced. However the Plankenbrug River is heavily contaminated and it can cause adverse health effects in the residents living in Kayamandi who use the water for domestic and recreational purposes. These effects can range from skin disease, diarrhea to opportunistic infections in immumunocompromised patients and possibly cancer. Secondly farmers who rely on this river for irrigation of crops, which are later, exported, are faced with a serious problem.

Recommendations

Surface water is an essential resource that should be maintained for sustainable development as scarcity during dry season can result in the recycling of the water from the Plankenbrug River for use in various areas. Thus future studies need to determine the identity and the type of contaminants in this river so as to limit further contamination. Secondly *in vivo* studies should to see if the immunotoxicity is not transferred maternally. In addition the residents of Kayamandi and nearby farms should be cautioned not to use the river for any purpose. Instead they should find an alternative water source. Professionals should be trained to do routine monitoring on the immunotoxicity of the river. Lastly resident's inhabiting this area should be educated on adverse health effects associated with pollution and exposure or direct contact with polluted water. The education should be done in such a way that communities along the river take up the custodianship of the river and protect it for future generations.