THE ROLE OF REGULATORY T CELLS IN ADULTS IN SOUTH AFRICA WITH ACTIVE TUBERCULOSIS

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A research report submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in the Branch of Pathology (Haematology)

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DECLARATION

I, Elizabeth Sarah Mayne, declare that this thesis is my own work. It is being submitted for the degree of Master of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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_____ Day of _____, 2009

Dedicated to my husband, Paul and my parents.

And in memory of my sister, Alexandra (1982-2003).

PUBLICATIONS AND PRESENTATIONS

Part of this work was presented as a poster at the Keystone Symposium on HIV Immunology, March 2007.

ABSTRACT

Introduction

Regulatory T cells (Tregs) are increasingly being recognized as key immunological players in immunosuppression and have been seen to be permissive for certain infections.

Aim

This study aimed to elucidate the role that Tregs play in symptomatic infection with *Mycobacterium tuberculosis* (TB), both with and without co-infection with human immunodeficiency virus type 1 (HIV 1) by quantification of these cells at *ex vivo*. It was then attempted to characterise the behaviour of FoxP3 positive cells in culture with stimulation.

Methods

Peripheral blood mononuclear cells were purified from uninfected controls, patients with active TB, patients with HIV infection and patients with HIV infection and active TB. The frequencies of Tregs were assessed by flow cytometry at *ex vivo* and again after four days of culture with stimulation with anti-CD3, Purified protein derivative, tetanus toxoid and HIV peptide superpools (gag and nef). These frequencies were compared between the four groups of patients. The ability of Tregs and effector T cells to proliferate was also assessed. Interferon-γ secretion was used as a measure of effector T cell response to stimulation.

Results

Frequencies of Tregs were significantly reduced in patients with active TB as compared with HIV infected patients and uninfected controls. Co-infected individuals showed a broad range of frequencies which were not significantly different from controls. These frequencies remained stable in culture with the exception of those individuals infected with HIV who showed a decline in the frequency of those cells expressing FoxP3 over the period. Cells expressing FoxP3 were not anergic and responded to stimulation. HIV specific proteins, in addition, resulted in specific effects on the Tregs with a positive interferon response to gag correlating with increased Treg frequencies and FoxP3 expression in $CD4^+$ T cells correlated with the proliferative response of $CD4^+$ T cells to Nef in HIV infected individuals.

Conclusions

This study shows significant differences of frequencies of FoxP3 positive producing cells in the peripheral blood at *ex vivo* in patients with active TB. The function of these cells in this population is uncertain and further functional data and long-term clinical follow-up is required. In addition, the frequencies of these cells remained constant over time and showed proliferative response to stimuli (most notably CD3) suggesting that these cells may be generated in the periphery.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1.0 Introduction:

1.1 Basic Immunology

The immune system is the body's defence against infectious organisms. It is classically divided into two distinct systems – the innate immune system and the adaptive immune system. The innate immune system, the first line of defence, is non-specific and responds to pathogen-associated molecular patterns. Pathogen-associated molecular patterns are distinctly foreign molecular signatures which include double-stranded RNA and prokaryoytic structural proteins like flagellin amongst others (Gordon 2002, Stenger and Modlin 2002). Some of the receptors which detect these foreign signatures are the toll-like receptors and the complement protein cascade. Cells which are intimately involved with the innate response to infection include phagocytes (macrophages, neutrophils and dendritic cells) and innate-like lymphocytes (natural killer cells, natural killer T cells and B1 B lymphocytes). These cells are responsible for priming the adaptive immune response and introducing the foreign organism to the lymphocytes (Bendelac et al 2007, Kronenberg and Havran 2007)

Lymphocytes are the key effector cells of the adaptive immune response. Two distinct types of lymphocytes are recognized – B cells and T cells. B cells are responsible for the humoral or antibody-mediated immune response. Antibodies are small protein molecules which are produced to respond to a small signature called an antigen. Once antibodies

bind to an antigen, they can trigger a series of processes which include stimulation of phagocytosis (opsonisation), stimulation of a cytolytic natural killer cell response (antibody dependent cellular cytotoxicity) and activation of the complement cascade which in itself results in phagocytosis, recruitment of immune cells to the area and cytolysis.

T lymphocytes are the central cell population of the adaptive immune response. T lymphocytes are divided into those which express the protein, CD4, on their cell surface and those which express the protein, CD8, on the cell surface (*Figure 1.1*). $CD4^+$ T cells are helper T cells – they coordinate the immune response predominantly by the secretion of humoral cellular signals called cytokines. $CD4^+$ T cells are classically divided into Th1 cells which stimulate primarily a cytotoxic response and Th2 cells which secrete cytokines which prime a B cell (antibody) response. In addition, regulatory classes of $CD4^+$ T cells have been described including Th3 and Tr1 cells which are thought to secrete predominantly immunosuppressive cytokines (transforming growth factor β and interleukin 10 respectively). $CD8^+$ T cells are cytotoxic T cells – they recognize cells with an internal infection through a unique molecule called the Major Histocompatibility Class (MHC) I molecule and cause them to undergo apoptosis or kill them through the perforin-granzyme pathway. (Lanzavecchia and Sallusto 2001). Recently, a third class of effector $CD4^+$ T cells with proinflammatory properties, has been described which develop under the influence of the cytokines IL-6 and IL-23 and produce the cytokine IL-17 which has been implicated in the development of autoimmune disease.

The immune system is necessarily under tight regulation in all stages of an immune response. The innate immune system, for example, is regulated in its zymogenic complement cascade through a number of complement inhibitors which work in an analogous way to the anticoagulants in the coagulation cascade. In addition, many of the

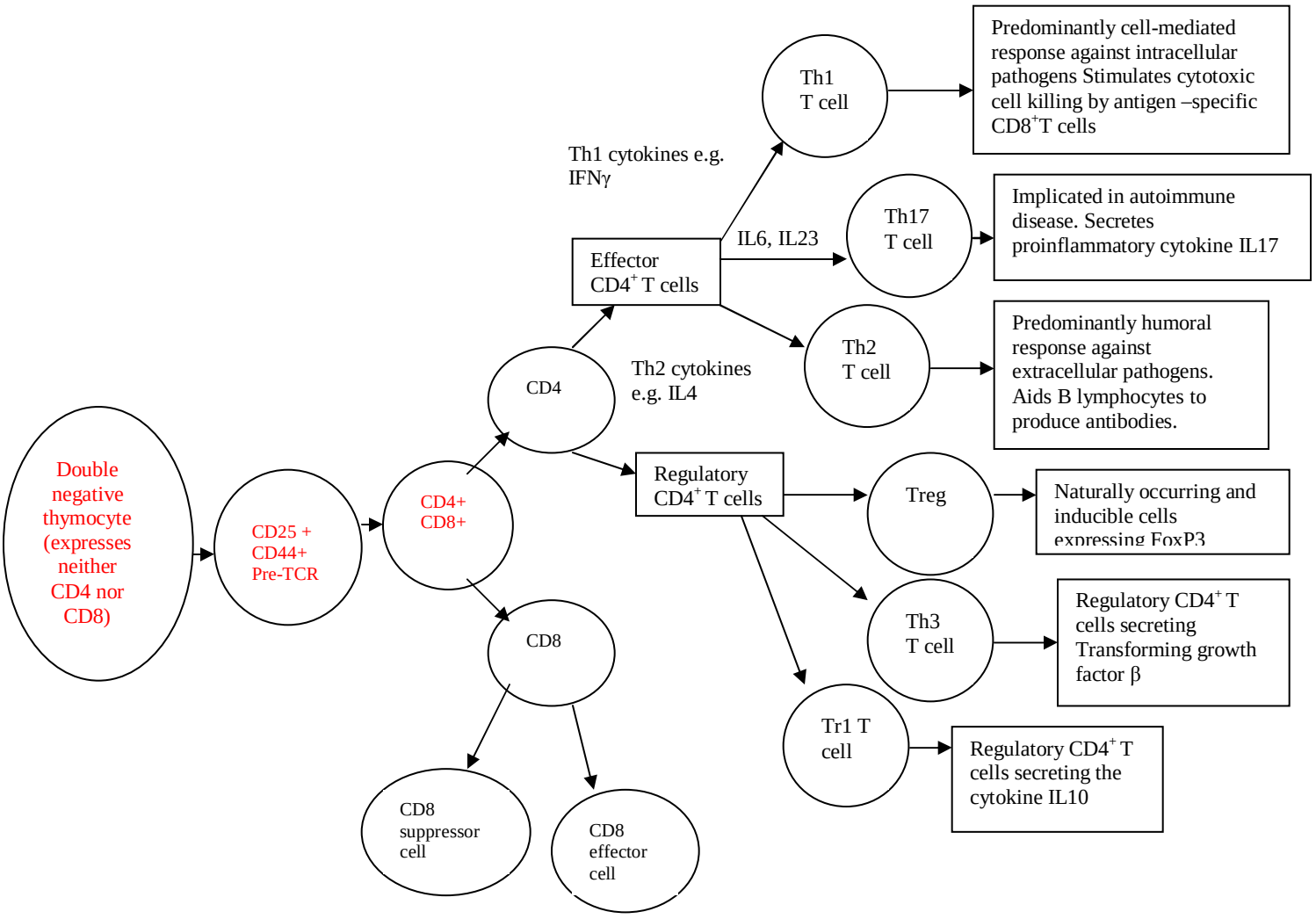


Figure 1.1

T cell ontogeny and T cell subsets. T cells proliferate in the bone marrow and move to the thymus where they undergo a process of conditioning which results in negative selection of self reactive T cells.

immune system cell populations contain regulatory subsets. For example, natural killer cells expressing no or dim CD56 tend to be regulatory rather than cytotoxic (Fan, Yang and Wu 2008). It is appropriate, therefore that the cell which coordinates the adaptive immune response, the helper T cell, receives tight regulation.

1.2 Characterisation of the Regulatory T cell

The Regulatory T lymphocyte appears to play a prominent role in regulation of the immune system. Although the existence of suppressor cells in the immune system has long been postulated, it was only in a recent study by Sakaguchi et al (2000) that a method for further characterizing a population of putative regulatory T cells utilizing the markers $CD4^+$ (a co-receptor for the T cell receptor utilized in stimulation) and high expression of CD25 (the gamma chain of the IL2 receptor), was described. Later, a transcriptional factor was identified, the Forkhead Box P3 factor or FoxP3 (a member of the winged/helix transcription family) which appears to be central to the activation, identification and function of the regulatory T cell (Hori, Nomura and Sakaguchi 2003, Ramsdell and Ziegler 2003). This factor was first identified in the Scurfy mouse. The Scurfy mouse, analogous to humans with the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) presents with a disease complex of uncontrolled lymphoproliferation, immune paresis and autoimmune phenomena, which is generally fatal (Hori, Nomura and Sakaguchi 2003, Hori and Sakaguchi 2004). Some of the postulated mechanisms of action of this factor include its direct effects on T cell

receptor signalling and its inhibition of other transcription pathways like the nuclear factor of activated T cells (NFAT) pathway.

Although FoxP3 has been described as best characterising the Treg population, it has limitations. FoxP3 measurement, by flow cytometry or PCR, requires permeabilisation of the cell. In addition, studies have suggested that FoxP3 (like CD25) may be upregulated as a marker of activation on human T cells as distinct from mice (Morgan et al 2005) albeit at lower levels than regulatory T cells and very temporarily (Allan et al 2007). This has recently been disputed – the suggestion being that co-culture of FoxP3+ and FoxP3-T cells confounds data because the regulatory population is overwhelmed by the FoxP3- population and that these cells which are FoxP3+ ex vivo have clear suppressive functions (Pillai et al 2008). Because of the controversy existing regarding the characterisation of regulatory T cells in humans, surrogate molecules are currently under evaluation including Cytotoxic T lymphocyte associated factor-4 (CTLA-4) and Glucocorticoid-induced tumour necrosis factor-like receptor (GITR) amongst others (Wing et al 2008, Yong et al 2006).

1.3 Ontogeny of Regulatory T cells

$CD4^+$ $CD25^+$ thymocytes display full regulatory T cell activity – the thymus hence appears to be an important nidus for Treg development (Thompson and Powrie, 2004). In addition, neonatal thymectomy is associated with a similar clinical picture to IPEX as a

result of Treg deficiency (Chatila 2005). The ability of Tregs to undergo development outside the thymus remains controversial (Bachetta et al 2005).

The development of this population appears to be strongly IL2-dependent as evidenced by the expression of CD25 and further confirmed by the proliferative response of Tregs following the administration of IL2 (Ahmadzadeh and Rosenberg 2006). Tregs have previously been described as an anergic population, which are unable to proliferate in the absence of the IL-2 (Hori and Sakaguchi, 2004) *in vitro.* Tregs could thus be a population that develops in response to an interaction between the T cell receptor and the MHC II antigen expressing self-antigens. Tregs cannot develop in recombination-activating gene (RAG) deficient mice (who lack a T-cell receptor) suggesting that the T cell receptor is vital for the development of this population of cells. Mice with MHC II expression limited to the thymic cortical epithelium (K14-Aβb mice) appear to be able to develop a functional Treg population suggesting that MHCII interaction outside of the thymus plays no major role in Treg maturation. Other studies have suggested that the thymic medullary epithelium may also play a role (Maggi et al 2005). The data support a high-affinity TCR-MHC interaction at avidities associated with deletion. Tregs cells appear to be subjected to positive selection in the thymus selecting autoreactive cells. They are also subject to negative selection as the thymus selects against cells which are strongly reactive to self-antigens (Hori and Sakaguchi, 2004, Chatila 2005). TCR variability in the population is as marked as in $CD4^+$ $CD25^-$ T cells suggesting that the population does not represent a recently activated set of lymphocytes (Fujisima et al 2005, Bosco et al 2005) An increased strength of the TCR signal is associated with a decreased suppressive

activity of Tregs (Baecher-Allan, Wolf and Hafler 2005). Although the role of self-antigens is probably paramount, it is certain that Tregs can recognise pathogenic antigens (notably *L. major*, an intracellular protozoan) through their T cell receptors - whether these pathogens are merely cross-reacting with similar self-antigens or play a role in Treg ontogeny is unclear (Hsieh et al 2004).

Studies in B7 deficient and CD28 deficient mice (NOD mice), which are unable to present antigen effectively to T cells because of an absence of co-stimulatory molecules, showed a marked numerical decrease in the population of Tregs, which suggested that these stimulatory pathways are necessary in the development of this population. These mice were prone to the development of diabetes, which was reversed by Treg transfusion (Salomon 2000). Nevertheless, the strength of the TCR signal appears to have an inverse relationship with the development of the Treg population (Baecher-Allan, Viglietta and Hafler 2005). Antibodies targeted at CD28 have strongly upregulated Treg production and have shown some promise in the context of animal models of autoimmunity (Beyersdorf et al 2005).

TGFβ appears to have a central role in the development of Tregs, which is now only beginning to be elucidated. It upregulates the expression of FoxP3 (Fantini et al 2004, Le et al 2005) and indirectly recruits the NFκB and MAP kinase signalling pathways and in some cases can attenuate the signalling pathways activated by other molecules e.g. lipopolysaccharides (LPS). Maintenance of the Treg population, as well as being dependent on IL2, requires TGF-β, which appears to maintain the levels of FoxP3

peripherally and plays an important role in their development (Fantini et al 2004). Toll-like Receptor proteins which play a crucial role in the innate system appear to be important in the development of Tregs (especially TLR5). Tregs appear to over-express some toll-like receptors and the ligation of TLR5 has been shown to increase the suppressive activity of the cells (Crellin et al 2005).

Although the Th1 response appears to be the primary target (through the $CD4^+$ T helper cell), Tregs also appear to inhibit several other important immune responses. The Th2 response may be inhibited through a contact-dependent mechanism on peripheral B lymphocytes– the Treg may directly inhibit B-cell somatic hypermutation and class-switch (Lim et al 2005). Studies in mice that overexpress FoxP3 have also suggested that the inhibition of the B lymphocytes may also be related to the inability of $CD4^+$ cells adequately to direct the antibody response in mice (Kasprowicz et al 2003)

Tregs appear to affect other antigen-presenting cells such as monocytes/macrophages that appear to downregulate their co-stimulatory molecules in response to Tregs and show reduced secretion of proinflammatory cytokines (TNF-α and IL-6) in response to stimulation with lipopolysaccharide (Taams et al 2005). Tregs also maintain dendritic cells in an inactive state in the absence of a large population of activated T cells or large amounts of antigenic stimulation (Serra et al 2003).

1.4 Function of Regulatory T cells

Regulatory T cells appear to have multiple functions associated both with the suppression of the immune response and paradoxically with its activation (in the interaction of Tregs with *H. pylori and Leishmania major* infection, these cells appear to play a role in a memory response recruiting cells to the environment – Thompson and Powrie 2004). The function of the Treg population could be characterised as a dominant tolerance mechanism – self-reactive T cells can become anergic in the thymus owing to the absence of a survival signal but Tregs actively destroy this population. (Sakaguchi 2000, Graca et al 2005).

1.4.1 Putative cell-contact mediated mechanisms of suppression.

Stimulation of Tregs *in vivo* resulted in an increase in granularity and increased binding of antibodies against granzyme but not granzyme B (Grossman 2004). Furthermore, inhibitors of the perforin pathway (e.g. EGTA and concanamycin A) seem to limit the cytotoxic capacity of Tregs (Grossman 2004). This argues that the Tregs utilise a cell-mediated cytotoxic process similar to that utilised by the Natural Killer Cell population of the innate immune system. In addition, it has been shown that Tregs express CD39, an ATPase, which neutralises the proinflammatory ATP in local inflammatory responses and generates adenosine which inhibits T effector cells through a cAMP mediated mechanism. This suppression results in decreased production of IL-2 by effector T cells (Bynoe and Viret 2008)

The role of CTLA-4 is uncertain. It has been suggested that CTLA-4, which binds strongly to B7 molecules (more strongly than CD28), may result in competitive inhibition – effector T cells can no longer access the co-stimulatory molecules (Sato et al 2005). CTLA-4 also triggers the induction of indoleamine 2,3-dioxygenase which catalyses tryptophan conversion into kinurenins, which are immunosuppressive (Maggi et al 2005). Recently, knockout studies in mice suggest that CTLA-4 function is integral to the function of Tregs but it is uncertain whether this translates to human Treg populations (Wing et al 2008).

Tregs also appear to inhibit a Th2 response through a contact-dependent mechanism on peripheral B lymphocytes post-activation – this suppression appears to be linked to an immunoglobulin class switch (Lim et al 2005). Interestingly, mice with a FoxP3 mutation resulting in a gain of function failed to express an adequate immunoglobulin response *in vivo* and showed markedly disrupted splenic architecture yet the function of the isolated B cells *in vitro* appeared to be normal. This was probably related to the inability of the $CD4^+$ T cells to secrete the necessary cytokines required for a class switch to IgG or IgE (INF-γ and IL4) and a failure to upregulate membrane ligands CD40 and CD69 ((Kasprowicz et al 2003). Tregs also affect other antigen-presenting cells – monocytes/macrophages downregulate their co-stimulatory molecules in response to Tregs and show reduced secretion of proinflammatory cytokines (TNF-α and IL-6) in response to stimulation with LPS (Taams 2004). Tregs also maintain DCs in an inactive state in the absence of a large population of $CD154^+$ $CD4^+$ $CD25^-$ T cells or large amounts of antigenic stimulation (Serra et al 2003).

The Glucocorticoid-induced TNF receptor-related protein (GITR) has a controversial role in the function of Tregs. Mouse studies suggest that the production of GITR ligand (GITR-L) by antigen presenting cells (by superantigen e.g *S. aureus*) results in the downregulation of the Treg response (Cardona et al 2006).

A final marker, which is constitutively expressed on Tregs, is CD137 (4-1BB) and ligation to $CD8^+$ T cells may have a role to play in their suppressive activity. In 4-1BB knock-out mice (a mouse model constitutively lacking CD137), however, Treg function appears not to be seriously compromised (Maerten et al 2005).

1.4.2 Cytokine secretion

A co-stimulatory pathway may involve secretion of cytokines – especially IL10, which is a potent downregulator of the immune response. This cytokine is secreted by other regulatory cells, including Th3 cells and Tr1 cells; but whether it plays a role in the function of naturally occurring Tregs is uncertain. (Vieira et al 2004). It was noted that FoxP3-transduced cells produced more IL10 mRNA but the exact reason remains unclear (O'Garra 2005). Furthermore, Tregs appear to dowregulate the secretion of other cytokines, particularly IFN-γ and TNF-α (although possibly not in the early stages of the suppressive response – Khazaie and Von Boehmer 2006). The downregulation of cytokine expression occurs both with and without suppression of proliferation of effector

T cells (Kelsen et al 2005). These are known functions of IL-10 further highlighting its possible importance in Tregs.

1.5 The role of Tregs in human disease

A strong Treg response can be beneficial in some human disease and detrimental in others.

Regulatory T cells play a fundamental role in the suppression of autoreactive T cells. They appear to function both in draining lymph nodes (attempting to prevent the activation of autoreactive cells) and in target organs. They migrate to the target organs as the disease progresses with eventual acquisition of adhesion markers. The Tregs are targeted at specific autoreactive cells – if there is no target effector T cell population directed at a specific antigen (like the pancreatic β islet cells) then Tregs have no function (Tonkin et al 2008). In the non-obese diabetic (NOD) mouse model of diabetes, for example, endocrine but not exocrine disease is halted, suggesting that the initial stimulation of the Treg is vital for its eventual function (Bluestone and Tang 2005).

Defects in FoxP3 have been discovered in multiple autoimmune diseases, suggesting that a primary pathology in Treg function or absolute number may be playing a role in the pathogenesis of the autoimmune disorders listed below.

Studies on mouse colitis as a model for inflammatory bowel disease demonstrate mutations in the FoxP3 gene which appear to be responsible for poor Treg function and decreased numbers of Tregs in the inflamed mucosa (Kelsen et al 2005)

The link between IPEX (the disease caused by congenital absence of the FoxP3 gene) and both eczema and food allergies suggests that these allergies may linked to primary Treg defects that both these diseases individually may be related to a primary Treg defect. Atopic dermatitis has been linked to abnormal T cell function in the presence of a superantigen (Thompson et al 2004) and the use of cyclophosphamide to treat contact dermatitis resulted in a paradoxical increase in the reaction owing to the effects on the Tregs (Ikezawa et al 2005). Mucosal tolerance in mice has been linked to the development of a specific set of Tregs for the antigen. (Winkler et al 2006)

Experimental autoimmune encephalomyelitis (EAE) is the mouse model for human demyelinating multiple sclerosis (anti-myelin basic protein). Studies in EAE mice have suggested that this disease is also related to poor Treg function and therapies targeting Treg proliferation rather than purely T cell depletion appear to hold promise for the treatment of this disease in humans (Kelsen et al 2005, Duplan et al 2006). These therapies include the use of oestrogen which has been shown to have a stimulatory effect on FoxP3 and specific TCR peptides. Another novel therapy is LF-15-0195, which also causes the Treg population to expand (Duplan et al 2006). Studies have suggested that Tregs may not be responsible for the primary remission of the disease but that secondary remissions do not occur in mice with EAE and ablated Tregs (Gartner et al 2006).

The pathogenesis of Rheumatoid arthritis appears to be linked to the inability of Tregs to control the secretion of inflammatory cytokines by $CD4^+$ T cells. Thus, although the absolute numbers of Tregs are normal in most subjects, the disease can be markedly improved by adding a suppressor against cytokine secretion like TNF-α. Higher numbers of Tregs in joint fluid is linked to an improved prognosis in Rheumatoid Arthritis (Chatila 2005).

Clinical improvement in asthmatics which is associated with treatment with glucocorticoids is associated with an increase in IL10 secretion, FoxP3 mRNA levels and with the upregulation of secretion of TGF-β (Karagiannidis et al 2004, Till et al 2004)

Some of the disadvantageous effects of Tregs include their propensity to facilitate evasion of the immune system. Two primary pathological processes, namely malignancy and infection, have been shown to utilise an expanded regulatory T cell population to suppress specific cytotoxic responses. In tumours, a strong cell-mediated immune response is associated with improved survival and a less aggressive phenotype. It is therefore not surprising that many tumours specifically and non-specifically activate Tregs as a strategy for immune evasion. Higher levels of Tregs have been found in many haemopoietic malignancies. Non-Hodgkin's Lymphomas appear to secrete chemotactic factors for Tregs including CD122 so that there is a markedly elevated population throughout the body (approximately 17% Tregs were found in diseased nodes and 7% in non-diseased nodes as opposed to the normal 2-5% - Yang et al 2006). Certain T cell

leukaemias express a Treg phenotype, which may be linked to a more aggressive course although the exact functional significance in these cells remains unknown (Chen et al 2006). Gastric and oesophageal carcinomas show an increased size of the population of Tregs compared with healthy donors; this correlated positively with increased stage of the tumours and with the risk of tumour recurrence (Perrone et al 2008). Higher levels of FoxP3 mRNA expression correlate with a poorer prognosis in ovarian cancer (Wolf et al 2005). Studies in mice have shown that certain tumours e.g. pancreatic adenocarcinoma can induce a regulatory phenotype in previously $CD25^{-}$ $FoxP3^{-}$ $CD4^{+}$ cells. Any immunomodulatory treatment, for example with dendritic cells focused at cancers (as is being considered for melanoma), may have to be associated with immunosuppressive therapy in order to eliminate pre-existing population of Tregs (Lopez et al 2005). An example would be the administration of IL-2 which is associated with massive proliferation of the Treg population (Ahmadzadeh and Rosenberg 2006)

Tregs also limit the response of the immune system to infections. This can result in infection persistence but may also prevent a neutralising response with loss of antigen-maintained memory (Gavin and Rudensky 2003, O'Garra 2005). In *Leishmania spp.* infections, it has been demonstrated that 50% of the involved T cell populations are Tregs with failure of the immune response to eradicate the pathogen (Gavin and Rudensky 2003). Certain pathogens are associated with the induction of immature dendritic cells (DCs), which favours the induction of a regulatory T cell population. *Plasmodium falciparum* scavenges CD36, which prevents the maturation of DCs by LPS and studies have demonstrated that an expanded regulatory T cell population is associated with faster

rates of parasite growth (Good 2005, Walther et al 2005). Mannosylated lipoarabinomannans (immunogenic glycolipids) from *Mycobacterium spp.* also inhibit Treg maturation and the secretion of IL-12. Ebstein-Barr virus (EBV) expressing cells which over-express the Notch ligand, Jagged-1, have been shown to induce the development of a Treg population that suppresses function and proliferation of effector cells with possible effector function in transplantation (Vigoroux et al 2003).

1.6 *Mycobacterium tuberculosis:* the immune response

The estimated prevalence of active infection with *Mycobacterium tuberculosis* in 2005 was approximately 14 million infected individuals, of which 3, 77 million cases were based in Sub-Saharan Africa with 340 000 new cases in South Africa alone in 2004 (World Health Organisation). Owing to the link between TB and HIV, the epidemic of tuberculosis continues to escalate.

In high prevalence settings, like Sub-Saharan Africa, many individuals are infected with *Mycobacterium tuberculosis*, but not all develop active disease. The WHO Global tuberculosis control - surveillance, planning, financing report (2005) indicates that 1.7 billion people are infected with TB worldwide. Latent tuberculosis is defined by a positive skin induration to purified protein derivative in the absence of signs or symptoms or typical radiological changes (Jasmer et al, 2004). Latent tuberculosis represents a treatment dilemma. For this reason it is important to define distinguish active tuberculous disease from asymptomatic infection and the correlates of protection.

The host cell for the mycobacterium is the macrophage. In order to survive within the phagosome, the bacterium must subvert the host immune system. The organism gains access to the macrophage through the complement receptor 3, which prevents proinflammatory activation of this cell (Houyen, Nguyen and Pieters 2006). One of the key strategies utilized by the bacillus is inhibition of fusion of the phago-lysosome. The mycobacterium appears to disrupt the essential signaling sequence which enables fusion of the early and late endosome - this includes the secretion of substances which mimic host signal transduction molecules, in particular protein kinase G (Walburger et al 2004) and inhibition of calcium signaling by inhibition of the production of sphingosine-1 phosphate (Thompson et al 2005). Mycobacteria also inhibit the expression of proteins with the FYVE domain (a specific zinc finger pattern) including early endosome autoantigen 1 (EAA-1) and hepatocyte growth-factor regulated tyrosine kinase substrate (Gruenberg and Stenmark 2004) – these proteins are permissive of fusion of the phago-lysosome. Other immunoevasive strategies include resistance against nitric oxide and its

toxic intermediaries and prevention of antigen presentation by attenuating the expression of HLA class II molecules (Chan and Flynn 2004).

One of the most important cell populations in the control of tuberculosis infection is the $CD4^+$ T cell population – it is clear that an intact $CD4^+$ T cell population correlates with a better outcome as studies in patients with compromised helper cell populations have shown including those with HIV infection (North and Jung 2004, Kaufmann 2005). Absent levels of Th1 cytokines (interferon-γ and IL12) are associated with an inability to control tuberculosis disease (Flynn et al 1993, Cooper et al 1993, Cooper et al 1997). This suggests that the regulatory T cell population may possibly have a pivotal role in determining the outcome of the disease process by inhibiting a TB specific $CD4^+$ T cell response. Currently, the role of Tregs is uncertain in the pathogenesis of tuberculosis. A small pilot study suggested that the numbers of Tregs are increased in active tuberculosis disease (Guyot-Revol et al 2006). These preliminary findings were confirmed by further studies (Hougardy et al 2007, Roberts et al 2007). These cells may aid the bacterium (in addition to the mechanism described above) in immune evasion. Further investigations (Chen et al 2007) suggest that regulatory T cells, as defined by their expression of CD4, CD25 and FoxP3 are increased in absolute number and frequency in the blood of patients with active tuberculosis which compared with uninfected controls and patients with latent tuberculosis. The effects of these cells appear to be diverse including secretion of IL-10 and suppression of tuberculosis specific interferon-γ secretion (Chen et al 2007). It seems unclear as to the role of these cells in suppression of infection. Studies in mice that have

had preferential depletion of the FoxP3-expressing $CD4^+$ T cell population showed a log reduction in the colony forming units of *Mycobacterium tuberculosis* (Scott-Browne et al 2007). In addition, adoptive transfer of FoxP3 expressing $CD4^+$ T cells into mice resulted in suppression of a tuberculosis-specific effector $CD4^+$ T cell response (Kursar et al 2007).

Infection of a patient with HIV and active tuberculosis disease further complicates studies of immune regulation in South African populations. It is estimated by the 2005 report by UNAIDS that 60% of patients with tuberculosis infection are co-infected with the virus (www.unaids.org). HIV infection itself has been associated with multiple effects on the Treg population. Depletion of $CD25^+$ cells from a PBMC population results in massive upregulation of IFN-γ secretion by the remaining T cells suggesting that this population, even in HIV positive individuals, is directly responsible for suppressing function of HIV specific $CD4^+$ and $CD8^+$ T cells(Nixon, Aandahl and Michaelsson 2005). This may be beneficial in downregulating the immune activation which may be partially responsible for CD4+ T cell apoptosis and depletion (Oswald-Richter et al 2004, Eggena et al 2005). Nevertheless, because this subset expresses the co-receptor CCR5 and the CD4 molecule, they are highly susceptible to infection and are also susceptible to the cytotoxic effects *in vitro* (Chase et al 2007, Oswald-Richter et al 2004). In addition, their effect on HIV pathogenesis *in vivo* remains to be elucidated.

2.0 Aim

2.1 Primary Aim

The primary aim of this study was to quantify the number of Tregs, expressing CD25 and intracellular FoxP3 in patients with active tuberculosis, patients with HIV infection alone or patients with HIV infection and active TB and to compare these to healthy controls, in order to determine whether this population of cells could be contributing to the pathogenesis of HIV infection or tuberculosis disease by suppressing the immune response to these infections.

2.2 Secondary aims

The secondary aims of the study included elucidating the ability of Tregs to proliferate in response to both antigen specific and non-specific stimulation in patients with infections mentioned above. It has been suggested that only centrally produced regulatory T cells express FoxP3 and that these cells are anergic. Adult patients with depletion of CD4 T cells would therefore be unable to regenerate their regulatory T cell population.

This was compared with the ability of $CD4^+$ and $CD8^+$ T cells which did not express FoxP3 to secrete interferon gamma (IFN-γ) and to proliferate in response to the same stimulation in culture in order to demonstrate a possible regulatory phenotype in co-culture in the absence of formal depletion studies.

The study also aimed to explore the use of additional surface immunophenotypic markers of regulatory T cells including CTLA-4 and GITR and to compare them to FoxP3 in order to evaluate their utility as possible surrogate markers for this population.

3.0 Methods

3.1 Patient selection

Patients were sourced from the Johannesburg Hospital, Hillbrow Primary Health Clinic and the Alexandra Primary Health Care Clinics. Each patient gave full informed consent and the study was approved by the Ethics Committee of the University of the Witwatersrand (Ethics number M060313). Up to 60 ml of blood was collected from a peripheral vein using the vacutainer system and anticoagulants: acid citrate dextrose (ACD) and Ethylenediamine tetra-acetic acid (EDTA) (*Table 3.1)*

HIV infected group

The patients with documented HIV infection (n=10) were sourced from the Hillbrow Wellness Clinic and the Johannesburg Hospital (both the anti-retroviral clinic and the wards). The diagnosis had been made by the managing physicians using a Determine HIV-1/2 rapid tests (Abbott Laboratories, USA) and confirmed in the majority of patients by a fourth generation ELISA (Abbott Laboratories, USA). The patients were antiretroviral drug naïve at time of enrolment and were not taking anti-tuberculous chemotherapy. They had no obvious clinical symptoms and signs of active tuberculosis. A small subpopulation (N=3) had bone marrow trephine biopsies which were negative for the presence of TB granulomata.

Active tuberculosis group

The patients with active *Mycobacterium tuberculosis* infection (n=14) were diagnosed microbiologically by the presence of acid-fast bacilli in the sputum. These patients were sourced primarily from the Johannesburg Hospital. The patients with HIV co-infection (n=9) were anti-retroviral drug naïve at enrolment. The diagnosis of HIV infection was made in a similar manner to that described above. No patient had taken more than 2 doses of anti- tuberculous chemotherapy at the time of collection of the samples.

Uninfected group

The uninfected controls (n=10) were sourced from clinically well hospital and laboratory staff. The samples were tested anonymously after collection for HIV infection using the Abbott determine HIV rapid test. No results of the testing were revealed to the participants.

Table 3.1: Demographic characteristics of patient sub- populations

Patient group	**Ethnic group (B=black, C=coloured, I=Indian, W=white)**	**Age group (1<20yrs, 2 =20-29yrs; 3=30-39yrs; 4=>40yrs)**	**Gender (M=male; F=female)**	**CD4 count ($x10^6$/l)**
Uninfected population	B	3	F	831
	B	3	F	973
	B	3	F	1503
	B	1	M	671

	B	3	M	1231
	B	2	F	694
	C	3	M	950
	I	3	M	1332
	B	3	M	818
	B	3	M	915
HIV infected population	B	3	F	523
	B	2	F	223
	B	4	F	118
	B	2	F	432
	B	3	M	40
	B	3	F	169
	B	3	M	712
	B	3	F	191
	B	2	F	219
	B	2	F	452
Active TB	C	4	M	1503

population	B	2	M	743
	B	1	F	859
	B	4	F	494
	B	3	F	801
HIV infected population with active TB	B	4	F	8
	B	2	F	131
	B	2	M	563
	B	3	M	1
	B	3	M	2
	B	3	M	43
	B	3	F	59
	B	4	F	655
	B	3	M	68
	B	4	M	258

3.2 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells were isolated by a standard Ficoll-Hypaque technique (Amersham Biosciences, UK). The blood was centrifuged at 2200rpm for 15 minutes using a Ficoll density gradient. The buffy coat was removed and washed twice with HBSS (Hank's Balanced Saline Solution – Gibco, Scotland). The cells were then counted using a Guava Viacount (Guava Technologies, California) according to manufacturer's instructions. Those PBMCs which were not stained with CFSE were resuspended in RPMI 1640 medium with added GlutaMAX and 25mM HEPES (Gibco, Scotland), supplemented with 10% human serum AB (HuAB) (Gemini Bio-Products, USA) and 0.1% gentamycin (R10) at a concentration of 2 million cells/ml.

3.3 Cell cultures and stimulations

The PBMCs were cultured for 96 hours in 24 well round-bottom plates using appropriate aseptic techniques. Approximately 2 million cells were cultured in every well. The culture medium utilized was RPMI 1640 with glutaMAX and 25mM Hepes with 10% HuAB.

Appropriate stimuli were added to each well. The following stimuli were added:

1. Anti-CD3 (0.1ug/ml) monoclonal antibody – positive control
2. Tetanus toxoid (2ug/ml) – an antigen to which most South Africans have been exposed in the form of vaccination

3. Purified protein derivative (0.01ug/ml – Statens Serum Institut) – a construct of peptides derived from mycobacterial species
4. Gag clade C peptide superpool (2ug/ml – NMI Peptides, Netherlands) – peptides of 15 amino acids in length derived from the p55 protein
5. Nef clade C peptide superpool (2ug/ml – NMI Peptides, Netherlands) – peptides of 15 amino acids in length derived from the negative regulatory factor of HIV clade C virus

An unstimulated culture was utilized as a negative control.

A separate culture was established in tubes for intracellular cytokine staining for interferon-γ. An hour after the culture was established, 10ug of Brefeldin A was added. These samples were incubated with the above-mentioned stimuli and in the culture medium overnight at a slight angle at 37^{o}c in 5% CO_2.

3.4 Intracellular cytokine staining for interferon-γ

Intracellular cytokine staining was performed using a protocol adapted from BD Bioscience(http://www.bdbiosciences.com/pharmingen/protocols/Intracellular_Cytokine) The samples were centrifuged in the culture tubes at 1800 rpm for 5 min. EDTA was then added to the samples which were then incubated for 15 min in the dark at room temperature. Contaminating red blood cells were lysed with appropriate FACS lysing solution for 10 minutes and then samples were centrifuged and resuspended. The cells were then washed with 2ml of FACS wash buffer. The sample was centrifuged and 0.5ml

FACS Perm solution was added. The sample was incubated for a further 10 minutes in the dark and then washed with FACS wash buffer.

The following antibody panel was utilized for staining using concentrations established during the project by formal antibody titration:

1. CD3 PerCP (15ul) – T cell receptor co-signalling chain
2. CD4 FITC (10ul) – co-receptor for the T cell receptor in T helper cells, acting to recruit downstream signalling molecules
3. CD8 PE (5ul) – co-receptor for the T cell receptor in cytotoxic T cells, acting to recruit downstream signalling molecules
4. IFN-γ APC (5ul) – T helper 1 cytokine associated with a pro-inflammatory response

3.5 Intracellular staining for FoxP3

The protocol utilized for FoxP3 staining was that recommended by the manufacturer (eBiosciences, UK). The samples were stained at 2 time points i.e. after resting overnight and at 96 hours. Briefly, samples rested overnight were centrifuged within their tubes. Those samples which had been cultured were harvested by gentle pipetting up and down and then decanting into standard tubes. The samples were washed with FACS wash solution, centrifuged at 1800 rpm for 5 minutes and the supernatant was discarded. 1ml of Fix/Perm Solution (eBiosciences, UK) was then added and the sample was vortexed and then incubated at 4°C for 30 minutes. Samples were washed with wash buffer and

washed a further two times with 2 ml permeabilisation buffer (eBiosciences, UK) with centrifugation and discard of supernatant after each wash.

Antibodies were then added in the following panels:

1. Panel 1 – CD4 FITC and CD3 PerCP(BD Biosciences, USA), FoxP3 PE (eBiosciences, UK) and GITR APC (R&D Systems, USA)
2. Panel 2 – CD4 FITC, CD3 PerCP and CD25 APC(BD Biosciences, USA) and FoxP3 PE (eBiosciences, UK)
3. Panel 3 – CTLA-4 FITC (R&D Systems, USA), CD3 APC, CD4 PerCP (BD Biosciences, USA) and FoxP3 PE (eBiosciences, UK)

The samples were incubated for 30 minutes in the dark at 4^{o}C. 2ml of Permeabilisation buffer were then added and the sample centrifuged. This wash step was repeated. The sample was then resuspended in 200ul of cell fixative (4% paraformaldehyde in PBS).

3.6 Carboxyfluorescein succinimidyl ester (CFSE) staining

The samples to be stained for CFSE were harvested. The cells were placed in a 15ml Falcon tube and wrapped in foil to avoid light contamination. 2ml of CFSE was added and the samples were incubated in the dark for 7 minutes. The reaction was then stopped by adding 4ml ice-cold Fetal Bovine Serum (Gibco, Scotland). The samples were then washed twice with R10 to remove excess CFSE. PBMCs to be stained with CFSE were

transferred to a 15 ml falcon tube wrapped in foil. Additional markers were then added including FoxP3 PE (eBioscience, UK) and CD3 APC and CD4 PerCP (BD Biosciences, USA).

3.7 Acquisition

Samples stained with CFSE were acquired solely on a multi-colour LSR II (BD Biosciences, USA) utilizing FacsDiva acquisition software (BD Biosciences, San Jose). The IFN-γ and remaining FoxP3 panels were acquired both on the LSR II and on the FacsCalibur which utilizes CellQuest acquisition software (BD Bioscience, San Jose). Both the LSR II and the FacsCalibur underwent daily calibration utilising Rainbow Calibration Beads and FacsComp beads (BD Biosciences, USA) as advised by the manufacturer. Separate samples were analysed as compensation controls utilizing CD8 FITC, PE, PerCP and APC stained cells which were prepared with the samples. Compensation was done digitally utilising the computer programme FlowJo (Tree Star, Stanford USA). A Propodium Iodide control was analysed to assess adequacy of cellular permeabilisation. Monoclonal antibody controls were utilised to exclude non-specific binding of fluorescent antibodies.

3.8 Analysis and Gating strategies

Analysis was accomplished utilizing FlowJo 6.4.2 software. The samples were analysed utilizing the negative control to exclude non-specific binding. In the case of the CFSE analysis, the Mississippi Gating strategy (developed by Mark Connors, of the NIH Immune Regulatory Laboratory) was utilized to exclude the presence of dead cells. $CD4^{+}$T cells were identified initially by separating the lymphocytes on the basis of their low intracellular complexity (low side scatter), small size (low forward scatter) and then on the basis of their expression initially of CD3 and then CD4. Gates were established on the unstimulated and negative (unstained) controls

3.9 Statistical Analysis

The data were analysed utilizing the following statistical tests on the STATA statistical programme (STATACORP 2005. *STATA Statistical Software: release 9*. College Station: STATCORP LP):

1. Testing of the samples for normality and homoskedasticity (equal variances) using a Shapiro-Wilk (Shapiro and Wilk 1965)
2. A paired t-test for comparison of means
3. Sidak adjustment for multiple comparisons
4. Linear regression and pairwise correlation for the variables of interest.

4.0 Results

4.1 *Ex vivo* Regulatory T cell frequencies

An immediate *ex-vivo* analysis was performed on samples from all four patient groups to analyse Treg frequencies. Although previous data (Guyot-Revel et al 2005, Chen et al 2007) has suggested that the frequencies of Tregs are increased in patients with active tuberculosis, these data show a reduced frequency *ex vivo* in patients with confirmed tuberculosis disease ($p<0.006$) compared with uninfected controls.

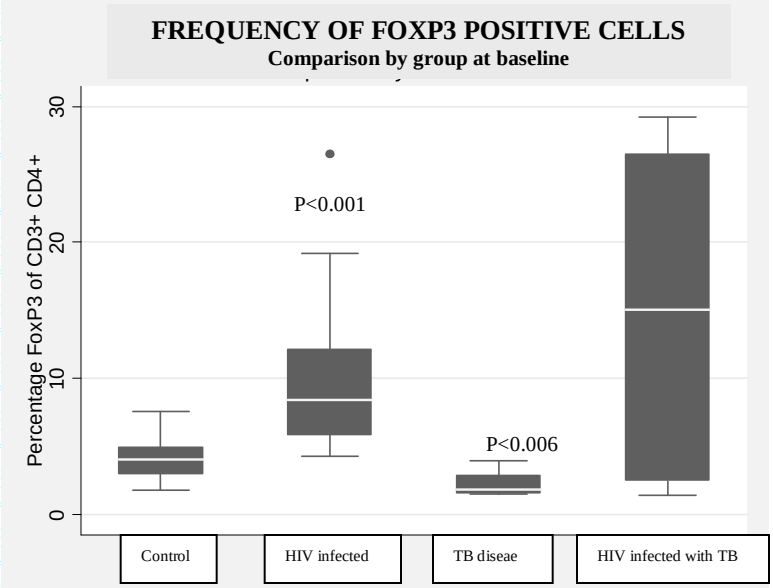


Figure 4.1

A comparison of Regulatory T cell frequencies ex vivo in the 4 groups with no exogenous stimulation (significantly increased in HIV infected individuals p<0.001)

The frequencies of Tregs *ex vivo* were increased in patients with HIV ($p<0.001$) compared with uninfected controls, in keeping with the previously described

immunoevasive strategy of the virus. There was a broad range of Treg frequencies in the group infected with HIV with active TB, but the overall frequency was not significantly different from the controls (*Figures 4.1 and 4.2)*

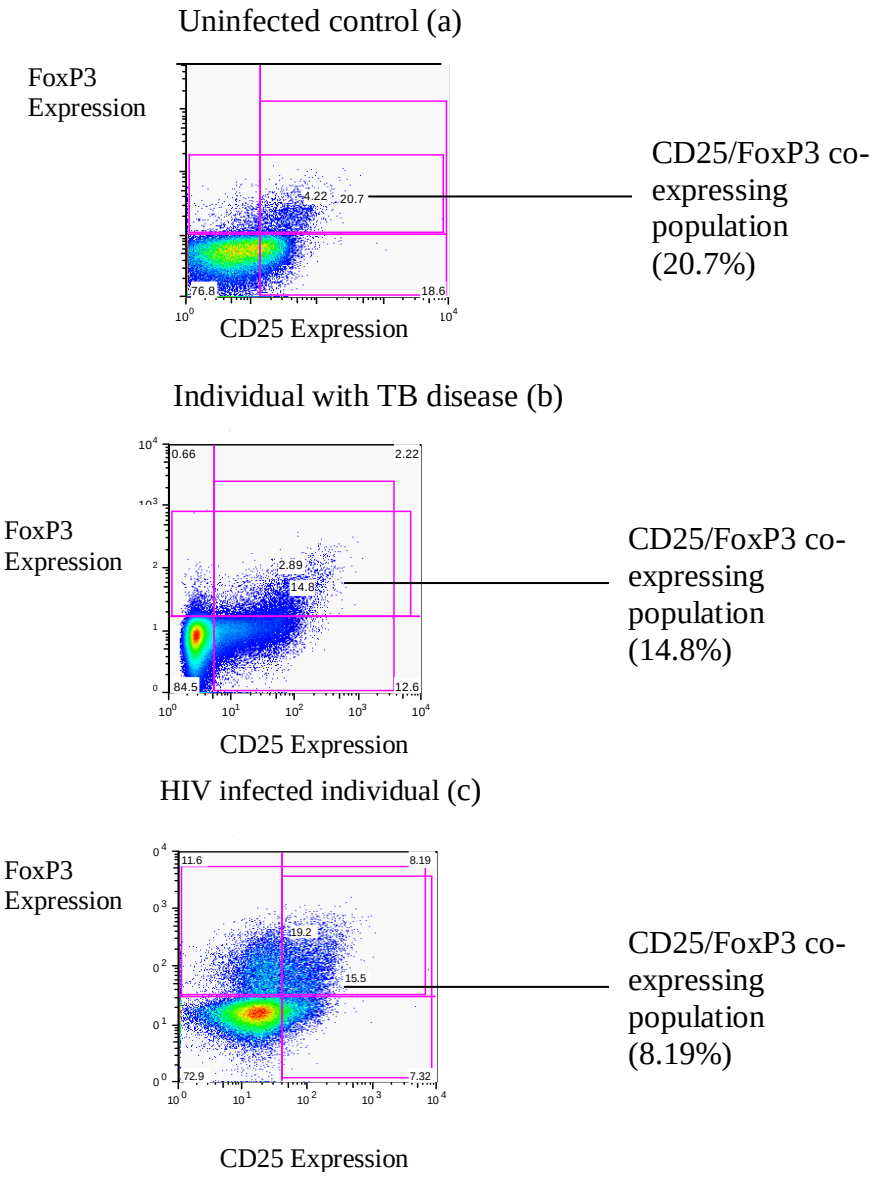


Figure 4.2

Comparison of FoxP3 expression (y axis) against CD25 expression in CD3+ CD4+ T lymphocytes ex vivo in uninfected (a), TB disease (b) and HIV infected (c) individuals

4.2 Alterations in Treg frequencies after culture

The samples were cultured for four days in order to assess whether these populations were anergic in culture. In addition, if, as has been suggested, FoxP3 merely represents an activation marker, it would be likely that the cells expressing FoxP3 would undergo apoptosis. There was indeed a significant decline in the frequency of $CD4^+$ T cells expressing FoxP3 after 4 days of culture in the HIV-infected individuals suggesting that these cells may have undergone apoptosis although no markers of apoptosis were utilised to measure this directly. The frequencies with culture remained stable (compared with frequencies immediately *ex vivo*) in all other population groups suggesting that there was no significant proliferation or apoptosis in the absence of stimulation. *(Figure 4.3)*

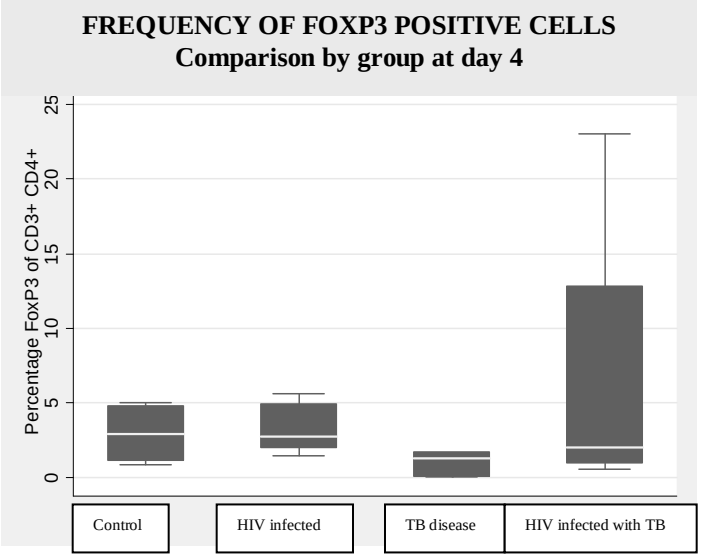


Figure 4.3

Frequencies of Tregs after 4 days of culture with no stimulation (no significant change was noted in frequency except in the group infected with HIV only)

Anti-CD3, which engages the T cell receptor signalling chain, is a potent T cell stimulant. In most groups, stimulation with anti-CD3 resulted in a significant proliferative response and a significant increase in the frequency of Tregs compared with frequencies *ex vivo*. The exception was the group of patients who were infected HIV and had active TB disease who failed to show a proliferative response even to this stimulant. Tetanus (against which, many of the South African population have been immunised), which was used as a second positive control, failed to produce a significant proliferative response in any of the four population groups (*Figure 4.4)*.

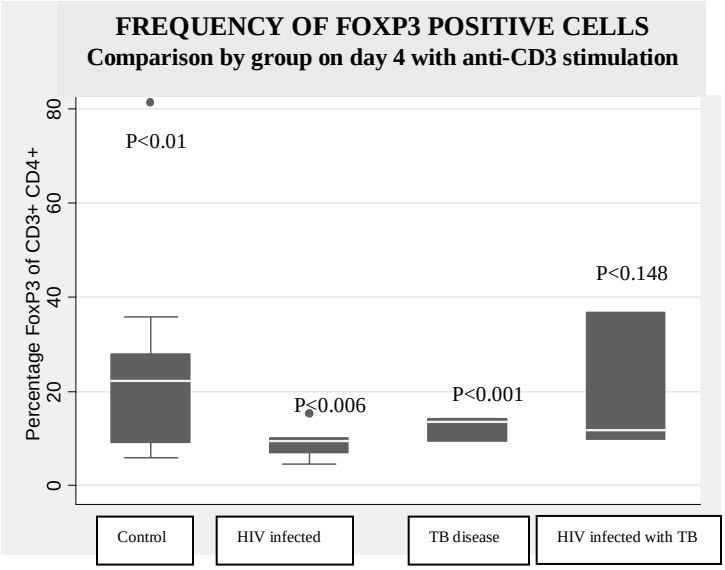


Figure 4.4

Comparison of the effect of anti-CD3 stimulation on Treg frequency after 4 days of culture (significant differences compared with ex vivo for control population, HIV infected population and patients with TB disease p<0.01, p<0.006 and p<0.001 respectively)

PPD (Staten Serum Institute, Denmark) is a tuberculous protein derivative and was used in all 4 groups to try to stimulate an antigen-specific response, particularly in the groups with active tuberculosis. There was, however, no significant response in any population group. A single HIV positive patient showed a significant response to PPD stimulation at day 4 (103% increase between PPD stimulation and no control) although the overall effect on the class as a whole was not significant (*Figure 4.5*).

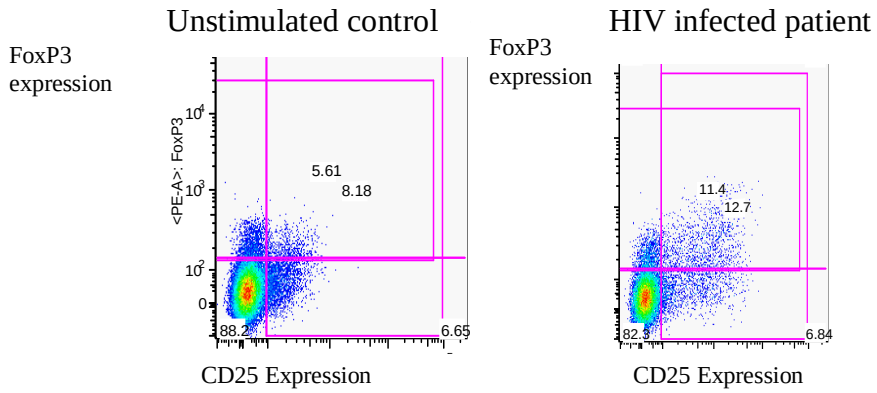


Figure 4.5

Response to PPD stimulation at day four by Treg frequency in an HIV infected individual compared with unstimulated control (12.7% versus 8.18%)

Two HIV peptide pools were utilised for stimulation – a Gag superpool composed of 70 peptides and a Nef superpool composed of 50 peptides (NMI peptides). Both superpools comprised peptides 15 amino acids in length, with an 11 amino acid overlap. Gag proteins include the structural proteins of the viral core (capsid, matrix, and nucleocapsid proteins). Nef (negative replication factor) protein appears to be important in viral replication and immunomodulation (Pennington et al 1997, Noviello et al 2007, Schindler et al 2007) although the extent to which the peptide superpool reflects this function is

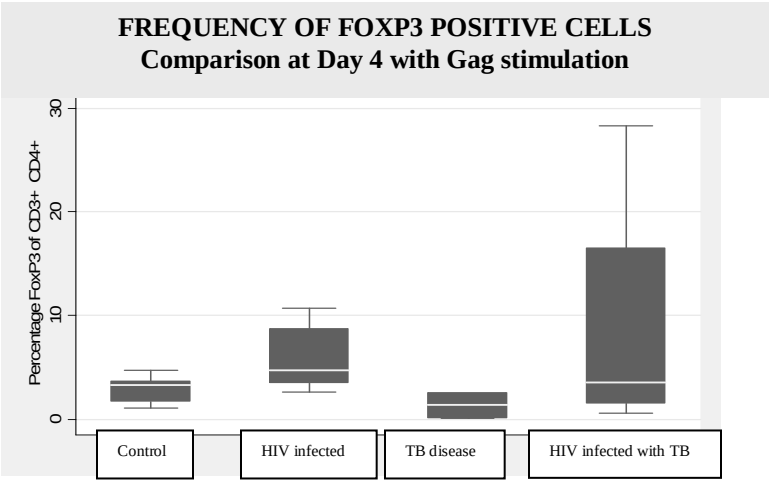


Figure 4.6

A comparison of the effect of stimulation with Gag superpool on Treg frequency in the 4 patient populations after 4 days of culture– Gag stimulation resulted in a significant increase in frequency in the HIV infected patients compared with the other patient populations ($p<0.05$)

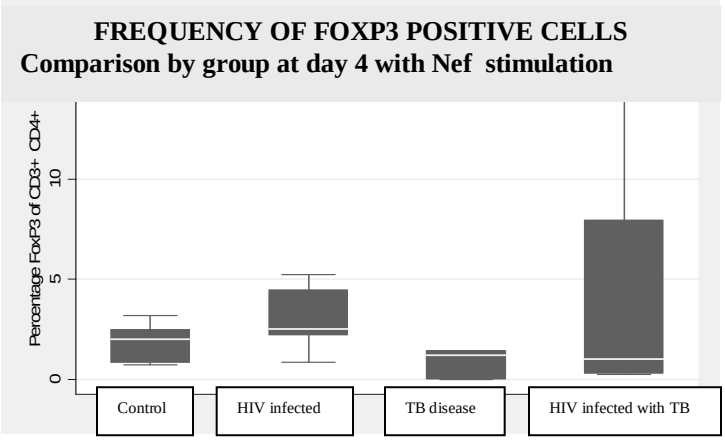


Figure 4.7

A comparison of the effect of stimulation with Nef superpool on Treg frequency in the 4 patient populations after 4 days of culture – Nef stimulation appeared to reduce the frequency of cells expressing FoxP3 in the uninfected controls but this trend failed to reach significance

uncertain. Gag stimulation resulted in a significant increase in Tregs in the patients infected with HIV alone ($p<0.05$ - *Figure 4.6*). A trend was noted in the uninfected controls for a decrease in Treg frequencies compared with *ex vivo* frequencies in response to Nef stimulation but this failed to reach significance (*Figure 4.7*). No trend was noted for a positive or a negative change in Treg frequencies in any other population group.

4.3 Correlation of GITR, CTLA-4 and CD25 high expression as markers of Tregs

Staining for FoxP3 necessitates permeabilisation of cells (which kills them) – this makes it difficult to assess function in FoxP3 positive cells. In addition, additional markers which may be associated with suppressive function have been described on cells with a regulatory function. For this reason, this study assessed three markers which have been associated with Tregs and correlated their expression with FoxP3. Bright expression of CD25 has traditionally been used as a surrogate marker in cases where the Treg population was needed intact. These data failed to show a significant correlation between CD25 and FoxP3 either at *ex vivo* or after culture in any patient population. Two other putative markers were also assessed in this study – CTLA-4 which is the regulatory ligand for B7 (a costimulatory molecule expressed by professional antigen presenting cells) and GITR which is a glucocorticoid induced receptor. CTLA-4 blockade has previously been utilised to promote a

proinflammatory response (for example in malignancy – Maker et al 2005). Only GITR showed a significant correlation with FoxP3 and only in the uninfected control group at *ex vivo* (a trend was noted after 4 days of culture but this failed to reach significance). GITR and CD25 expression levels correlated well at baseline and after 4 days of culture in the uninfected control group, but this was not reproducible in the other groups. (*Figure 4.8 and Figure 4.9*)

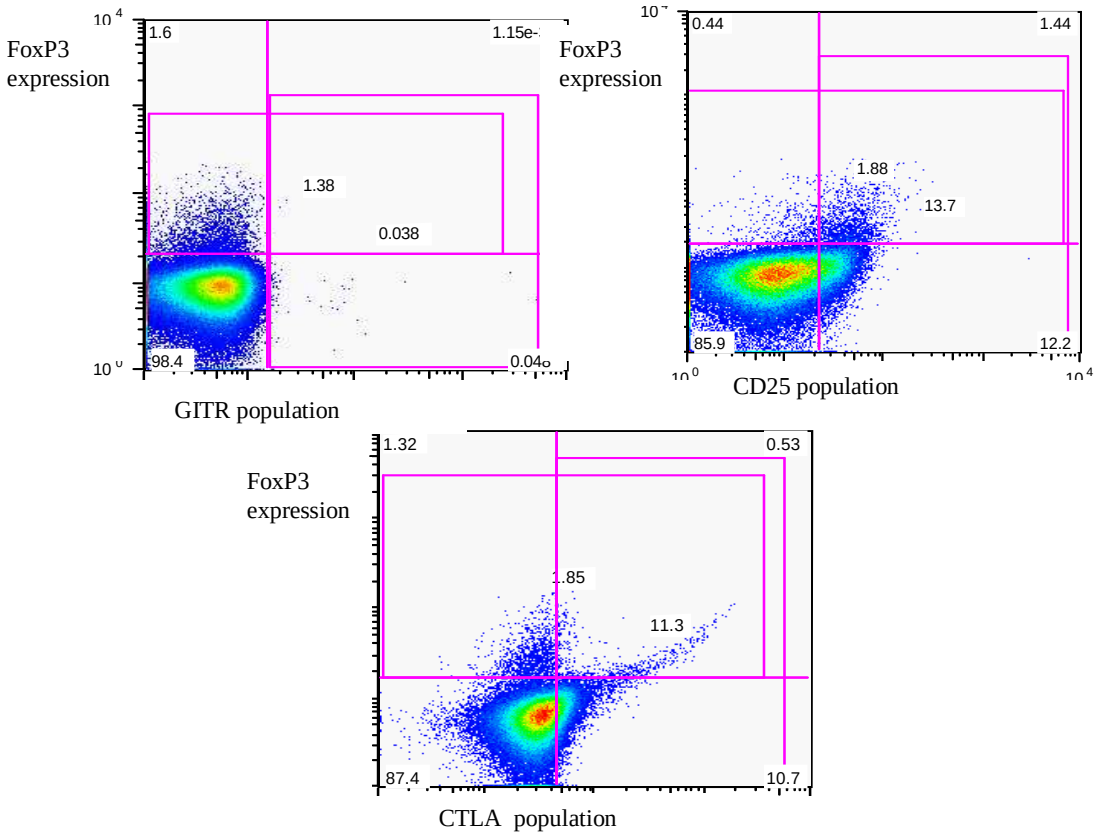
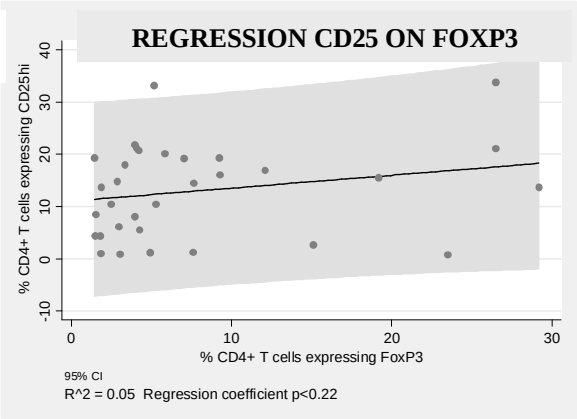


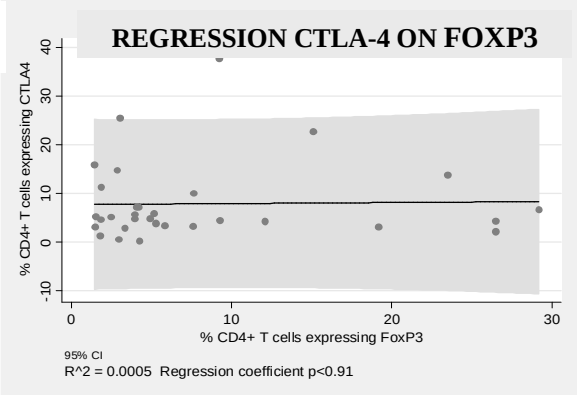
Figure 4.8

A comparison of CD25 high staining, CTLA-4 staining and GITR staining in a tuberculosis infected individual at ex vivo – the correlation amongst these markers and between these markers individually and FoxP3 was unreliable

(a)



(b)



(c)

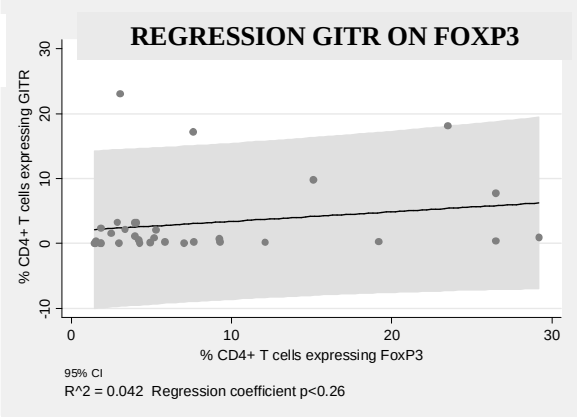


Figure 4. 9

Regression of CD25hi (a), CTLA-4 (b) and GITR (c) expression on FoxP3 failed to show a statistically significant correlation with FoxP3 for any of the patient groups ($p<0.22$, $p<0.91$, $p<0.26$ respectively)

4.4 CFSE staining

CFSE is an intracellular dye which binds DNA and is shared among daughter cells in a predictable manner allowing for assessment of cell proliferation. It was utilised in this study to assess the proliferative response of cells which expressed FoxP3 and CD4 to the exogenous stimuli and to assess whether these cells are anergic in culture. As expected, the positive control, anti-CD3, stimulated a strong proliferative response in $CD4^+$ T cells expressing FoxP3 and $CD4^+$ T cells which did not express FoxP3 after 4 days in most patients (*Figure 4.10*). The exception was the cohort of patients with HIV infection and active TB who showed a significant decrease in frequencies of $CD4^+$ T cells and no $CD4^+$ T cell proliferation in response to anti-CD3. This was unexpected and suggested that strong stimulation in this population group may have resulted either in anergy or in apoptosis in this T cell subset. The possibility that this may have been a result of the toxicity of the CFSE dye was excluded – there was no reduction in T cell frequency when these cells were exposed to the dye in the absence of anti-CD3 stimulation. It was noted, however, that the presence of the dye itself resulted in a significant loss of Tregs in both the healthy controls and HIV infected individuals.

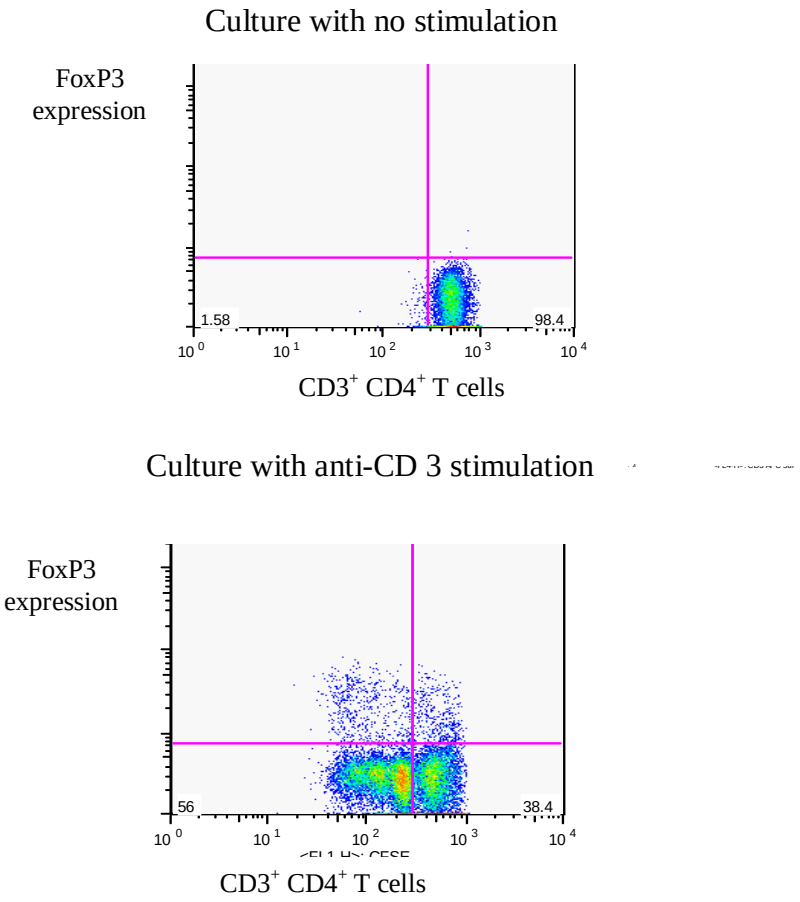


Figure 4.10

CFSE dye dilution showing significant proliferation of $CD3^+$ $CD4^+$ $FoxP3^+$ cells in response to anti-CD3 stimulation

Proliferation of the non-Treg $CD4^+$ T cells was also assessed to see whether there was a response to stimuli. IFNγ secretion by $CD4^+$ $FoxP3^-$ T cells and $CD8^+$ T cells was also measured and compared with proliferation.

As expected, there was a significant proliferative response in CD4 T cells in all populations to anti-CD3 stimulation (except in co-infected subjects as discussed above)

which correlated well with IFNγ production by both CD4 and CD8 T cells. There was no significant difference in proliferative response to other stimuli, although individual patients did show responses to PPD and tetanus, which correlated with their ability to upregulate production of IFNγ. Gag and Nef produced weak proliferative responses and expression of IFNγ with some exceptions in individual patients (*Figure 4.12*).

Interestingly, the proportion of $FoxP3^+$ $CD4^+$ CFSElow T cells correlated positively with capability of CD4 cells to express IFN-γ and negatively with the ability of CD8 cells to express IFN-γ when stimulated with Gag in uninfected individuals (*Figure 4.12*).

The ability of CD8 T cells to express IFN-γ correlated negatively with the frequency of cells expressing FoxP3 in HIV infected individuals in response to anti-CD3 ($p<0.03$). FoxP3 expression in $CD4^+$ T cells correlated with the proliferative response of $CD4^+$ T cells to Nef in HIV infected individuals (*Figure 4.13*).

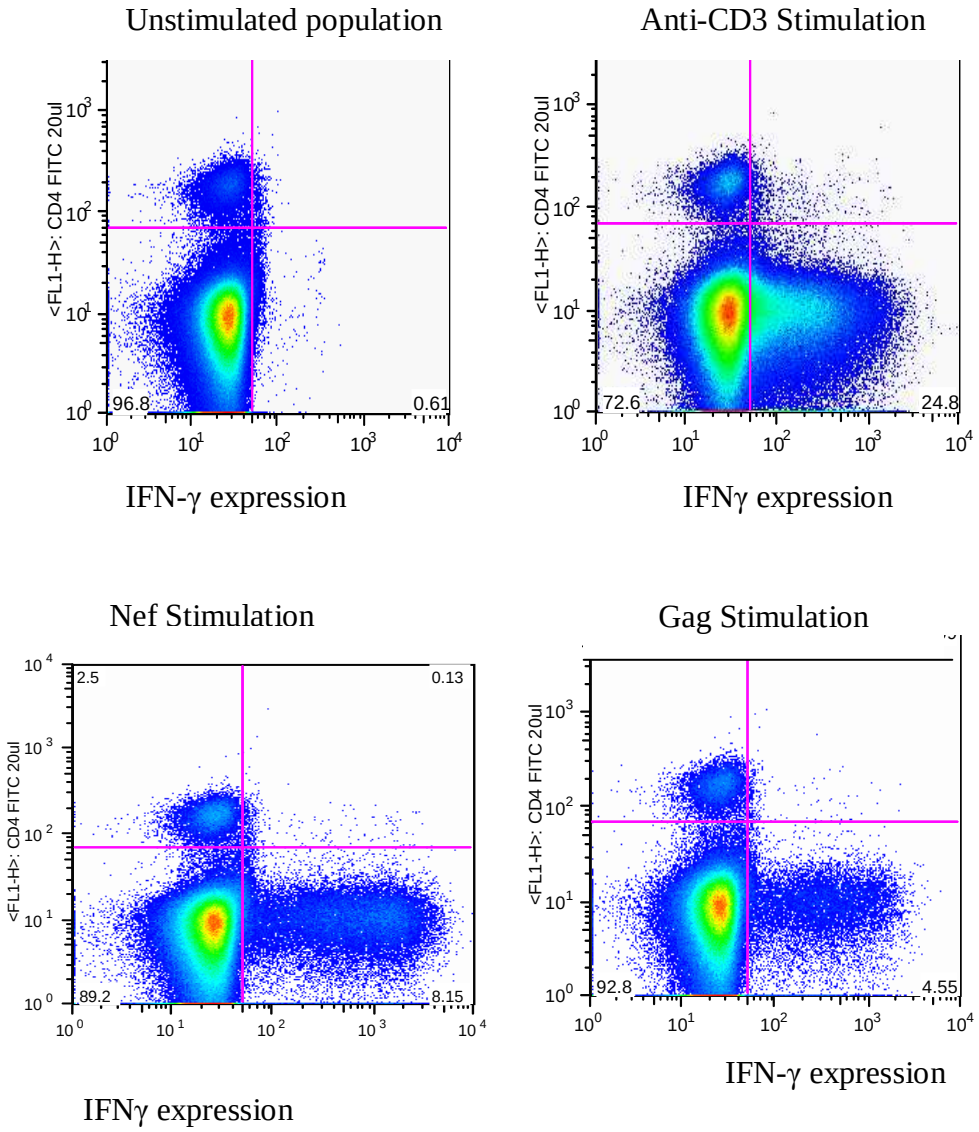


Figure 4.11:

Interferon- gamma expression at day 1 by CD4$^+$ T cells in an individual with HIV and active TB in response to no stimulation, anti-CD3, Gag superpool and Neg superpool (CD4$^+$ T cells on the y-axis and IFN-γ on x-axis). A significant response is shown to the HIV specific peptides and to anti-CD3 by the non- CD4$^+$ T cells (defined by their expression of CD3)

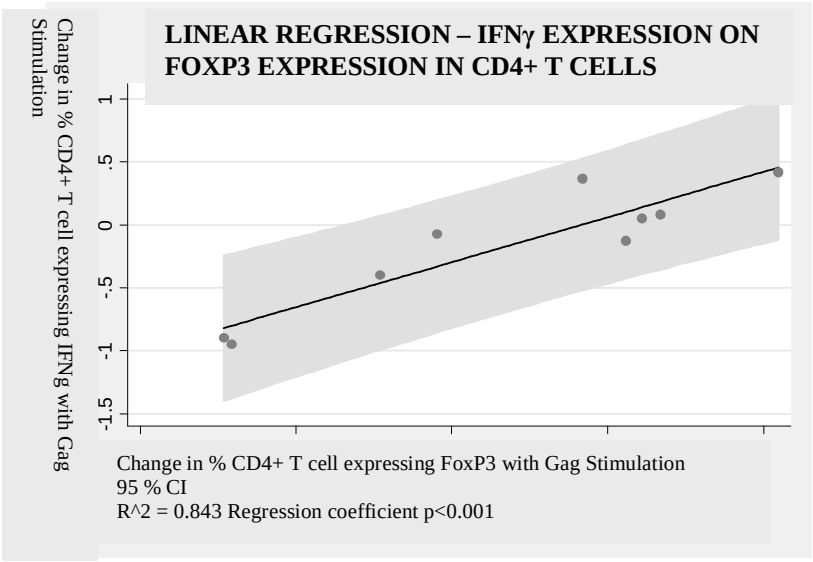


Figure 4.12

A regression of change in FoxP3 on change in IFNγ expression in CD4+ T cells in uninfected individuals with Gag stimulation showing a significant correlation (p<0.001)

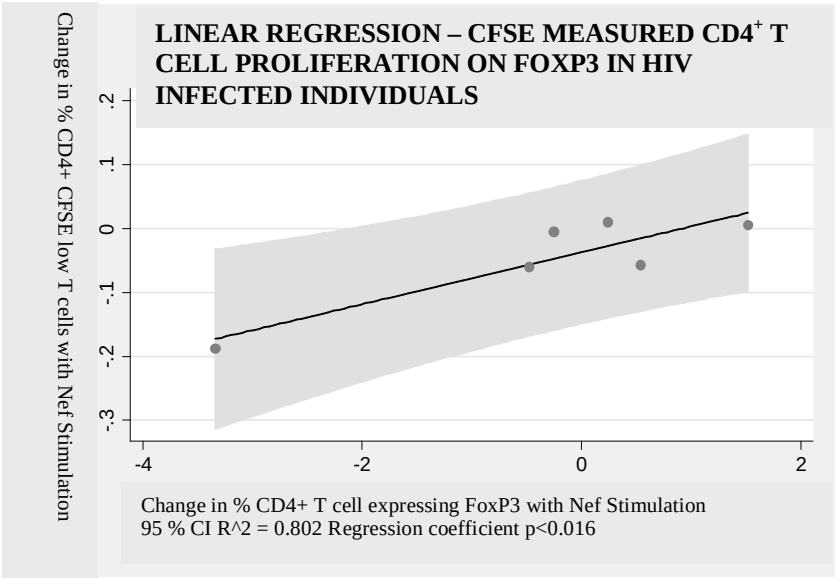


Figure 4.13

A linear regression of change in CFSE low on change in FoxP3 in CD4+ T cells in HIV infected individuals showing a significant correlation between proliferation and Treg frequencies with Nef stimulation after 4 days of culture (p<0.016)

5.0 Discussion

5.1 *Ex vivo* regulatory T cell frequencies are markedly lower in patients with tuberculous disease than in normal controls and in patients with HIV infection.

In contrast to recent findings suggesting that elevated Treg frequencies may be responsible for the pathogenesis of symptomatic TB infection (Hougardy et al 2007, Guyot-Revel et al 2006) , this study showed significantly reduced levels of Tregs as a proportion of the $CD3^+$ $CD4^+$ T cells compared with uninfected controls. This may suggest that symptomatic infection in the patient population under investigation may be related to a failure to suppress an overactive immune response. Since the primary site of infection of the tubercle bacillus is the macrophage, it is possible that suppression of the immune response by the bacterium is counterproductive to its survival. Although Tregs appear to be increased in frequency in HIV infected individuals, this effect is largely ablated when these patients develop active TB disease. This *ex vivo* reduction in frequency was maintained with culture in the patients with tuberculosis showing that the proportion may be sustained with time. There was attrition in the frequencies of Tregs in HIV infected individuals with culture compared with frequencies *ex vivo*. This effect has been previously described although it is uncertain whether this represents effects of HIV proteins or susceptibility of these cells to culture.

5.2 Regulatory T cells are not anergic in culture

It has previously been suggested that naturally occurring Tregs are anergic (Hori, 2004) and that there is no response to stimulation in this population in the periphery. This is important in patients who have lost a high number of $CD4^+$ T cells as is the case in advanced HIV infection because it suggests that a functional thymus will be required to replenish the population. The stimulated Treg population (generated in the periphery) must therefore consist predominantly of IL-10 or TGF-β secreting cells. This study showed significantly higher frequencies of $CD4^+$ T cells expressing FoxP3 with anti-CD3 stimulation. With CFSE dye dilution, it was clear that this represents in part proliferation to this stimulus. In addition, there was significant proliferation of $CD4^+$ T cells expressing FoxP3 to tetanus in the uninfected control group.

5.3 Neither GITR nor CTLA4 are reliable markers for assessment of Tregs

CTLA-4 (Cytotoxic T-lymphocyte associated protein 4) has been associated with Treg populations (Ramsdell and Ziegler 2003, Thompson and Powrie 2004, Salomon et al 2000). This molecule is postulated to have a suppressive function accomplished by binding to the B7 antigens on the antigen-presenting cells, thus ablating the co-stimulatory pathways for T cell activation. Nevertheless, its function in the Treg population still remains controversial as monoclonal antibodies directed against this marker do not appear to impact the progression of autoimmune disease in mice (in this case NOD mice or non-obese diabetic mice – Salomon et al 2000). In addition, it does not

appear to inhibit $CD4^+$ CD25hi T cell suppressive function in humans' *in vivo* or *in vitro culture,* even though CTLA-4 polymorphisms have been associated with autoimmune disorders in humans.

Glucocorticoid-induced tumour-necrosis factor receptor-related protein or GITR has been linked to the regulatory activities of Tregs (Hisaeda et al 2005, Cardona et al 2006). A transfer of T cells, which are depleted for GITR, into mice lacking a thymus results in more aggressive autoimmunity than if only CD25 depleted T cells are transferred into these mice (Uraushihara et al 2003)

In this study, CTLA-4 and GITR were correlated with FoxP3 and CD25high expression. Both FoxP3 and CD25 have been used to define the regulatory population in mice and humans, but certain authors have suggested that FoxP3 may be upregulated in response to activation (Morgan et al 2005). An attempt was made to characterise these 2 markers, with established regulatory functions, on the cell populations in this study to attempt to establish a regulatory phenotype in the absence of depletion studies. Neither of these markers correlated consistently with FoxP3 expression in this study and could thus not be considered useful as a surrogate marker of this population. In this study, both molecules were assessed by flow cytometric analysis following the manufacturer's instructions for the monoclonal antibody staining. Because CTLA-4 and FoxP3 were assessed by intracellular staining, compensation was suboptimal and the data regarding this marker were treated with reserve. It is possible that it would be preferable to assess these molecules by quantitative reverse transcriptase PCR.

Other molecules are currently being explored as surface markers for Tregs. Tregs express the other 2 chains of the IL2 receptor, CD122 (IL2-R beta chain) and CD132 (IL2-R gamma chain). In addition they express the intercellular adhesion molecule, LFA3 or CD58. Recent studies have also demonstrated that downregulation of the IL-7α chain (CD127) may have utility in characterizing this population (Hartigan-O'Connor et al 2007).

5.4 Tregs can respond to specific stimuli

In HIV infected individuals, stimulation with an HIV specific peptide resulted in an increase in the frequency of FoxP3-expressing cells as a proportion of $CD3^+$ $CD4^+$ T cells. This may represent apoptosis in the non-Treg population rather than active proliferation of the Gag specific Tregs. Gag specific proliferation was not significantly different in the HIV infected population compared with either unstimulated cells or with uninfected individuals. It is also interesting to note that IFN-γ production in CD4 cells in uninfected individuals stimulated with Gag correlated strongly with the frequency of cells *ex vivo* in individuals with active TB disease compared with uninfected controls, no similar response was noted in response to stimulation with PPD, which contains mycobacterial antigens. It must be noted that the concentration of PPD was suboptimal and that at higher concentrations, a response might have been noted.

5.5 $CD4^+$ T cell proliferation correlates with interferon-gamma production in $CD4^+$ T cells

As expected, there was a significant correlation between CD4+ proliferation and IFNγ production by $CD4^+$ T cells in response to all stimuli in uninfected controls. A significant correlation was not present in individuals with active TB for any stimulation apart from with PPD. The response to HIV specific peptides within HIV infected individuals with and without active TB was broadly divergent from patient to patient. This probably suggests that there is no clear uniform pattern and that a larger sample size will be needed for greater clarification of the response.

5.6 HIV specific peptides exert an immunomodulatory role in Tregs with prolonged exposure

The frequency of $CD4^+$ T cells expressing FoxP3 in uninfected controls correlated positively with the response of these cells by IFNγ secretion at day 1 in response to Gag stimulation . In conjunction with the response of cells from an HIV infected individual to Gag at day 4, this suggests that this peptide may have a function in selecting out $CD4^+$ T cells which respond to this HIV peptide.

There was a highly significant correlation between FoxP3 expression after 4 days of culture and CD4 T cell proliferation in HIV patients in response to Nef stimulation. There was no significant proliferation of FoxP3 positive cells or increase in the proportion of cells which expressed FoxP3 in these individuals although it must also be noted that there

was a trend for Nef to reduce FoxP3 expression in uninfected controls. These data suggest that Nef may not influence FoxP3 expression directly as an immunosuppressive strategy although Nef stimulation also resulted in a significant negative correlation between IFNγ secretion by CD8+ T cells and the frequency of FoxP3 cells in uninfected controls. Some of the previously described immunological effects of Nef expression include CD4 molecule down-regulation and impaired thymocyte proliferation in vivo (Pennington et al 1997) and MHC class I and II down-regulation (Schindler et al, 2007; Noviello et al 2007) which are all associated with an abnormal immunological synapse formation. Polymorphisms in the Nef gene have been associated with differential progression of HIV infection to AIDS (Walker et al 2007). These results were obtained with a Nef peptide pool, rather than a complete Nef protein and it is unclear what effects would be obtained with the full protein structure.

6.0 Conclusion

Tregs have been described as a population of cells which regulate the adaptive immune response. Clearly, these cells will be of vital importance in diseases which affect the immune system as profoundly as HIV and tuberculosis. This study contains preliminary work using flow cytometric assays to assess the frequency of these cells in patients affected with these two diseases and attempts to answer further questions regarding the behaviour of this population in culture and under conditions of stimulation.

The data are subject to some important limitations. A small sample was investigated and thus generalization of these results to the patient population as a whole not feasible and requires confirmation in a larger study. In addition, controversy exists as to whether FoxP3 is the best marker to characterise human regulatory T cells. Certain studies (Morgan et al 2005, Allan et al 2007) suggest that effector T cells may upregulate FoxP3 transiently in response to activation. It is unclear whether the increased frequency of FoxP3-expressing cells in HIV-infected patients in this study represent T cells which are subject to chronic activation. Depletion studies using $CD25^{hi}$ to isolate regulatory T cells, followed by co-culture with $CD25^{lo}$ cells would have been useful to confirm that the cells expressing FoxP3 in this study had regulatory properties. The data does, nevertheless, support the findings of other groups that the Treg population is upregulated in HIV infected individuals. As the data regarding the role of Tregs in tuberculosis are more controversial, the meaning of the reduced frequency of FoxP3 expressing cells in this population of patients is uncertain. The data do show conclusively, however, that this

population is not anergic *in vitro* and this may indicate that FoxP3 expressing Tregs can be induced by specific stimuli *in vivo*.

Studies of proliferation of antigen-specific T cells and IFNγ production were performed to assess the function of FoxP3 expressing cells on other cells in culture – it has been suggested that FoxP3 may be an activation marker (Morgan et al 2005). These did not show consistent results although some observations including the significant negative correlation between FoxP3 expression and IFNγ production by CD8+ T cells in response to Nef stimulation suggest that there may have been a direct suppressive function from this population. Future studies using co-cultures of Tregs and antigen-specific cells from HIV infected individuals and individuals with active tuberculosis may be of value, particularly if it can be shown that Tregs can be isolated and cultured. These data question whether bright CD25 expression is a good surrogate marker for FoxP3 and it is still unclear how these cells would best be separated in a viable state. The data showing the correlation of CD4 proliferation after 4 days of culture with stimulation with Nef and the frequency of FoxP3 positive cells at this time point is interesting and the role of Nef in this population may be worth exploring further.

Future directions include mapping the pathways of the stimulants, use of other techniques to demonstrate the upregulation of FoxP3 in the population (in particular, reverse-transcription PCR) and exploration of other markers of this population. In addition, a longitudinal study to assess whether FoxP3 expression correlates with clinical outcome

may be of use in individuals with active tuberculosis or HIV infection. It is possible that Treg frequency may be an important clinical marker of progression or response to therapy but this will still need to be determined.

APPENDIX

Statistical results for experimental data

Table 4.1:

Treg frequencies following stimulation compared with unstimulated cells at day 4 in all four classes (ND= no statistical difference, I=increased compared with control, D=decreased compared with controls)

	Uninfected controls	**TB diseased subjects**	**HIV infected subjects**	**Co-infected subjects**
Anti-CD3 (0.1ug/ml)	I (p<0.011)	I (p<0.006)	I (p<0.001)	ND (p<0.148)
PPD (0.01ug/ml)	ND (p<0.138)	ND (p<0.154)	ND (p<0.123)	ND (p<0.194)
Tetanus toxoid (2 ug/ml)	ND (p<0.071)	ND (p<0.125)	ND (p<0.051)	ND (p<0.304)
Gag superpool (2ug/ml)	ND (p< 0.497)	ND (p<0.367)	I (p<0.05)	ND (p<0.406)
Nef superpool (2ug/ml)	ND (p<0.065)	ND (p<0.422)	ND (p<0.341)	ND (p<0.340)

Table 4.2

CFSE measured proliferation of Tregs to stimulation compared with no stimulation on day 4 (ND=no statistical difference, I=increased, D=decreased)

	Uninfected controls	**TB diseased subjects**	**HIV infected subjects**	**Co-infected subjects**
Anti-CD3 (0.1ug/ml)	I (p<0.020)	I (p<0.008)	I (p<0.002)	D(p<0.05)
PPD (0.01ug/ml)	ND (p<0.194)	ND (p<0.184)	ND (p<0.098)	ND (p<0.278)
Tetanus toxoid (2 ug/ml)	ND (p<0.372)	ND (p<0.474)	ND (p<0.258)	ND (p<0.427)
Gag superpool (2ug/ml)	ND (p<0.310)	ND (p<0.269)	ND (p<0.356)	ND (p<0.400)
Nef superpool (2ug/ml)	ND (p<0.215)	ND (p<0.269)	ND (p<0.404)	ND (p<0.384)

Table 4.3

Comparison of CFSE measured regulatory T cell proliferation in TB, HIV and coinfected subjects compared with uninfected controls (ND=no statistical difference, S=suppressed with respect to controls, I=increased with respect to controls

	TB diseased subjects	**HIV infected subjects**	**Co-infected subjects**
Unstimulated	ND (p<0.261)	ND (p<0.156)	ND (p<0.352)
Anti-CD3 (0.1ug/ml)	ND (p<0.325)	ND (p<0.105)	S (p<0.012)
PPD (0.01ug/ml)	ND (p<0.283)	ND (p<0.234)	ND (p<0.366)
Tetanus toxoid (2 ug/ml)	ND (p<0.357)	S (p<0.031)	ND (p<0.354)
Gag superpool (2ug/ml)	ND (p<0.232)	ND (p<0.251)	ND (p<0.372)
Nef superpool (2ug/ml)	ND (p<0.358)	ND (p<0.430)	ND (p<0.256)

Table 4.4

Correlation between $CD4^+$T cell proliferation and gamma interferon expression by $CD4^+$T cells

	Uninfected controls	**HIV infected subjects**	**TB diseased subjects**	**Coinfected subjects**
Anti-CD3	Correlation coefficient=0.767, (p<0.047)	Correlation coefficient=0.451, (p<0.671)	Correlation coefficient=-0.906, p<0.622	Correlation coefficient=-0.108, (p<0.9987)
PPD	Correlation coefficient=0.960, (p<0.000)	Correlation coefficient=0.678, (p<0.094)	Correlation coefficient=-0.999, (p<0.035)	Correlation coefficient=-0.989, (p<0.031)
Tetanus toxoid	Correlation coefficient=0.929, (p<0.003)	Correlation coefficient=0.631, (p<0.448)	Correlation coefficient=-0.136, p<0.999	Correlation coefficient=-0.220, (p<0.997)
Gag	Correlation coefficient=0.892, (p<0.042)	Correlation coefficient=0.213, (p<0.956)	Correlation coefficient=0.650, (p<0.909)	Correlation coefficient=0.621, (p<0.761)
Nef	Correlation coefficient=0.965, (p<0.001)	Correlation coefficient=0.405, (p<0.746)	Correlation coefficient=0.993, (p<0.07)	Correlation coefficient=0.734, (p<0.605)

Table 4.5

Proliferation of FoxP3$^+$ CD4$^+$ T cells correlated with interferon γ production by CD8$^+$ T cells

	Uninfected controls	**HIV infected subjects**	**TB infected subjects**	**Coinfected subjects**
Anti-CD3	Correlation coefficient=0.544, (p<0.342)	Correlation coefficient=-0.854, (p<0.030)	Correlation coefficient=-0.970, (p<0.365)	Correlation coefficient=-0.982, (p<0.094)
PPD	c=-0.178, p<0.956	c=-0.022, p<1.00	c=-0.985, p<0.460	c=-1, p<0.017
Tetanus	c=0.284, p<0.874	c=-0.032, p<1.00	c=-0.414, p<0.980	c=-1, p<0.09
Gag	c=-0.323, p<0.934	c=-0.393, p<0.825	c=0.972, p<0.386	c=-0.906, p<0.623
Nef	c=-0.881, p<0.026	c=-0.484, p<0.700	c=-0.403, p<0.982	c=-0.403, p<0.982

Table 4.6

FoxP3 frequency correlated against CFSE measured proliferation and secretion of interferon gamma by $CD4^+$ T cells

	Uninfected controls	HIV infected individuals	TB infected individuals	Coinfected individuals
Anti-CD3 (2ug/ml)				
FoxP3 frequency compared with CD4 proliferation	Correlation coefficient t=0.68, (p<0.872)	Correlation coefficient =252, p<0.995	Correlation coefficient=-0.895, (p<0.357)	Correlation coefficient= -0.744, p<0.852
FoxP3 frequency compared with Interferon secretion	Correlation coefficient =-0.259, (p<0.1128)	Correlation coefficient =0.252, (p<0.995)	Correlation coefficient =-0.969, (p<0.404)	Correlation coefficient=-0.529, p<0.852
PPD (0.01ug/ml)				
FoxP3 frequency compared with CD4 proliferation	Correlation coefficient= 0.400, (p<0.615)	Correlation coefficient = 0.021, (p=1)	Correlation coefficient =-0.972, (p<0.387)	Correlation coefficient =-0.760, (p<0.834)
FoxP3 frequency compared with Interferon secretion	Correlation coefficient =0.5, (p<0.444)	Correlation coefficient=0.021, (p=1)	Correlation coefficient =-0.185, (p<0.998)	Correlation coefficient=-0.784, (p<0.519)
Tetanus toxoid (2ug/ml)				
FoxP3 frequency compared with CD4 proliferation	Correlation coefficient =0.605, (p<0.232)	Correlation coefficient=0.500, (p<0.671)	Correlation coefficient=0.757, (p<0.838)	Correlation coefficient =0.124, (p<1)
FoxP3 frequency compared with Interferon secretion	Correlation coefficient=0.59, (p<0.260)	Correlation coefficient =0.149, (p<0.984)	Correlation coefficient=0.537, (p<0.988)	Correlation coefficient=0.231, (p<0.988)
Gag superpool (2ug/ml)				

FoxP3 frequency compared with CD4 proliferation	Correlation coefficient=0.576, (p<0.298)	Correlation coefficient=-0.256, (p<0.947)	Correlation coefficient=0.791, (p<0.804)	Correlation coefficient=-0.731, (p<0.858)
FoxP3 frequency compared with Interferon secretion	Correlation coefficient=0.921, (p<0.001)	Correlation coefficient=-0.173, (p<0.98)	Correlation coefficient=-0.186, (p<0.99)	Correlation coefficient=0.806, (p<0.476)
Nef superpool (2ug/ml)				
FoxP3 frequency compared with CD4 proliferation	Correlation coefficient=0.364, (p<0.707)	Correlation coefficient=0.896, (p<0.046)	Correlation coefficient=-0.473, (p<0.969)	Correlation coefficient=0.863, (p<0.709)
FoxP3 frequency compared with Interferon secretion	Correlation coefficient=0.421, (p<0.594)	Correlation coefficient =-0.274, (p<0.910)	Correlation coefficient=-0.981, (p<0.329)	Correlation coefficient=-0.151, (p<0.997)

REFERENCES

ONLINE REFERENCES

www.who.int/mediacentre/factsheets

www.unaids.org

http://www.bdbiosciences.com/pharmingen/protocols/Intracellular_Cytokine

1. Ahmadzadeh, M and Rosenberg S. *Il-2 administration increases CD4+CD25hiFoxP3+ regulatory T cells in cancer patients.* Blood. 2006 Mar 15;107(6):2409-14
2. Allan SE, Crome SQ, Crellin NK, Passerinin L, Steiner TS, Bacchetta R, Roncarolo MG and Levings MK. *Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production.* International Immunology 2007 Apr; 19 (4): 345-54
3. Baccheta, R, Gregori, S and Roncarolo, M. *CD4+ regulatory T cells: Mechanisms of induction and effector function.* Autoimmunity reviews 4:8 (2005) 491-496
4. Baecher-Allan, C, Wolf, E and Hafler, D. *Functional analysis of highly defined FACS-isolated populations of human regulatory CD4+ CD25+ T cells.* Clinical immunology 115:1 (2005) 10-18
5. Baecher-Allan, Viglietta, V and Hafler, D. *Human CD4+CD25+ regulatory T cells* Seminars in Immunology 16:2 (2004) 89-9
6. Bendelac, A, Savage, P and Teyton, L *The biology of NKT Cells.* Annual Reviews in Immunology (2007)
7. Beyersdorf, N, Hanke, T, Kerkau, T and Hunig, T. *CD28 superagonists but a break on autoimmunity by preferentially activating CD4+CD25+ regulatory T cells.* Autoimmunity Reviews 5:1 (2006) 40-45
8. Bluestone, J and Tang, Q. *How do CD4+CD25+ regulatory T cells control autoimmunity.*Current opinion in immunology 17:6 (2005) 638-642
9. Bosco N, Hung H, Pasqual N, Jouvin-Marcdhe E, Marche P, Gascoigne N and Ceredig R. *Role of the T cell receptor alpha chain in the development and phenotype of naturally arising CD4+CD25+ T cells* Molecular immunology 43:3 (2006) 246-254

10. Bynoe MS and Viret C. Foxp3+CD4+ T cell-mediated immunosuppression involves extracellular nucleotide catabolism Trends in Immunology (2008) Mar; 29(3) 199-102
11. Cardona, I, Goleva, E, Ou, L and Leung, D. *Staphylococcal enterotoxin B inhibits regulatory T cells by inducing glucocorticoid-induced TNG receptor-related protein ligand on monocytes* Journal of Allergy and Clinical Immunology (2006) Mar;117(3):688-95.
12. Chan, J and Flynn, J. *The immunological aspects of latency in Tuberculosis* Clinical Immunology (2004) 110 2-12
13. Chase AJ, Sedaghat AR, German JR, Gama L, Zink MC, Clements JE, Siliciano RF. *Severe depletion of CD4+ CD25+ regulatory T cells from the intestinal lamina propria but not peripheral blood or lymph nodes during acute simian immunodeficiency virus infection.* Journal of Virology. (2007) Dec;81(23):12748-57.
14. Chatila, T. *Role of regulatory T cells in human diseases.* Journal of allergy and clinical Immunology (2005) 116:5 949-959
15. Chen S, Ishii N, Ine S, Ikedam S, Fujimura T, Ndhlovu L, Soroosh P, Tada K, Harigae H, Kameoka, J, Kasai N, Sasaki T and Sugamura K. *Regulatory T cell-like activity of FoxP3+ adult T cell leukaemia cells.* International Immunology (2006) Feb;18(2):269-77.
16. Chen X, Zhou B, Li M, Deng Q, Wu X, Le X, Wu C, Larmonier N, Zhang W, Zhang H, Wang H, Katsanis E. *CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease*. Clinical Immunology. (2007) Apr;123(1):50-9. 17
17. Chen, Z, Herman, A, Matos, M, Mathis, D and Benoist, C. *Where CD4+CD25+ Treg cells impinge on autoimmune diabetes.* Journal of Experimental Medicine (2005) 21:202 1387-1397
18. Cooper AM, Dalton DK, Stewart TA, Griffen JP, Russell DG, Orme IM. *Disseminated tuberculosis in IFN-γ gene-disrupted mice*.. Journal of Experimental medicine (1993). 178: 2243–47
19. Cooper AM, Magram J, Ferrante J, Orme IM.. *Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with Mycobacterium tuberculosis.* Journal of Experimental Medicine (1997). 186:39–45
20. Cortesini, R and Suciu-Foca, N. *The concept of "partial" clinical tolerance.* Transplant Immunology (2004) 13:2 101-104

21. Crellin N, Garcia R, Hadisfar O, Allan S, Stenier T and Levings M. *Human CD4+ T cells express TLR5 and its Ligand Flagellin enhances the Suppressive Capacity and Expression of FoxP3 in CD4+CD25+ T regulatory cells.* Journal of Immunology (2005) 175 8051-8059

22. Duplan V, Beriou G, Heslan J, Bruand C, Dutartre P, Mars L, Liblau R, Cuturi M and Saoudi A. *LF15-0195 treatment protects against central nervous system autoimmunity by favoring the development of FoxP3-expressing regulatory CD4 T cells.* Journal of immunology (2006) 15:176 839-847

23. Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, Kityo C, Mugyenyi P, Cao H. *Depletion of regulatory T cells in HIV infection is associated with immune activation* Journal of Immunology (2005) Apr 1; 174 (7): 4407-14

24. Fan Y, Yang B and Wu C *Phenotypically and functionally distinct subsets of natural killer cells in human PBMCs* Cell Biology International (2008) Feb;32(2):188-97.

25. Fantini, M, Becker, C, Monteleone, G, Pallone, F, Galle, P and Neurath, M. *Cutting edge: TGF-β induces a regulatory phenotype in CD4+ CD25- T cells through FoxP3 induction and downregulation of Smad7.* Journal of Immunology 172 (2004) 5149-5153

26. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. 1993. *An essential role for interferon-γ in resistance to Mycobacterium tuberculosis infection.* Journal of Experimental Medicine 178:2249–54

27. Fujisima, M, Hirokawa, Fujisima, N and Sawada, K. *TCRαβ repertoire diversity of human naturally occurring CD4+CD25+ regulatory T cells.* Immunology Letters (2005) 99:2 193-197

28. Gartner, D, Hoff, H, Gimsa, U, Burmester, G, Brunner-Weinzierl, M. *CD25 regulatory T cells determine secondary but not primary remission in EAE: Impact on long-term disease progression.* Journal of Neuroimmunology (2006) Mar;172(1-2):73-84

29. Gavin, M and Rudensky, A. *Control of immune homeostasis by naturally arising regulatory CD4+ T cells* Current opinion in Immunology 15:6 (2003) 690-696

30. Goleva, E, Cardona, I, Ou, L and Leung, D. *Factors that regulate naturally occurring T regulatory cell-mediated suppression.* Journal of Allergy and Clinical Immunology 116:5 (2005) 1094-1100

31. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ.*Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism.*Journal of Immunology (2005) Feb 15;174(4):1783-6.
32. Good, M. *Identification of Early Cellular immune factors regulating growth of malaria parasites in humans.* Immunity (2005) 23:3 241-242
33. Gordon, S. *Pattern Recognition receptors. Doubling up for the innate immune response* Cell (2002) 111:7 927-930
34. Graca, L, Chen, T, Le Moine, A, Cobbold, S, Howie, D and Waldmann, H. *Dominant tolerance :activation thresholds for peripheral generation of regulatory T cells*. Trends in Immunology 26:3 (2005) 130-135
35. Grossman W, Verbsky J, Barchet W, Colonna M, Atkinson J and Ley T. *Human T regulatory cells can use the perforin pathway to cause autologous target cell death.* Immunity 21:4 (2004) 589-601
36. Gruenberg J and Stenmark H*: The biogenesis of multivesicular endosomes.* Nature Reviews Molecular Cell Biology (2004) 5:317-323.
37. Guyot-Revol, V, Innes, J, Hackforth, S, Hinks, T and Lalvani, A. *Regulatory T cells are expanded in blood and disease sites in Tuberculosis patients.* American Journal of Respiratory Critical Care Medicine 2006 Apr 1;173(7):803-10.
38. Hartigan-O'Connor DJ, Poon C, Sinclair E, McCune JM. Human CD4+ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells. Journal of Immunological Methods. (2007) Jan 30;319(1-2):41-52.
39. Hisaeda H, Hamano S, Mitoma-Obata C, Tetsutani K, Imai T, Waldmann H, Himeno K and Yasutomo K. *Resistance of regulatory T cells to glucocorticoid-inducted TNFR family-related protein (GITR) during Plasmodium yoelli infection.* European Journal of Immunology (2005) 35:12 3516 - 3524
40. Hoffmann, P and Edinger, M. *CD4+CD25+ regulatory T cells and Graft-versus-host disease.* Seminars in haematology (2006) 43:1 62-69
41. Hori, S and Sakaguchi, S. *FoxP3: a critical regulator of the development and function of regulatory T cells.* Microbes and Infection (2004) 6:8 745-751

42. Hori, S, Nomura, T and Sakaguchi, S. *Control of regulatory T cell development by the transcription factor FoxP3.* Science (2003) 299:5609 1057-1061

43. Houben E, Nguyen L and Pieters J. *Interaction of pathogenic mycobacteria with the host immune system.* Current Opinion in Microbiology (2006), 9:76–85

44. Hougardy JM, Place S, Hildebrand M, Drowart A, Debrie AS, Locht C, Mascart F. *Regulatory T cells depress immune responses to protective antigens in active tuberculosis.* American Journal of Respiratory Critical Care Medicine (2007) Aug 15;176(4):409-16.

45. Houyen EN, Nguyen L, Pieters J Interaction of pathogenic mycobacteria with the host immune system. Current Opinion in Microbiology (2006) Feb; 9 (1); 76-85

46. Hsieh, C, Liang, Y, Tyznik, A, Self, S, Liggitt, D and Rudensky, A. *Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors.* Immunity (2004) 21:2 267-277

47. Huehn, J and Hamann, A. *Homing to suppress: address codes for Treg migration.* Trends in Immunology (2004) 25:12 632-636

48. Ikezawa, Y, Nakazawa, M, Tamura, C, Takahashi, K, Minami, M and Ikezawa, Z. *Cyclophosphamide decreases the number, percentage and the function of CD25+ CD4+ regulatory T cells, which suppress induction of contact hypersensitivity*. Journal of Dermatological Sciences 39:2 (2005) 105 – 112

49. Jasmer, RM, Nahid P and Hopewell PC. *Latent Tuberculosis Infection* New England Journal of Medicine (2002) Dec; 347 1860

50. Karagiannidis C, Akdis M, Holopainen P, Woolley N, Hense G, Ruckert B, Mantel P, Menz G, Akdis C, Blaser K and Schmidt-Weber C. *Glucocorticoids upregulate FoxP3 expression and regulatory T cells in asthma.* Journal of Allergy and Clinical Immunology (2004) 114:6 1425-1433

51. Kasprowicz D, Smallwood S, Tyznik A and Ziegler S. *Scurfin (FoxP3) controls T-dependent immune response in vivo through regulation of $CD4^+$ T cell effector function.* Journal of Immunology (2003) 171 1216-1223

52. Kasprowicz, D, Droin, N, Soper, D, Ramsdell, F, Green, D and Ziegler, S. *Dynamic regulation of FoxP3 expression controls the balance between $CD4^+$ T cell activation and cell death.* European Journal of Immunology (2005) 35:12 3424 - 3432

53. Kauffman, S *Recent findings in immunology give tuberculosis vaccines a new boost* Trends in Immunology (2005) Dec; 26(12): 660-7

54. Kaufmann S *Tuberculosis: Back on the Immunologists' Agenda* Annual. Reviews. In Immunology (2004) 22:599–623

55. Kelsen J, Angholt J, Hoffman H, Romer J, Hvas C and Dahlerup J. *FoxP3$^+$CD4$^+$CD25$^+$ T cells with regulatory properties can be cultured from colonic mucosa of patients with Crohn's disease.* Clinical and Experimental Immunology (2005) 141 549-557

56. Khazaie, K and von Boehmer, H. *The impact of CD4$^+$ CD25$^+$ Treg on tumor specific CD8$^+$ T cell cytotoxicity and cancer.* Seminars in cancer biology (2006)16:2 124-136

57. Kronenberg M and Havran W. *Frontline T cells: gammadelta T cells and intraepithelial lymphocyte.* Immunology Reviews (2007) Feb 215:5-7

58. Kursar M, Koch M, Mittrücker HW, Nouailles G, Bonhagen K, Kamradt T, Kaufmann SH.Cutting Edge: *Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis.*Journal of Immunology (2007) Mar 1;178(5):2661-5.

59. Lanzavecchia, A and Sallusto, F. *Understanding the generation and function of memory T cell subsets.* Current opinion in Immunology (2005) 17:3 326-332

60. LeY, Yu X, Ruan L, Wang Qi D, Lu X, Kong Y, Cai K, Pang S, Shi X and Wang J. *The immunopharmacological properties of transforming growth factor beta.* International Immunopharmacology. (2005) 5:13-14 1771-1782

61. Lim H, Hillsamer P, Banham A and Kim C.H. *Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells.* Journal of Immunology (2005) Oct 1;175 (7) 4180-3

62. Lopez M, Aguilera R Perez X, Mendoza-Naranjo A, Pereda C, Ramirez M, Ferrada C, Aguillon J and Salazar-Onfray F. *The role of regulatory T lymphocytes in the induced immune response mediated by biological vaccines.* Immunobiology (2006) 211(1-2):127-36.

63. Maerten, P, Kwon, B, Shen, C, De Hertogh, G, Cadot, P, Bullens, D, Overbergh, L, Mathieu, C, Van Assche, G, Geboes, K, Rutgeerts, P and Ceuppens, J. *Involvement of 4-1BB (CD137)-4-1Bbligand interaction in the modulation of CD4+ T cell-mediated inflammatory colitis.* Clinical and Experimental Immunology 143 (2005) 228-236

64. Maggi, E, Cosmi, L, Liotta, F, Romagnani, S and Annunziato, F. *Thymic regulatory T cells.* Autoimmunity Reviews (2005) 4:8 579-586

65. Maker, A, Attia, P and Rosenberg, S. *Analysis of the cellular mechanism of antitumour responses and autoimmunity in patients treated with CTLA-4 blockade.* Journal of Immunology 1:175 (2005) 7746-7754

66. Morgan M, Van Bilesen J, Bakker A, Heemskerk B, Schilham M, Hartgers F, Elfrink B, Van der Zanden L, De Vries R, Huizinga T, Ottenhoff T and Toes R. *Expression of FoxP3 mRNA is not confined to CD4+25+ T regulatory cells in humans.* Human Immunology (2005) 66:1 13-20

67. Morgan ME, van Bilsen JH, Bakker AM, Heemskerk B, Schilham MW, Hartgers FC, Elferink BG, van der Zanden L, de Vries RR, Huizinga TW, Ottenhoff TH, Toes RE. *Expression of FoxP3 mRNA is not confined to CD4+ CD25 T regulatory cells in humans.* Human immunology (2005) Jan: 66(1): 13-20

68. Nixon D, Aandahl E and Michaelsson J. *$CD4^{+}CD25^{+}$ regulatory T cells in HIV infection.* Microbes and Infection 7:7-8 (2005) 1063-1065.

69. North, R.J. and Jung, Y *Immunity to tuberculosis* Trends in Immunology (2005) Dec 26:12

70. Noviello CM, Pond SL, Lewis MJ, Richman DD, Pillai SK, Yang OO, Little SJ, Smith DM, Guatelli JC. *Maintenance of Nef-mediated modulation of major histocompatibility complex class I and CD4 after sexual transmission of human immunodeficiency virus type 1*: Journal of Virology (2007) May;81(9):4776-86.

71. O'Garra, A. *Development and function of IL-10-secreting regulatory T cells: Comparison with naturally occurring CD4+CD25+ regulatory T cells.* International Congress Series (The Innate System: Strategies for Disease Control. Proceedings of the Uehara Memorial Foundation Symposium on the Innate Immune System: Strategies for Disease Control, *T*okyo) 1285 (2005) 160-168

72. Oswald-Richter K, Grill SM, Shariat N, Leelawong M, Sundrud MS, Haas DW, Unutmaz D. *HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells.*PLoS Biol. (2004)Jul;2(7):E198.

73. Pennington DJ, Jenkins SA, Brady HJ, Miles CG, Dzierzak EA, Abraham DJ. *HIV- I Nef severely impairs thymocyte development and peripheral T-cell function by a CD4-independent mechanism.*Genes and Function. (1997) Dec;1(5-6):321-35

74. Perrone G, Ruffini PA, Catalano V, Spino C, Santini D, Muretto P, Spoto C, Zingaretti C, Sisti V, Alessandroni P, Giordani P, Cicetti A, D'Emidio S, Morini S, Ruzzo A, Magnani M, Tonini G, Rabitti C, Graziano F. *Intratumoural FOXP3-positive regulatory T cells are associated with adverse prognosis in radically resected gastric cancer* Eur J Cancer. (2008) Sep;44(13):1875-82.

75. Pillai, V, Baughman, E and Karandikar *F.22. Suppressive Property of Activated FOXP3+ T Cells Revealed by a Novel Suppression Assay* Clinical Immunology, Volume 127, Supplement 1, 2008, Page S50

76. Ramsdell, F and Ziegler, S. *Transcription factors in autoimmunity*. Current opinion in Immunology (2003) 15:6 718-724

77. Rieger K, Loddenkemper C, Maul J, Fietz T, Wolff D, Terpe H, Stenier B, Berg E, Miehlke S, Bornhauser M, Schneider T, Zeitz M, Stein H, Duchmann T and Uharek L. *Mucosal FoxP3 regulatory T cells are numerically deficient in acute and chronic GvHD.* Blood 2006 Feb 15;107(4):1717-23.

78. Roberts T, Beyers N, Aguirre A, Walzl G. *Immunosuppression during active tuberculosis is characterized by decreased interferon- gamma production and CD25 expression with elevated forkhead box P3, transforming growth factor- beta , and interleukin-4 mRNA levels*.Journal of Infectious Disease (2007) Mar 15;195(6):870-8.

79. Romagnani, S. *Introduction: characterisation and functions of human T regulatory cells.* Microbes and Infection (2005) 7:7-8 1015-1016

80. Sakaguchi, S. *Regulatory T cells: Key controllers of Immunologic Self-tolerance*. Cell (2000) 101:5 455-458

81. Salomon, B, Lenschow, D, Rhee, L, Ashourian, N, Singh, B, Sharpe, A and Bluestone, J. *B7/CD28 Costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control Autoimmune diabetes.* Immunity (2000) 12:4 431-440

82. Sato K, Tateishi S, Kubo K, Mimura T, Yamamoto K and Kanda H. *Downregulation of IL-12 and a novel negative feedback system mediated by $CD25^{+}CD4^{+}$ T cells.* Biochemical and Biophysical Research Communications (2005) 220:1 226-232

83. Schindler M, Wildum S, Casartelli N, Doria M, *Kirchhoff F Nef alleles from children with non-progressive HIV-1 infection modulate MHC-II expression more efficiently than those from rapid progressors.* AIDS (2007) May 31;21(9):1103-7.

84. Scott-Browne JP, Shafiani, S, Tucker-Heard G, Ishida-Tsubota K, Fontenot JD, Rudensky A, Bevan MJ, Urdahl, K *Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis.* Journal of Experimental Medicine (2007) Sep 3; 204(9): 2159-69

85. Serra P, Amrani A, Yamanouchi J, Han B, Thiessen S, Utsugi T, Verdaguer J and Santamaria P. *CD40 ligation releases immature dendritic cells from the control of regulatory CD4+CD25+ T cells.* Immunity (2003) 19:6 877-889

86. Shapiro S and Wilk M *An analysis of variance test for normality(complete samples)* Biometrika (1965) 52:591-611

87. Stenger, S and Modlin, R. *Control of Mycobacterium tuberculosis through mammalian Toll-like receptors* Current opinion in immunology (2002) 14: 452-457

88. Taams, L, Van Amelsfort, J, Tiemessen, M, Jacobs, K, De Jong, E, Akbar, A, Bijlsma, J and Lafeber, F. *Modulation of monocyte/macrophage function human CD4+CD25+ regulatory T cells.* Human Immunology (2005) 66:3 222-230

89. Thompson CR, Iyer SS, Melrose N, Van Oosten R, Johnson K, Pitson SM, Obeid LM, Kusner DJ: *Sphingosine kinase 1 (SK1) is recruited to nascent phagosomes in human macrophages: inhibition of SK1 translocation by Mycobacterium tuberculosis.* Journal of Immunology (2005) 174:3551-3561.

90. Thompson, C and Powrie, F. *Regulatory T cells.* Current opinion in Pharmacology (2004) 4:4 298-414.

91. Till, S, Francis, J, Nouri-Aria, K and Durham, S. *Mechanisms of immunotherapy.* Journal of Allergy and Clinical Immunology (2004) 113:6 1025-1034

92. Tonkin D, He J, Barbour G and Haskins K. *Regulatory T cells prevent transfer of type 1 diabetes in NOD mice only when their antigen is present in vivo.* Journal of Immunology (2008) Oct 1; 181 (7): 4516-22

93. Uraushihara K, Kanai T, Ko K, Totsuka T, Makita S, Iiyama R, Nakamura T, Watanabe M. *Regulation of murine inflammatory bowel disease by $CD25^+$ and $CD25^-$ $CD4^+$ glucocorticoid-induced TNF receptor family-related gene+ regulatory T cells*.Journal of Immunology (2003) Jul 15;171(2):708-16.

94. Vieira, P, Christensen, J, Minaee, S, O'Neill, E, Barrats, F, Boonstra, A, Barthlott, T, Stockinger, B, Wraith, D and O'Garra, A. *IL-10-secreting regulatory T cell do not express FoxP3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells.* Journal of Immunology 172 (2004) 5986-5993

95. Vigoroux S, Yvon E, Wagner H, BiagE, Dotti G, Silli U, Lira C, Rooney C and Brenner M. *Induction of antigen-specific regulatory T cells following overexpression of a Notch ligand by human B lymphocytes.* Journal of Virology (2003) 77:20 10872-10880

96. Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, Huygen K, Klebl B, Thompson C, Bacher G, Pieters J.*Protein kinase G from pathogenic mycobacteria promotes survival within macrophages.*Science (2004) Jun 18;304(5678):1800-4

97. Walker PR, Ketunuti M, Choge IA, Meyers T, Gray G, Holmes EC, Morris L.*Polymorphisms in Nef associated with different clinical outcomes in HIV type 1 subtype C-infected children.* AIDS Research and Human Retroviruses. 2007 Feb;23(2):204-15.

98. Walther M, Tongren J, Andrews L, Korbel D, King E, Fletcher H, Andersen R, Bejon P, Thompson F, Dunachie S, Edele F, De Souza J, Sinden R, Gilbert S, Riley E and Hill A. *Upregulation of TGFβ, FoxP3 and CD4$^+$CD25$^+$ regulatory T cells correlates with more rapid parasite growth in human malaria infection.* Immunity (2005) 23:3 287-296

99. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. *CTLA-4 control over Poxp3+ regulatory T cell function.* Science (2008) Oct 01; 322 (5899): 271-5

100. Winkler B, Hufnagl K, Spittler A, Ploder M, Kállay E, Vrtala S, Valenta R, Kundi M, Renz H, Wiedermann U. *The role of Foxp3+ T cells in long-term efficacy of prophylactic and therapeutic mucosal tolerance induced in mice* Allergy. 2006 Feb;61(2):173-80.

101. Wolf D, Wolf A, Rumpold H, Fiegl H, Zeimet A, Muller-Holzner E, Deibl M, Gastl G, Gunsilius E and Marth C. *The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer.* Clinical Cancer Research (2005) 1:11 8326-8231

102. Yang, Z, Novak, A, Stenson, M, Witzig, T and Ansell, S. *Intratumoral CD4+CD25+ regulatory T-cell-mediated suppression of infiltrating CD4+T-cells in C-cell non-Hodgkin lymphoma.* Blood 2006 May 1;107(9):3639-46.

103. Yong Z, Chang L, Mei YX, Yi L.*Role and mechanisms of $CD4^{+}CD25^{+}$ regulatory T cells in the induction and maintenance of transplantation tolerance*.Transplant Immunology (2007) Feb;17(2):120-9.