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Characterisation of regulatory T cells in HIV-infected adults in South Africa

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1. INTRODUCTION

1.1 Regulatory T cells

Regulatory T cells (Tregs) are $CD4^+$ T lymphocytes that suppress other cells of the immune system such as $CD4^+$ and $CD8^+$ T lymphocytes. In 1995 Sakaguchi *et al*. demonstrated that these cells expressed the surface markers CD4 and CD25 (the α subunit of the interleukin-2 receptor) allowing them to be studied flow cytometrically and by adoptive transfer experiments. It has recently been shown that the gene responsible for suppressor activity in these Tregs is *Foxp3* (forkhead box transcription factor P3, written "Foxp3" in animals and "FOXP3" in humans) (Bennett *et al*., 2001; Brunkow *et al.*, 2001; Wilden *et al*., 2001; reviewed by Ziegler, S.F., 2006)

Tregs exert their suppressor function primarily by cell-cell contact mechanisms, although they may also secrete suppressive cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). They are thought to develop in the thymus and are thus referred to as "naturally occurring Tregs" in order to differentiate them from other suppressor subsets that develop in the periphery and are not FOXP3 dependent such as Tr1 (T-regulatory 1) cells which act primarily via IL-10 (Interleukin 10) secretion and Th3 (T helper 3) cells which act primarily via TGF-β secretion (reviewed by Goleva *et al.*, 2005; Taylor *et al.*, 2005).

Cell surface molecules postulated to be involved in Treg function include CTLA-4 (cytotoxic T lymphocyte-associated antigen-4) and GITR (glucocorticoid-induced TNF receptor). There are numerous other postulated markers which may be helpful such as CD44 (Firan *et al.*, 2006) absence of CD127 (Legrand *et al.*, 2006; Liu *et al.*, 2006) and CD62L (Eggena *et al.*, 2005). Tregs are generally thought to be of memory

phenotype expressing CD45RO (Sereti *et al.*, 2005; reviewed by Weiss *et al.*, 2004; Levy, 2006) although there have been reports of naïve CD45RA Tregs (Valmori *et al.*, 2005).

In order to act, Tregs need to be stimulated in an antigen specific way via their T cell receptor, although their suppressive effector effects are antigen non-specific, that is Tregs suppress bystander $CD4^+$ and $CD8^+$ T cells (reviewed by Oswald-Richter *et al.*, 2004). The precise mechanism(s) of action of Tregs is yet to be elucidated. Tregs may act directly on effector cells through binding of CTLA-4 to B7 molecules (CD80 and CD86) on effectors. Alternatively, Tregs may induce increased expression of the tryptophan-catabolizing enzyme indoleamine deoxygenase (IDO) in antigen presenting cells (reviewed by Goleva *et al.*, 2005; Nilsson *et al.*, 2006). Tregs may also exert their suppressor function by down-modulating costimulatory signals such as CD80 and CD86 on dendritic cells (reviewed by Kornbluth, 2006).

Tregs have been shown to be involved in suppressing autoimmune disease and in inducing transplantation tolerance (Green, 2002; Chatila, 2005; Liu *et al.*, 2005; Miyara *et al.*, 2005). They have, however, also been shown to suppress the immune response to tumours, allowing greater risk of metastasis (Liyanage *et al.*, 2002; Ormandy *et al.*, 2005; Wolf *et al.*, 2005; Beyer *et al.*, 2006). The role of Tregs in infectious disease seems to be double-sided. Tregs seem to be responsible for dampening cytotoxic T cell responses to infection. This has various effects. On the one hand it may allow pathogen survival. Alternatively, it prevents excessive tissue destruction. For example, in hepatitis C disease, chronic infection is strongly associated with the presence of virus-specific Tregs (MacDonald *et al*., 2002). Suvas

et al. (2003) showed that depleting mice of Tregs (using anti-CD25 antibodies) prior to infection with Herpes Simplex Virus (HSV) resulted in an enhanced $CD8^+$ T cell response.

1.2FOXP3

FOXP3 mutations leading to loss of function of FOXP3 suppressor activity cause a disease in humans known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in which patients suffer both from autoimmune manifestations (type 1 diabetes, thyroiditis, inflammatory bowel disesase, atopic dermatitis, food allergies) as well as from overwhelming infection and usually die before the age of 2 years (Chatila *et al.*, 2000; Wildin *et al.*, 2001; Bennett *et al.*, 2001; Owen *et al*., 2003). IPEX corresponds to a similar disease in mice known as scurfy and FOXP3 was originally called scurfin (Bennet *et al.*, 2001; Brunkow *et al.*, 2001; Wildin *et al.*, 2001)

FOXP3 is a transcriptional repressor which associates with NFAT and NFκB and decreases expression of their target genes (Bettelli *et al.*, 2005). FOXP3 exists as two isoforms in humans, one of which lacks the second coding exon (FOXP3Δ2). Both isoforms appear to be uniformly expressed and both act as transcriptional repressors, decreasing expression of multiple genes including interleukin 2. The two isoforms expressed concurrently appear to have additive function, increasing expression of Treg associated markers (CD25, CTLA-4, GITR), decreasing cytokine expression and decreasing proliferation of responder cells (Allan *et al.*, 2005).

1.3Tregs in HIV

With regards to HIV, new findings on Tregs have excited interest, as it is known that progression to AIDS is correlated with excessive immune activation. Tregs are $CD4^+$ T cells and can therefore be infected with the HIV virus (Oswald-Richter *et al*, 2004). It is plausible that preferential destruction or inactivation of Tregs over other subsets of T cells by HIV could lead to excessive immune activation. This has been suggested by Oswald-Richter *et al*. (2004) and Apoil *et al.* (2005) who showed that HIV positive subjects had significantly lower expression of FOXP3 mRNA than HIV negative subjects. Kinter *et al*. (2004) have also asserted that individuals with strong HIV-specific Treg function *in vitro* had lower levels of viraemia and higher $CD4^+CD8^+$ T cell ratios and that HIV-specific Treg function may be compromised in certain individuals before $CD25^-$ HIV-specific $CD4^+$ T cell responses. In 2007 Kinter *et al.* found that Treg mediated suppression of HIV specific responses *in vitro* was more effective with cells isolated from relatively healthy HIV-infected patients compared with later stage AIDS patients, suggesting that Tregs (total or HIV-specific) were depleted or dysfunctional later in HIV disease.

It has also been suggested by some authors that preferential preservation of Tregs over other subsets of T cells could lead to suppression of immune responses to viral infections, leading to a high viral load (Aandahl *et al*., 2004; Weiss *et al.*, 2004). Montes *et al.* (2006) found increased Tregs in HIV positive patients, particularly in those with low $CD4^+$ T cell counts (CD4 counts). Nilsson *et al.* (2006) found high levels of Tregs in tonsillar biopsies from HIV progressors but normal levels in non-progressors.

It is uncertain whether Treg activity in the blood correlates with Treg activity in the tissues, for example the lymph nodes. Andersson *et al.* (2005) found Tregs at high levels in tonsils of HIV infected individuals but low levels of FOXP3 mRNA in the peripheral blood. The high level of Tregs in the tonsils corresponded with a high viral load. Estes *et al.* (2006) showed in macaques that the frequency of FOXP3 positive Tregs increased in lymph nodes as early as 7 days after SIV infection with a dramatic increase by 28 days and postulate that this may limit SIV-specific immune responses. Kornfeld *et al.* (2005) however also found increased Treg levels in non-pathogenic SIV infection in African Green monkeys and suggest that this anti-inflammatory response occurs earlier than in pathogenic SIV infection, limiting aberrant immune activation.

It has been shown using macaque models that massive $CD4^+$ T cell depletion occurs in the gut mucosa within the first few weeks of primary infection. (Veazey R.S and Lackner, A.A., 2005). Epple *et al.* (2006) found accumulation of $FOXP3^+$ Tregs immunohistochemically in the duodenal mucosa of HIV-infected patients (unlike in patients with other viral infections such as norovirus infection) despite depletion of total gut $CD4^+$ T cells. Tregs were increased in absolute number as well as in frequency, implying that the increased proportion of Tregs was not just due to depletion of non-Treg $CD4^+$ T cells. Treg percentages (expressed as a percentage of $CD4^+$ T cells) in the blood however were only slightly increased and absolute values were within the normal range. In patients on HAART, mucosal Tregs returned to the level seen in controls.

Nilsson *et al.* (2006) compared Tregs in lymphoid tissue from HIV progressors with those of non-progressors. Progressors had 5-fold higher FOXP3 levels than non-progressors, seemingly due to increased Treg survival. This supports the hypothesis that increased Treg levels may be a contributing factor to the immune suppression and disease susceptibility seen in AIDS patients. The causative role of Tregs in disease pathogenesis remains unproven however, as elevated Tregs may also be simply a response to immune activation driven by HIV or intercurrent infectious agents.

1.4 Technical and analytical issues

Analytic difficulties in determining Treg numbers in humans have been encountered due to the absence of an ideal Treg marker. CD25 is known to be an activation marker on effector T cells and is not a specific marker of T regulatory cells. Neither CTLA-4 nor GITR expression is confined to Tregs. Even the applicability of FOXP3 has been questioned, as it has been shown that in humans FOXP3 can be induced in $CD25^-$ $FOXP3^-$ cells by stimulation of cells through the T cell receptor, for example using anti-CD3 monoclonal antibody stimulation. Whether these new $FOXP3^+$ cells acquire suppressive function and act as "induced" Tregs is still unclear (Walker *et al.* 2003; Allan *et al.*, 2005; Walker *et al.*, 2005; Ziegler, 2005). The level of FOXP3 induced also seems to be lower than that of natural Tregs (Allan *et al.*, 2005).

Certain discrepant results may be explained by the use of different markers to identify Tregs. CD25 does not give clear positive and negative cell populations in the human as it does in the mouse and different investigators have used different definitions of Tregs (involving $CD25^+$ or $CD25^{++}$), many not including flow cytometric analysis of FOXP3 in their definition. CD25 may also vary in different groups of patients, for

example Nilsson *et al.* (2006) report co-expression of CD25 on FOXP3 positive cells varies from 19% in HIV progressors to 75% in uninfected donors. They also showed in an *in vitro* model that while most HIV exposed $FOXP3^+$ cells expressed CTLA-4 (75%) and CD25 (71%), most did not express GITR (43%), suggesting that these molecules are differently regulated.

In the analysis of FOXP3 mRNA by quantitative reverse-transcriptase PCR there are also technical issues that may result in discrepant results between studies. Choice of a suitable reference gene as an internal control is crucial to avoid biasing data. Many of the commonly used reference genes may in fact be more variable than previously thought, and may vary with cell type and activation state. Apoil *et al.* (2005) obtained different results using TATA box binding protein 1 as a reference gene compared with CD3γ or β-actin. Dheda *et al.* (2005) have recently shown that the commonly used genes β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) are more variable (7.5-fold and 3.5-fold, respectively) than human acidic ribosomal protein (which varied 3-fold) and can give false results. Bas *et al.* (2004) also showed that β-actin and GAPDH were more variable than 18sRNA as a reference gene.

Due to the unavailability of an ideal Treg marker, Treg studies should include more than one modality of Treg measurement as well as functional assays. This manuscript will report on the immunophenotypic analysis of Treg percentages as well as the molecular analysis of FOXP3 mRNA expression using two reference genes (GAPDH and 18sRNA). Functional studies including measurement of gamma interferon production and cell proliferation using carboxyfluorescein succinimidyl ester dye

(CFSE) were conducted on the same samples by a different member of the group and will be reported separately.

1.5 Aims and Objectives

It is critical to the understanding of HIV pathogenesis, as well as for rational vaccine design, to understand more about this suppressive arm of the immune system. Much work is therefore needed to elucidate the role of Tregs in HIV, both in susceptibility to disease as well as in rate of progression to terminal AIDS.

The effect of HIV on Treg proportions is likely to be a fundamental factor in HIV disease pathogenesis and progression. This study aimed to investigate Treg levels in the blood of treatment-naïve HIV-infected patients using the best available methods and to compare these with levels of Tregs in healthy controls. We hypothesized that there would be a significant difference in Treg levels between these two groups.

We also aimed to correlate Treg levels with markers of disease progression such as $CD4^+$ T cell count and therefore included patients with a wide range of CD4 counts. Viral loads were not assessed for the purposes of this study and will form part of future analysis of these samples.

As part of the study we assessed the utility and agreement of two techniques in the measurement of Tregs - intracellular cytokine staining for FOXP3 protein and RT-PCR for *FOXP3* mRNA. We also assessed the utility of the fluorescent monoclonal antibodies anti-CD25, anti-GITR and anti-CTLA-4 for Treg analysis.

The findings from this research are particularly relevant in a field where many previous studies have used outdated methods and shown conflicting results. The findings also provide important information on the levels of Tregs in healthy South Africans as well as in subtype C infected patients. The data demonstrate elevated Treg levels in HIV positive patients, particularly those with low CD4 counts, a sample group that has often been neglected in previous studies. Additionally, the analytical insights gleaned give direction for future monitoring of Treg levels in a diagnostic setting.

2. Materials and Methods

2.1 Sample collection

HIV seropositive patients were recruited from the antiretroviral clinics of the New Johannesburg hospital and Helen Joseph Hospital prior to their commencing antiretroviral therapy. HIV subtyping was not performed. Most subjects were expected to be harbouring HIV subtype C, as other subtypes are rare in this setting. Controls were selected from amongst health care workers from various institutions and blood donors from the South African National Blood Transfusion Service. All subjects gave informed consent for the study. Ethics approval was granted by the University of the Witwatersrand Human Medical Ethics Committee as well the Ethics Committee of the South African National Blood Transfusion Service. The day of sample collection was regarded as Day 0.

The HIV positive sample group comprised 17 Black patients between age-group 2 (20-29 years) and age-group 4 (40-49 years) (actual ages were not recorded according to ethics committee wishes in order to protect anonymity, particularly of controls). Thirteen participants were female and four were male. CD4 counts of the HIV positive subjects ranged from 17 cells/μl to 712 cells/μl (median 223 cells/μl).

Twenty-two healthy controls were recruited from amongst healthcare workers and blood donors. Ten were male, twelve female, eleven Black, eight White, two Coloured and one Indian. Their age groups ranged from 1 (10-19 years) to 6 (60-69

years). CD4 counts of controls ranged from 539 to 1503 cells/μl (median 873 cells/μl). For demographics please see Appendix A.

2.2 HIV tests and CD4 counts

The HIV status of controls was confirmed using HIV rapid testing (*Determine HIV-1/2*, Abbott Laboratories, Abbot Park, Il 60064,USA). CD4 counting was performed at the Department of Molecular Medicine and Haematology, National Health Laboratory Services, using the PanLeucogating method (Glencross *et al.*, 2002).

2.3 Isolation of Peripheral Blood Mononuclear cells

Peripheral blood mononuclear cells were isolated within 3-5 hours of sample collection using Ficoll Hypaque in LeucoSep tubes. Cells were washed twice in Hanks Buffered Salt Solution with 0.1% gentamycin and counted using a Guava cytometer. Cells (2×10^6 cells/ml) were then rested overnight at 37˚C in a 5% CO_2 atmosphere in RPMI 1640 medium with GlutaMAX and 25mM HEPES (Gibco, Scotland) supplemented with 20% fetal bovine serum (Gemini Bio-Products, USA) and 0.1% gentamycin.

2.4 RNA extraction and RT-PCR

Total RNA was extracted from one million peripheral blood mononuclear cells per sample using QIAamp RNA mini kit (QIAGEN, Germany). The mRNA was immediately converted to cDNA using the Applied Biosystems High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). A blank tube was treated similarly with each run and served as a negative control.

cDNA was frozen at –20ºC until needed. Multiplexed real-time reverse transcriptase PCR was then performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA.) on the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). FoxP3 expression was assessed using a fluorescently labelled probe (TaqMan Gene expression assays, probe Hs00203958_m1, Applied Biosystems, Foster City, CA) and compared with that of constitutively expressed housekeeping genes. Endogenous control probes used were 18sRNA and GAPDH (Pre-Developed TaqMan Assay Reagents Control Kit, Applied Biosystems Foster City, CA). All PCR reactions were run in triplicate in the presence of a blank control tube.

2.5 Intracellular cytokine staining of unstimulated cells

After sample collection, isolated PBMCs were rested in RPMI with 20% foetal calf serum overnight (Day 0) and stained the following day (Day 1) for flow cytometric analysis. Fluorochromes used included CD3 APC; CD3 PerCP; CD4 PerCP; CD4 FITC; CD25 APC; (BD Biosciences, San Jose, CA), CTLA-4 FITC; GITR APC (R&D Systems, Minneapolis) and FoxP3 PE (eBiosciences, UK).

Samples were stained using the FOXP3 staining set (eBiosciences, UK) according to the manufacturer's instructions. Briefly, surface stains (CD3, CD4, CD25 and GITR) were added and cells were incubated in the dark for 15 minutes, spun and washed with commercial wash buffer (FOXP3 staining set, eBiosciences, UK). They were then fixed and permeabilised using commercial buffers (FOXP3 staining set, eBiosciences, UK). Cells were then incubated with intracellular stains (FOXP3 and CTLA-4) for 30 minutes at 4˚C after which cells were washed again in

permeabilisation buffer and resuspended in fixing solution (Cell Fix, BD Biosciences, San Jose, CA.).

Initial titration of fluorescently labelled antibodies was conducted on control cells in order to determine optimum staining volumes (data not shown). Propidium iodide staining was used to determine efficacy of cell permeabilisation (see Appendix B). Isotype controls were not used. Fluorescence minus one colour experiments were performed initially (that is three colours added without FOXP3) to confirm accuracy of FOXP3 gating strategy (see Appendix C).

Cells were acquired using the FACScalibur flow cytometer (BD Biosciences, San Jose, CA.) and CellQuest Pro software or the BD LSR II flow cytometer (BD Biosciences, San Jose, CA.) and FACSDiva Software. Analysis, including digital compensation, was performed using FloJo software (FloJo 6.4.2 TreeStar, USA). At least 50 000 and up to 1 000 000 events were recorded per sample.

2.6 Cell stimulation and culture

After isolation, a portion of the peripheral blood mononuclear cells (2×10^6 cells) were added to each well of a 24-well culture plate (Nunc, Denmark). RPMI 1640 medium $^+$ GlutaMAX $^+$ 25mM HEPES (Gibco, Scotland) supplemented with 10% human serum AB (Gemini Bio-Products, USA) and 0.1% gentamycin (R10) was added such that the total volume in the well was 2 ml. Cells were stimulated with 0.1 ug/ml anti-CD3 monoclonal antibody (12F6). Plates were incubated at 37° in a 5% CO_2 atmosphere for 4 days. Cells were harvested on day 4, washed in RPMI 1640 medium $^+$

GlutaMAX $^{+}$ 25mM HEPES (Gibco, Scotland) supplemented with 10% human serum AB (Gemini Bio-Products, USA) and 0.1% gentamycin (R10) and stained as above.

2.7 Statistical Analysis

2.7.1 Statistical tools

Statistics were calculated using Microsoft Excel, SAS Enterprise Guide 3.0, SPSS version 14 and GraphPad Prism version 4.0. Groups were compared using non-parametric one-way ANOVA and Dunn's multiple comparison test for post-hoc analyses. Correlations were calculated using Spearman's correlation coefficient. Significance was chosen as the 5% level.

2.7.2 Exclusions

Not all samples were available for analysis of all parameters, depending on CD4 count and numbers of PBMCs available for culture.

For PCR data triplicate measurements were averaged prior to analysis. Outliers (RQ values above 10) were excluded and remaining samples averaged. 3 samples (2 for GAPDH and a different sample for 18sRNA) were deemed to be erroneous outliers and disregarded during analysis.

3. RESULTS

3.1. Tregs are elevated in HIV positive patients compared with controls

Treg percentage was measured in the peripheral blood of 22 healthy controls and 17 HIV-infected patients in order to determine if levels of circulating Tregs differ between these two groups. Peripheral blood mononuclear cells (PBMCs) were isolated and cells were rested unstimulated overnight. The cultures were unstimulated in order to better reflect *in vivo* Treg levels. The next day (day 1) Tregs were assessed flow cytometrically by intracellular cytokine staining using labelled monoclonal antibodies to CD3, CD4, FOXP3, CD25, CTLA-4 and GITR (see methods section for more detail). All flow cytometric panels were run in triplicate to ensure reliability and results were averaged. Treg percentages were determined as percentage $FOXP3^+$ of $CD3^+CD4^+$ cells.

After PBMC processing there was sufficient material for analysis on 16 patients and 19 controls. Treg percentage was found to be significantly elevated in the HIV positive group (median 6.8% range 2.4-26.3% n=16) compared with control (median 3.72% range 1.3-7.5%, n=19, P=0.0031) (See Figure 3.1). Mean coefficient of variation of triplicate FOXP3 measurements was 15.14%.

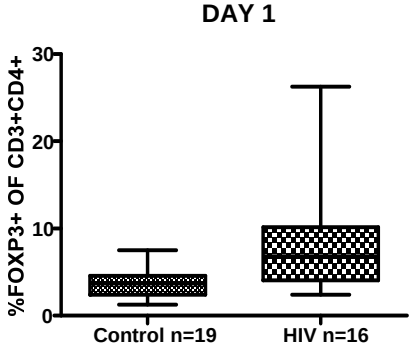


Figure 3.1: Unstimulated FoxP3 expression in the HIV and control groups.

Day 1Tregs (%$FOXP3^+$ of $CD3^+CD4^+$, averaged from triplicates) are significantly higher in the HIV group than controls.

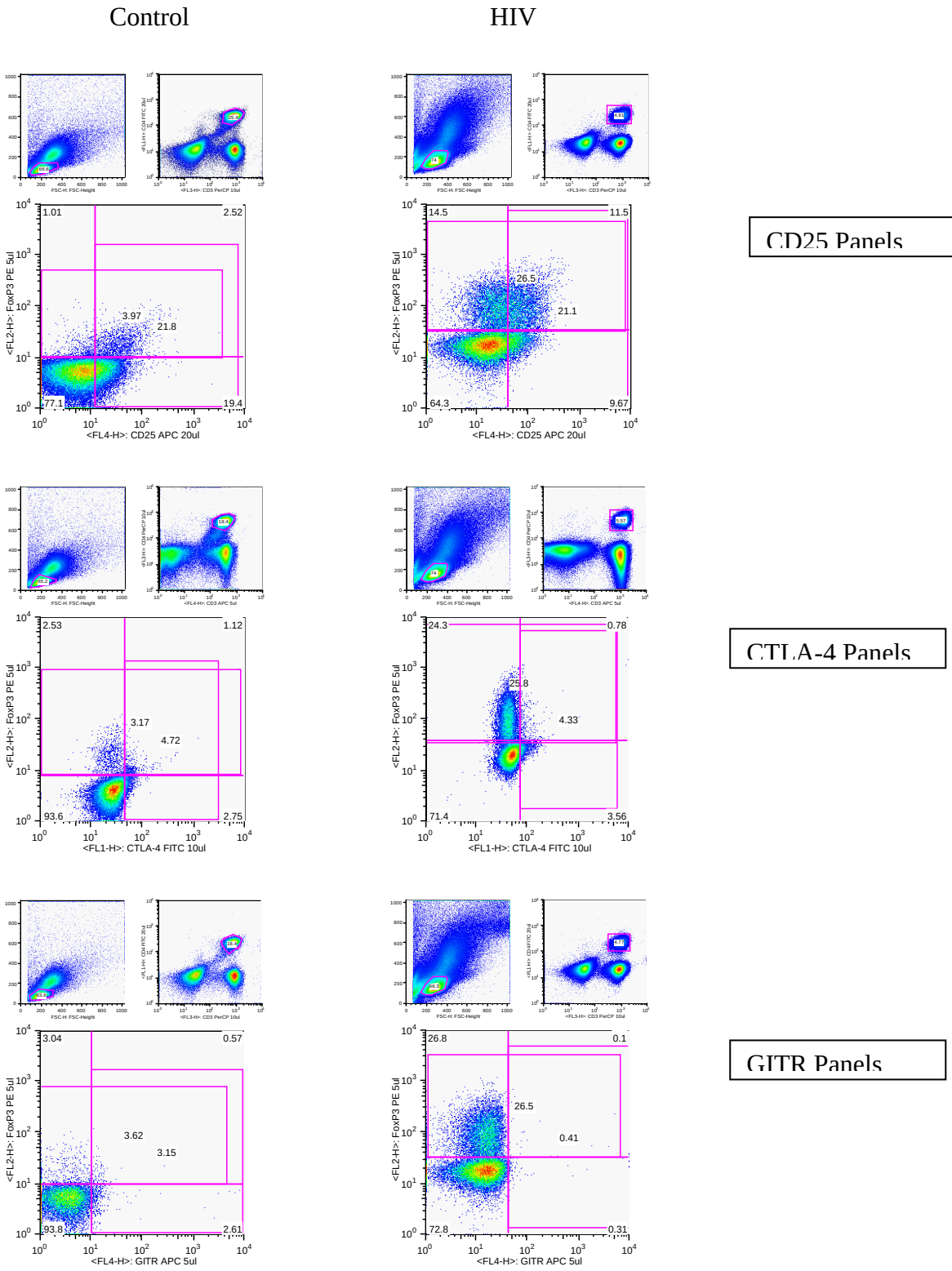


Figure 3.2: Representative flow cytometric plots for a control and a HIV-positive sample. (see next page for full annotation)

Gating strategy:

Figure 3.2 comprises 6 panels. Each panel comprises 3 separate plots.

Top left plot of each panel: Lymphocytes gated according to forward and side scatter

Top right plot of each panel: Dual positive $CD3^+CD4^+$ cells gated. Figure represents percentage of lymphocytes.

Lower large plot of each panel: x-axis represents CD25 (top two panels), CTLA-4 (central two panels) or GITR (lower two panels); y-axis represents FOXP3 positivity. Two rectangular gates are shown for each plot in addition to quadrant gates – a horizontal $FOXP3^+$ gate and a vertical $CD25/CTLA\text{-}4/GITR^+$ gate. Numerical percentages are given within respective gates. These percentages represent the $FOXP3^+$ events (left-most percentage within horizontal rectangular gate), the CD25/CTLA-4/GITR+ events (right-most percentage within vertical rectangular gate), or the dual positive events (percentage shown in upper right-hand quadrant), expressed as percentages of the $CD3^+CD4^+$ gate.

Comparison of control with HIV-positive samples:

Plots show FOXP3, CD25, CTLA4 and GITR positivity for a control sample (left) and an HIV-positive sample (right). FOXP3 percentage is noticeably elevated in the HIV-positive sample. $FOXP3^+$ cells are seen to lie in the $CD25^+$ subset but not in the $GITR^+$ or $CTLA4^+$ subsets in both the HIV positive and the control sample.

Other markers that have been proposed as Treg markers include CTLA-4, GITR and CD25. None of these markers is specific for Tregs and each can be found on other subsets (see Introduction). We measured these markers flow cytometrically and assessed whether there was a significant difference in any marker between the HIV and control group.

Cells that co-express FOXP3 with CTLA-4 were found to be a much smaller subset than CTLA-4 expressing cells. Similarly, despite low GITR expression overall, cells which co-expressed FOXP3 with GITR were a much smaller subset than $GITR^+$ cells.

We found no significant difference between groups in %CTLA-4^+; %CTLA-4^+FOXP3^+ co-expression; %$GITR^+$ or %$GITR^+FOXP3^+$ co-expression.

The $FOXP3^+$ cells were seen to lie in the $CD25^+$ and $CD25^{hi}$ subset. Percentage CD25 positivity is not reported however, due to the inherent inconsistencies of CD25 gating. (See first panel of Figure 3.2 – cut-off between $CD25^-$, $CD25^+$ and $CD25^{hi}$ subsets is somewhat arbitrary).

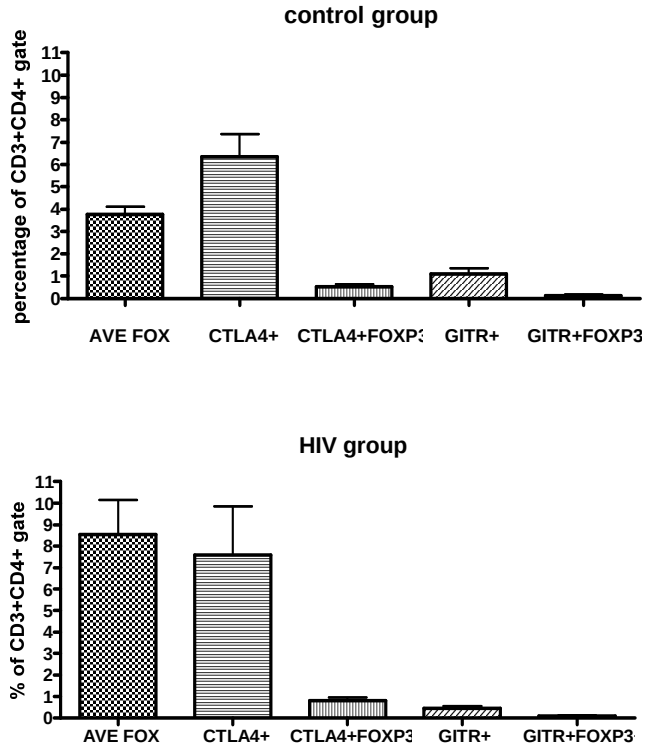


Figure 3.3: Expression of CTLA-4 and GITR as Treg markers in the control (top) and HIV positive (bottom) groups. FOXP3 expression is significantly higher in the HIV positive group than control. There is no significant difference between HIV and control groups in the $CTLA4^+$, $CTLA4^+FOXP3^+$, GITR or $GITR^+FOXP3^+$ expressing subgroups.

As GITR, CTLA-4 and CD25 were seen to mark different cellular subsets to FOXP3 and to each other, and given that FOXP3 is essential (if not sufficient) in the suppressor function of Tregs (reviewed by Ziegler, S.F., 2006), for the following results and discussion the term "Treg percentage" will denote the $FOXP3^+$ percentage of the $CD3^+CD4^+$ subset and will not refer to the other three markers.

As Tregs have been purported to alter in the elderly (Brusko *et.al*., 2005; Trzonkowski *et.al*., 2006) as well as with the female menstrual cycle (Polanczyk *et.al*., 2004; Arruvito *et.al*., 2007) , we assessed the association of Treg percentages by age and gender.

3.2. Increased Tregs are negatively correlated with CD4 count

In order to determine whether the elevated Tregs noted in HIV positive patients was dependent on CD4 count, we assessed the relationship between Treg percentage (as a percentage of the $CD3^+CD4^+$ subset) and CD4 count in both the HIV positive and control groups.

Considering both groups together, there was a significant negative correlation of Treg percentage with CD4 count (rho=-0.53; P=0.001) (See Figure 3.4). This correlation lost significance when each group was analysed separately, which may be due to the small sample number in each group, although a trend towards a negative correlation was noted in the HIV group only (Figure 3.5)

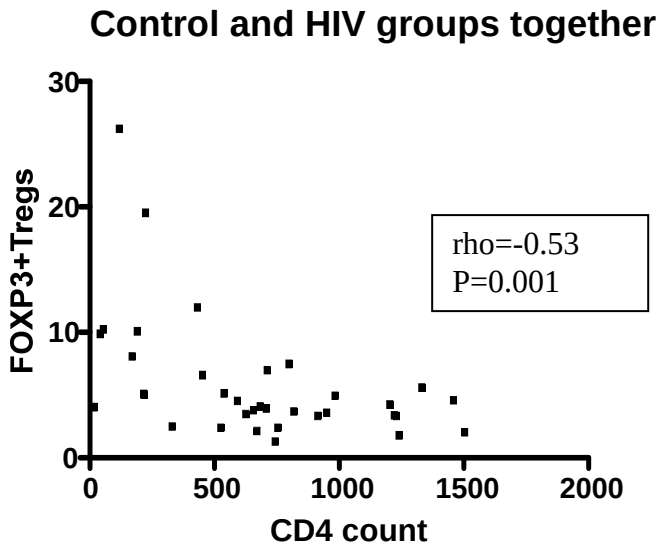


Figure 3.4: Correlation between Tregs and CD4 count – both groups analysed simultaneously.

There is a significant negative correlation between Day1 FOXP3$^+$ Tregs and CD4 count. Trend line has not been added as relationship visually appears to be curved rather than linear.

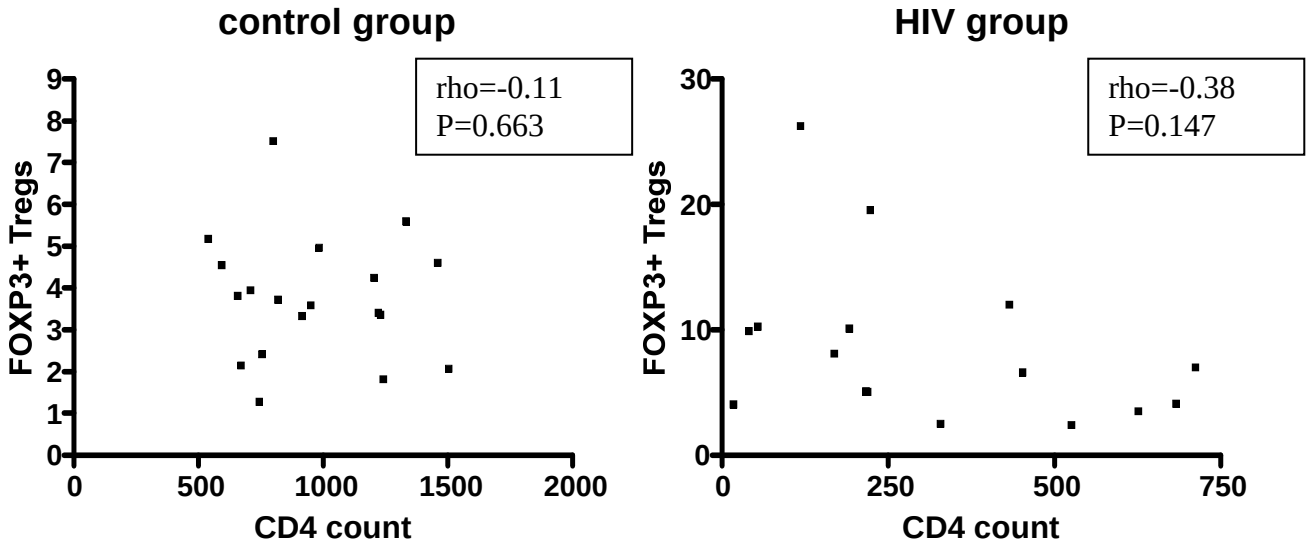


Figure 3.5: Correlation between Tregs and CD4 count – both groups analysed individually.

There is no significant correlation in either the control (left) or HIV (right) group, although a trend to a negative correlation is apparent in the HIV group.

The relationship depicted implies that Tregs are elevated in the later stages of HIV disease when the CD4 count is lower and raises the possibility that Tregs may be involved in opportunistic disease susceptibility late in the course of disease.

3.3 FOXP3 mRNA levels are dependent on choice of reference gene and do not correlate with FOXP3 protein levels

FOXP3 is the best current marker of Tregs (discussed above) but flow cytometric staining for FOXP3 protein has only recently become available. Many previous studies have explored *FOXP3* mRNA expression using the reverse transcriptase polymerase chain reaction (RT-PCR). We chose to use both methods (ie looking at both mRNA and protein expression) and to look for agreement between them. We repeated the PCR in triplicate with each of two distinct reference genes, GAPDH and 18sRNA, due to the unavailability of an ideal reference gene (discussed under Introduction).

Interestingly, the PCR results using GAPDH as a housekeeping gene showed poor agreement with that using 18sRNA. Agreement was poor at higher expression levels, with results using 18sRNA as a housekeeping gene being generally lower than those using GAPDH as a reference gene. This can be seen on the Bland-Altman plot for agreement between methods (Figure 3.6) (Bland, J.M. and Altman, D.G., 1986).

Percentage similarity between the two housekeeping genes was also calculated by averaging the two PCR results and expressing the average as a percentage of GAPDH

value (Scott *et al.*, 2003). The mean percentage similarity between the values was 64% (See percentage similarity plot for agreement between methods, Figure3.7).

The poor agreement between the results using GAPDH and those using 18sRNA as a housekeeping gene illustrate the pitfalls of relying on relative mRNA expression data to draw conclusions. Any condition affecting the housekeeping gene will heavily influence results (as discussed in the Introduction). Which is the most suitable reference gene to use is not always immediately apparent and requires intensive study on its own.

There was no significant correlation noted between *FOXP3* mRNA expression, as measured by PCR using either reference gene, and FOXP3 protein expression as assessed by flow cytometry (data not shown). This may be due both to biological variation between mRNA and protein expression, or due to methodological differences, such as the ability of flow cytometry to assess FOXP3 levels in $CD4^+$ cells specifically while RT-PCR was performed on a heterogeneous mixture of peripheral blood mononuclear cells.

Comparing the RT-PCR results for the control and HIV groups, there was no significant difference detected in expression of FOXP3 mRNA using either reference gene. Thus the difference detectable by flow-cytometric FOXP3 protein detection was not detectable by analysis of *FOXP3* mRNA in PBMCs. *FOXP3* mRNA expression also did not correlate with CD4 count when using either of the reference genes. The elevated Treg level noted in HIV-positive patients with low CD4 counts was thus only detectable using flow-cytometric methods in this study.

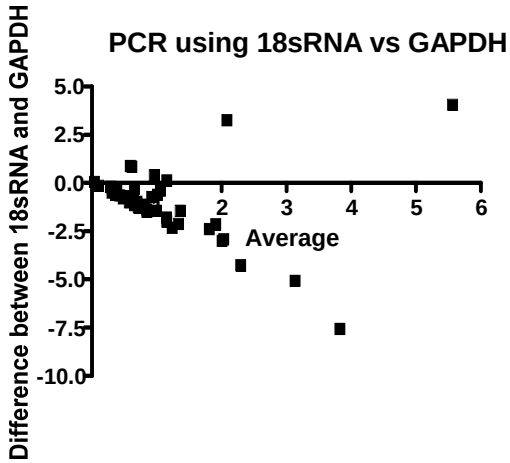


Figure 3.6: Bland-Altman Plot of agreement between PCR results using different reference genes. The average of the 18sRNA and GAPDH methods (shown on the x-axis) is plotted against the difference between the methods (y-axis). The plot shows poor agreement at higher expression levels, the results using 18sRNA being lower than corresponding results using GAPDH.

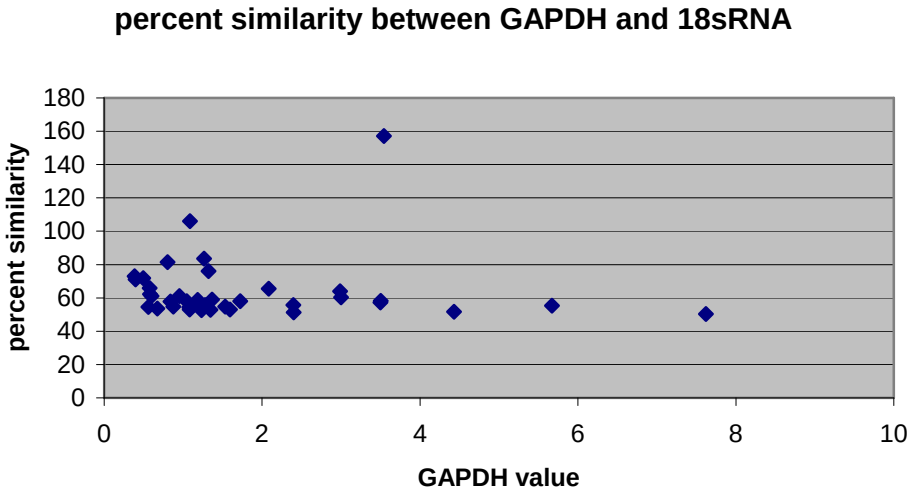
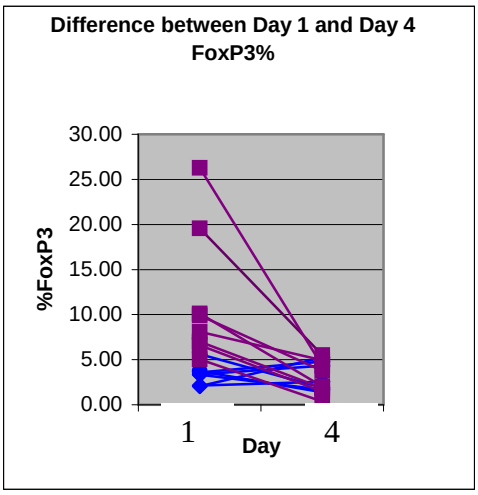


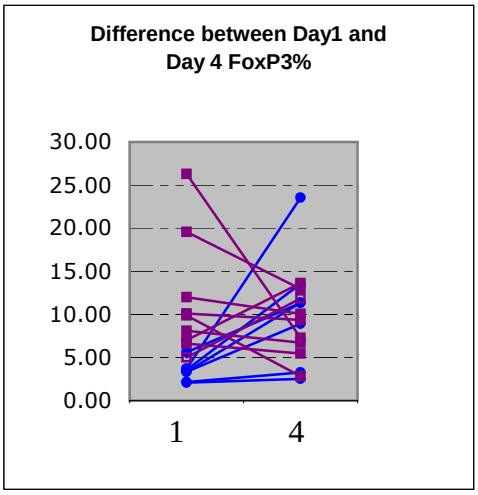
Figure 3.7: Percentage similarity plot between PCR results using different reference genes. Mean percentage similarity between GAPDH and 18sRNA results, expressed proportional to GAPDH results, is 64% over the range of GAPDH values

3.4 High Treg levels in HIV positive patients drop rapidly in unstimulated culture

Possible causes of the high Treg levels in HIV-positive patients might be due either to increased proliferation of Tregs or decreased apoptosis. As preliminary investigation we explored Treg levels from HIV and control patients in four-day cell cultures. We expected to see further elevation of Treg levels in the HIV positive patients at day 4, and were then planning to explore the factors leading to these high levels. Surprisingly however, we found that $FOXP3^+$ expression (as a percentage of $CD3^+CD4^+$cells) was significantly lower on day 4 than on day 1. This difference was found to lie in the HIV group primarily (significant difference between day 1 and day 4 $P<0.01$). This possibly implies that the stimulus maintaining high Treg levels *in vivo* was not found *in vitro*.

We postulated that the stimulus maintaining the high Treg levels *in vivo* may be a higher antigen load in HIV positive patients, or may be higher levels of cytokines or growth factors. In order to ascertain whether stimulation through the T cell receptor may be the stimulus maintaining the high *in vivo* levels, we cultured peripheral blood mononuclear cells in the presence of anti-CD3 stimulation for four days. We measured Treg levels flow cytometrically on day 4. In the control group anti-CD3 stimulation caused a statistically significant increase in Treg levels on day 4 compared with unstimulated day 1 ($P=0.031$). In the HIV group, however, stimulated Treg levels still trended to decreased levels compared with unstimulated day 1 samples, although there was no statistically significant difference between the day 1 (unstimulated) and day 4 (stimulated) levels.





Purple: HIV
Blue: Control

Figure 3.8A ***Figure 3.8B***

Figure 3.8 Comparison between Treg levels on day 1 and day 4, with and without stimulation.

In panel A there is a significant decrease in Treg levels in the HIV group (purple) between the first and fourth day of unstimulated culture. There is no significant drop in Treg levels in the control group (blue). In panel B when stimulated with *anti-CD3****, the control group exhibits significantly increased Tregs on day 4 compared with unstimulated day 1 baseline. The HIV group however still trends to decreased stimulated Treg levels on day 4 compared with the unstimulated baseline.***

4. DISCUSSION

Measurement of Tregs in humans is not standardised and results vary according to choice of technique and choice of markers. FOXP3 positivity is the most widely accepted marker of Tregs, being responsible for Treg suppressive function as well as identification (Fontenot *et al.*, 2003; Hori *et al.*, 20003; Khattri *et al.*, 2003, Fontenot t.al., 2005). Detection of FOXP3 by flow cytometry provides data that is reproducible and easy to gate in a standardized manner. FOXP3 positive T lymphocytes as measured immunophenotypically by intracellular cytokine staining will therefore be regarded as "Tregs" for the purposes of this discussion except where other markers are specifically mentioned.

Treg percentage (expressed as percentage $FOXP3^+$ cells of $CD3^+CD4^+$ cells) in controls was 1.3% to 7.5% of $CD4^+$ T cells (median 3.72%). This is in keeping with the ranges quoted in the literature which range between 5-10% of $CD4^+$ cells ($CD4^+CD25^+$ subset) to 1-2% of $CD4^+$ T cells ($CD4^+CD25^{hi}$ subset) (O'Garra and Vieira, 2004; Valmori et al., 2005).

We found a significantly higher percentage of Tregs in unstimulated HIV positive samples compared with controls. Analysing both groups together, we also found that Treg percentage was inversely correlated with CD4 count. The correlation was not statistically significant in the HIV positive group analysed separately, possibly due to the small sample size, but the same trend was apparent (see Figure 3.5). This is in keeping with results of Montes *et al.* (2006) who also reported Tregs to be inversely correlated with CD4 count and found highest levels in patients with CD4 counts less

than 200 cells/μl. Our data illustrates that variability in CD4 counts of study participants should be borne in mind when comparing data between studies.

While other authors have reported elevated percentages of Tregs in various tissues of HIV infected patients or SIV infected macaques, particularly in gut and lymphoid tissue, (Estes *et al.,* 2006; Nilsson *et al.,* 2006) most investigators have not found elevated levels in peripheral blood (Andersson *et al.* (2005), only moderate increase found by Epple *et al.*., 2006). The absence of elevated circulating Tregs may be due to the heterogeneous study groups, many of which analysed patient groups with relatively preserved CD4 counts or who were already on antiretroviral therapy.

The proportion of Tregs to non-regulatory $CD4^+$ T cells may be elevated either due to upregulated Treg proliferation, conversion of non-regulatory T cells to Tregs (by upregulation of FOXP3 expression) (Walker *et al.,* 2003; Oswald-Richter *et al.* 2004; Allan *et al.,* 2005); or disproportionate loss of the non-regulatory $CD4^+$ cells. Upregulated Treg proliferation may occur in response to generalized immune activation (Estes *et al.,* 2006) or in response to antigenic stimulation from viral proliferation itself (Weiss *et al.* 2004). Disproportionate loss of non-regulatory T cells is not in keeping with findings by Oswald-Richter *et al.* (2004) who showed that Tregs express CCR5 and are more susceptible to HIV infection *in vitro* than other $CD4^+$ T cells, or by Epple *et al.* (2006) who showed that there was an absolute elevation in gut mucosal Tregs in untreated HIV positive patients and that this elevation normalized with antiretroviral treatment. Also Eggena *et al.* (2005) and Montes *et al.* (2006) have shown that while Treg percentages are elevated in patients with lower $CD4^+$ T cell counts, the absolute numbers of Tregs do decline along with

other $CD4^+$ T cells during HIV progression. Nilsson *et al.* (2006), however, showed opposing findings, proving that exposure to HIV selectively promotes Treg survival via gp120 (even from non-infectious virions of both X4 and R5 strains) binding to CD4. Nilsson's group also found decreased levels of apoptosis in Tregs of HIV progressors suggesting increased Treg survival in HIV and not induction of FOXP3 in non-Tregs as a mechanism for increased Tregs levels.

Taken together, these findings suggest a relative accumulation of Tregs in late stage HIV. Tregs are elevated at a time characterised by generalised immune activation which leads to the question of why the Tregs do not suppress the concomitant immune activation. This calls into question their functionality, which needs to be further addressed. It may also be that Tregs are elevated as one additional feature of generalised immune activation, or possibly as a response to it. It will be interesting to elucidate how much of the immune activation associated with active disease is due to activation markers on activated Tregs themselves, although Nilsson *et al.* (2006) found no correlation between FOXP3 positivity and the activation marker CD69. Additionally it raises the possibility that elevated levels of functional Tregs may be responsible for the immune suppression characteristic of the disease.

The mechanism of action of Tregs in late stage HIV is still speculative as their physiological mechanisms of action are still disputed. It has been suggested that in healthy individuals Tregs suppress the ability of dendritic cells to upregulate CD80 and CD86. Stimulated $CD4^+$ T cells use CD40 ligand (CD40L) to override this suppression. HIV Env (even from non-infectious particles) binding to the CD4

molecule can suppress CD40L expression on activated T cells, leading to an inability to overcome Treg effects. (Kornbluth, 2006; Zhang, 2006).

Whether the large proportion of Tregs in HIV (both in lymphoid tissues in early infection and in circulation in later stages) retains its function, however, is still a matter of uncertainty. Certain authors have suggested that late stage Tregs may become dysfunctional (Oswald Richter *et al.*, 2004; Eggena *et al.*, 2005; Kinter *et al.*, 2007) while Nilsson *et al.* (2006) have reported that Tregs in HIV progressors maintain their suppressive function.

In terms of other immunophenotypic markers of Tregs, in this study use of GITR (singly or dual positive $GITR^{+}FOXP3^{+}$) or CTLA-4 (singly or dual $CTLA\text{-}4^{+}FOXP3^{+}$) did not reflect the differences in Treg percentage between groups. For GITR, this may due to expression on Tregs that is differently regulated to other Treg markers. This is in keeping with findings by Nilsson *et al.* (2006) who showed that most HIV-exposed $FOXP3^{+}$ cells did not express GITR. For CTLA-4 the reason may be that differences in intracellular CTLA-4 may not reflect the surface CTLA-4 active on Tregs. Intracellular staining rather than surface staining was used due to the short time that CTLA-4 is expressed on the cell surface. Mead *et al.* (2005) have shown that CTLA-4 is predominantly localized to intracellular compartments and deployed to the cell surface rapidly when needed, followed by rapid endocytosis. While surface CTLA-4 may be a vital functional protein for Tregs, its transient expression on the surface of the cell makes it a poor surface marker. Intracellular CTLA-4 is not specific to Tregs however and can be found in activated T cells (Mead *et al*, 2005).

FOXP3 positivity was found to correspond with $CD25^{bright}$ lymphocytes (see Figure 3.2) as expected. CD25 however is a suboptimal Treg marker in humans for two reasons. Firstly, it is also an activation marker found on non-regulatory $CD4^+$ T cells and thus is found on a larger subset than that delineated by FOXP3. Also, there is no clear separation in many cases between $CD25^-$, $CD25^{dim}$ and $CD25^{bright}$ subsets. This leads to inconsistencies in gating and we thus chose not to report our CD25 frequencies. CD25 in humans should be used primarily for sorting Tregs, as if one sorts only $CD25^{bright}$ cells, these will contain many Tregs. The utility of CD25 however in terms of quantitative analysis is severely limited due to the arbitrary nature of a negative/positive/high cut-off and a poor signal:noise ratio.

There was no difference detected between the HIV positive and control groups using RT-PCR for *FOXP3* mRNA expression. This may be due either to biological or technical reasons. Biologically, kinetics of FOXP3 expression may play a role. After activating non-regulatory $CD4^+$ T cells, Allan *et al.* (2005) found that *FOXP3* mRNA was increased within 24 hours while FOXP3 protein was only detectable by Western blot after 3 days. It is possible that some activation occurred during PBMC isolation leading to *ex vivo* induction of *FOXP3* mRNA.

Technically, it is possible that neither GAPDH nor 18sRNA is an ideal reference "housekeeping gene" with which to compare FoxP3 expression. Any differential expression of the housekeeping gene in different cell subsets or between HIV^+ patients and controls could skew the results. Also, we did not separate $CD4^+$ T lymphocytes from other peripheral blood mononuclear cells prior to mRNA extraction. While FOXP3 expression has not been reported in monocytes,

granulocytes or B lymphocytes, some expression has been noted in $CD8^+$ T lymphocytes, possibly marking a $CD8^+$ suppressor subset (Singh *et al.* 2007). Thus our mRNA findings may be affected by the mix of $CD4^+$ and $CD8^+$ T lymphocytes present while the flow cytometric data analysed only $CD4^+$ lymphocytes. Relative proportions of the non-FOXP3 expressing cells (such as granulocytes, monocytes and B lymphocytes) that also express the housekeeping genes may be reflected in results. Thus quantitative *FOXP3* mRNA expression data is difficult to interpret and should ideally only be applied to a sorted population of interest. Flow cytometry alleviates this problem by analysing FOXP3 expression at a single cell level.

The elevated Treg levels in the HIV group were seen to decline rapidly in culture conditions, differing significantly on day 4 from levels on day 1. There was no similar decrease noted in the control group. This implies that the factors maintaining high Treg levels *in vivo* are not present *in vitro*. In order to ascertain if this may be due to prevalence of antigen *in vivo,* peripheral blood mononuclear cells were stimulated with anti-CD3 prior to culture. After stimulation levels on day 4 were not significantly different from day 1 levels, there was in fact a non-significant decrease. The control group on the other hand manifested a significant *increase* in levels after anti-CD3 stimulation as expected. This may imply that one of the differences in Tregs from HIV infected individuals compared with those from controls lies in their response to T cell receptor stimulation. What drives the high *in vivo* Treg levels is still unclear, however. Other humoral factors such as the prevailing cytokine milieu in the blood stream of HIV infected patients may be playing a role. Alternatively Tregs in the HIV group may be prone to apoptosis under specific culture conditions or due to the virus itself. This however is not in keeping with the resistance to apoptosis proposed by

Nilsson *et al.* (2006) as a mechanism of Treg elevation. Apoptosis assays would be of value in future studies.

One also needs to bear in mind that FOXP3 is not the perfect Treg marker and its expression may be increased in activated T cells (non-Tregs) (see introduction, section 1.4 page 6). The raised FOXP3 expression noted in the HIV group on day 1 may relate to the immune activation found in HIV infection (Brenchley et.al. 2006). It is not clear however why FOXP3 expression would not increase in the HIV group after aCD3 stimulation if it is merely acting as an activation marker.

The culture data also have implications for future experiments exploring the role of antigen specificity of Tregs – in future experimentation with antigen-specific stimulation of Treg cultures (eg with HIV peptides, cytomegalovirus peptides, BCG etc) it should be borne in mind that Tregs from HIV-positive patients will be expected to decrease in number even after stimulation, as these stimuli are weaker and will stimulate fewer cells than anti-CD3.

These data illustrate the importance of monitoring Tregs as close to the *in vivo* situation as possible and demonstrate the need for caution when interpreting data from culture-based experiments.

5. CONCLUSION

In conclusion we have shown that Treg percentages, as measured immunophenotypically by the best available marker FOXP3, are significantly higher in the peripheral blood of HIV patients compared with controls. Levels of Tregs correlate negatively with $CD4^+$ T cell count. CTLA-4 and GITR were not expressed on the majority of FOXP3 positive cells in this study.

FOXP3 mRNA expression did not correlate with immunophenotypic results of FOXP3 protein expression. Caution is therefore necessary in interpreting results from mRNA data which may be confounded by post-transcriptional processing as well as analytical factors.

The finding of increased levels of Tregs in late stage disease contributes to a better understanding of the pathogenesis of the immune deficiency state seen in AIDS and may have therapeutic and prognostic implications. Intervention in Treg function may prove to be a therapeutic strategy. This has been shown in a macaque model where CTLA-4 blockade by monoclonal antibody decreased viral RNA levels and increased effector function of $CD4^+$ and $CD8^+$ T cells (Hryniewicz *et al.*, 2006). In humans, depletion of $CD4^+CD25^+$ Tregs and IL-10 neutralization dramatically increased the ability of α-IFN matured dendritic cells to induce p24-specific $CD4^+$ T cell responses *in vitro,* suggesting potential application in therapeutic vaccination strategies (Carbonneil *et al.* 2004). Legrand *et al.* (2006) have also reported augmentation of *in vitro* HIV-specific $CD4^+$ and $CD8^+$ T cells responses in HIV exposed uninfected neonates by depletion of Tregs from cord blood. Prognostically, it remains to be

shown in longitudinal studies whether Tregs may prove a better marker of disease progression than total CD4 count or immune activation markers.

This study differs from previous published observations where lower Treg or FOXP3 levels were found in HIV-infected patients compared with controls (Oswald-Richter *et al.* 2004; Apoil et al., *2005*) as well as from those studies which showed elevated Treg levels in mucosal sites but not peripheral blood of HIV-infected patients (Andersson *et al.* (2005) Epple *et al.*., 2006). This study draws attention to the effect of disease stage on peripheral blood Treg level and explains why studies including only patients with higher CD4 counts or patients on HAART may not detect any significant elevation in Treg levels.

Further directions for study include identification of the factors driving elevated Treg percentages *in vivo* in order to elucidate whether elevated Tregs are a cause or an effect of HIV-related disease. Further characterisation of factors affecting *in vitro* survival and proliferation of Tregs may shed light on this issue as well as explaining the drop noted in Treg levels in unstimulated culture. Additionally, the functionality of the Tregs needs evaluation, as it is plausible that the elevated levels of Tregs seen may be compensating for a loss in Treg functionality. The relationship of HIV viral load, as well as immune activation markers (such as CD38 positivity, $CD8^+$ T cell proliferation, $CD4^+$ T cell proliferation and serum immunoglobulin levels) with Treg levels also needs exploration.

The significant difference between HIV and control groups noted in this study, as well as the negative correlation with CD4 count, indicate that Tregs may prove a valuable

monitoring tool for laboratory follow-up of HIV-infected patients. Additionally Treg levels pre-and post vaccination should be monitored in all future HIV vaccine trials in order to assess the impact of Tregs on HIV susceptibility. The value of Tregs as a therapeutic target to prevent disease acquisition or progression is an exciting future prospect.

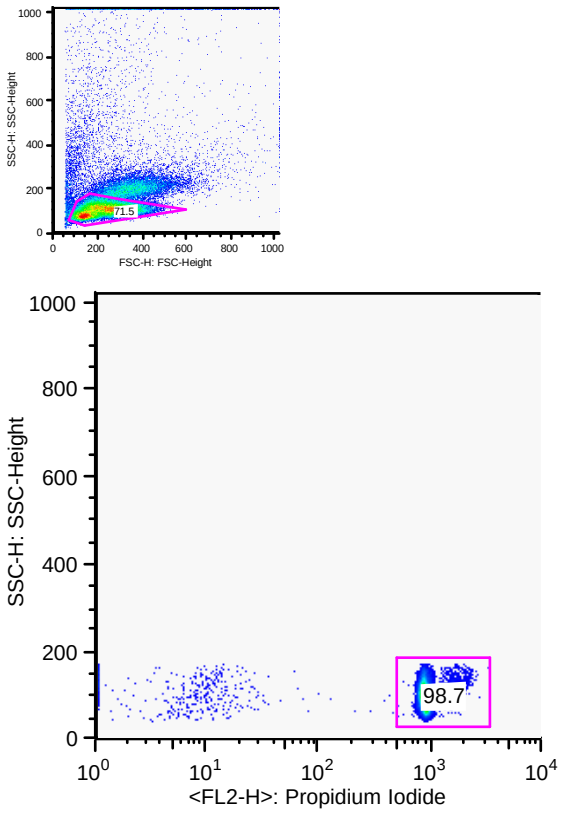
APPENDIX A: Demographics of sample group

RACE	SEX	AGEGROUP	CD4COUNT
Control Group			
B	F	3	831
B	F	3	973
B	F	3	1503
B	M	1	671
B	M	3	1231
B	F	2	694
C	M	3	950
I	M	3	1332
B	M	3	818
B	M	3	915
W	F	3	708
C	F	3	984
W	M	4	539
W	F	3	1204
W	F	5	798
W	M	4	1459
W	F	6	755
W	M	6	744
B	F	3	1240
W	F	5	657
B	F	2	1221
B	M	2	593
HIV group			
B	F	3	523
B	F	2	223
B	F	4	118
B	F	2	432
B	M	3	40
B	F	3	169
B	M	3	712
B	F	3	191
B	F	2	219
B	F	2	452
B	F	3	626
B	F	4	329
B	F	2	525
B	F	3	17
B	M	3	54
B	F	4	216
B	M	2	683

Table 1. Demographics of sample group.
B Black, W White, C coloured, I indian

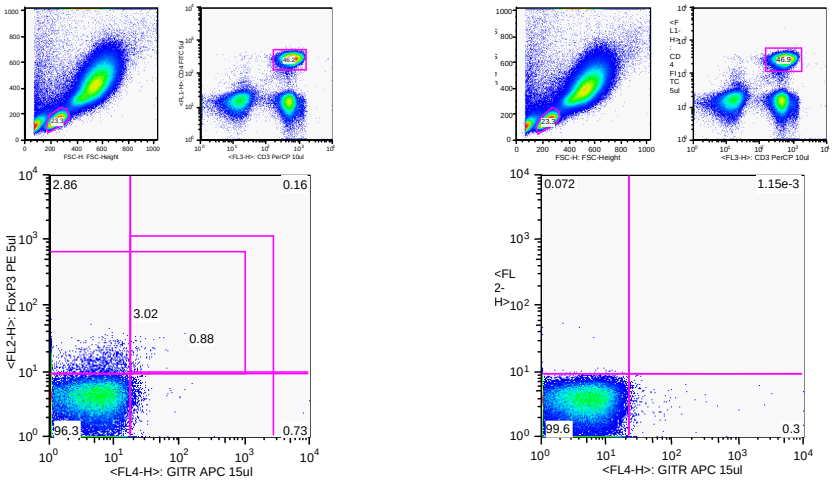
AGE GROUPS:	
1	10 to 19
2	20-29
3	30-39
4	40-49
5	50-59
6	60-69

APPENDIX B: Propidium Iodide staining to assess efficacy of cell permeabilisation



First plot shows lymphocytes gated according to forward and side scatter. Second plot shows 98.7% success of permeabilisation, allowing entry of propidium iodide into cells.

APPENDIX C: Fluorescence Minus One Control for FOXP3 staining



Fluorochromes added to cells in left hand panel: anti-CD3 PerCP, anti-CD4 FITC, anti-GITR APC and anti-FOXP3 PE. Right hand panel: same fluorochromes without anti-FOXP3 PE

APPENDIX D: Approval of Change of Title

Faculty of Health Sciences
Medical School, 7 York Road, Parktown, 2193
Fax: (011) 717-2119
Tel: (011) 717-2075/6

Reference: Mrs Alison Mclean
E-mail: mcleanam@health.wits.ac.za
16 July 2007
Person No: 9400721V
TAA

Dr MS Suchard
67 Kerkira
131 Katherine Street
Sandown
2196
Sandown, South Africa

Dear Dr Suchard

Master of Medicine in the specialty of Clinical Pathology: Change of title of research

I am pleased to inform you that the following change in the title of your Research Report for the degree of has been approved:

From:	**Characterisation of regulatory T cells in HIV-infected or exposed infants and HIV-infected adults in South Africa**
To	**Characterization of regulatory T cells in HIV-infected adults in South Africa**

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

University of the Witwatersrand
2007 -07- 16
JOHANNESBURG

APPENDIX E: Ethics Approval from Human Ethics Committee of the University of the Witwatersrand

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Suchard

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M060138**
PROJECT	Characterisation of Regulatory T Cells in HIV-Infected Adults in South Africa
INVESTIGATORS	Dr M Suchard
DEPARTMENT	Clinical Pathology (Microbiology)
DATE CONSIDERED	06.01.27
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 06.04.28 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof C Tiemessen

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX F: Patient Information sheet

You are invited to participate in a research study. The aim of the study is to learn more about how the body fights against HIV. We are studying the function of a type of T cell known as a Regulatory T cell (Treg) in HIV.

If you agree to be part of the study, we will draw 48ml (6 tubes or about 10 teaspoons) of blood from your arm. This blood will be used to study the cells involved in fighting HIV such as T cells and Tregs using a technique called flow cytometry. We will also study the genes (DNA) involved in how the body reacts to HIV. All results will be anonymous, that is we will not record your name. We will record some of your personal details such as your age, sex, race, CD4 count, viral load, other illnesses and medications etc. Your confidentiality is protected at all times and no-one will know your results. Neither you nor your doctor will receive the results of these tests. The results will be used for research purposes only.

You may choose to participate or not to participate. There is no benefit to you in being part of the study, except for helping researchers to know more about HIV. If you choose not to participate it will not disadvantage you in any way. The only disadvantage to you in participating in the study is the discomfort of having blood drawn from your arm and the possibility of bruising or infection (very unlikely) that may result, as for any blood test.

During our study we are going to use different methods in the laboratory to try to learn more about HIV. One area in which we are interested is how a person's genes (DNA) help them to fight HIV. If you give consent, some of the blood you give for the study will be used to study the gene called *foxP3*, which we believe is important for fighting HIV.

The results of the tests will be anonymous. Neither you, nor your doctor, nor anyone else will ever know your results. The results are to be used for research purposes only.

Some blood will also be frozen in case we need to come back and do further tests at a later stage. The blood will be stored without any record of your name, so that any further tests done will also be strictly confidential.

Please help us to learn more about this disease. We appreciate your contribution.
If you have any questions, please do not hesitate to ask.

Thank you
Dr Melinda Suchard

AIDS Research Unit
National Institute of Communicable Diseases
1 Modderfontein Rd
Sandringham
Johannesburg Tel: 0ffice: (011) 386 6370
2192 Cell: 083 671 3909

APPENDIX G: Patient Consent Form

Consent to participate in the Treg Study

I understand the information I have read in the "Patient Information Sheet". I have asked all questions I wanted to about the study and the study has been fully explained to me.

I give consent for my blood to be taken for laboratory tests for the Treg Study. These tests may include CD4 counting and immunological tests. I understand that I will not find out the results of these tests.

Name: ……

Consent to genetic testing

I understand that some of the tests to be done on my blood for the Treg Study include tests of my genes (DNA). I understand that no-one will find out the results of these tests. I agree to this testing.

Name: ……

Consent to storage of blood

I understand that some of my blood or DNA may be stored for future research tests. I understand that no-one will find out the results of these tests. I agree to the storage of my blood.

Name: ……

Date……. Place……..

Person conducting Informed consent……..

Witness……

APPENDIX H: Ethics Approval from the Ethics Committee of the South African National Blood Transfustion Service

SANBS

South African National Blood Service

RESEARCH AND MEDICAL ETHICS COMMITTEE

20th July 2006

Dr M Suchard
AIDS Research Unit
National Institute for Communicable Diseaese

Dear Melinda

Protocol: Characterization of regulatory T cells in HIV-infected or exposed infants and HIV-Infected adults in South Africa.

A review, by the SANBS Ethics Committee, of the ethical implications of the collections of donor samples as described in the above proposal was favorable with the following provisos;

1. The supply of blood will be dependant on blood availability.
2. Six monthly reports on the number of units provided by SANBS, including the unit numbers and collection dates, are to be submitted to the Committee

Subject to the above your application is approved.

A charge of R647-00 (inclusive of VAT) will be levied to cover expenses incurred in the collection and testing of the blood

Could you please let me have details of the method of payment that will be employed?

Could you please make the appropriate logistical and billing arrangements with Mr. Peter Nortman on 011-761-9005 or by e-mail at pnortman@inl.sanbs.org.za.

Please feel free to contact me telephonically on 082 449 2243 or by e mail at tscanes@inl.sanbs.org.za should you require assistance with any aspect of the project.

Yours sincerely

T Scanes (Committee Secretary)
pp Dr B Naidoo
CHAIRPERSON: RESEARCH & MEDICAL ETHICS COMMITTEE
SOUTH AFRICAN NATIONAL BLOOD SERVICE

Constantia Boulevard
Constantia Kloof Ext 22
1709

Private Bag X14
Weltevreden Park
1715

Tel.: +27 11-761-9211
Fax.: +27 11-761-9275
Email: tscanes@inl.sanbs.org.za

Members / Lede
Dr B Niadoo (Chairman), Prof T Jenkins, A Kleinsmidt, N Makitshi, A Ngobese,
T Scanes (Secretary)

APPENDIX I: Patient information form for Blood Donors at Blood Bank

You are invited to participate in research against HIV/AIDS conducted by the National Institute of Communicable Diseases (NICD) AIDS Research Unit. We are looking for blood from healthy blood donors to use in our ongoing research.

If you choose to participate, a sample of your blood (or the whole unit in certain cases, provided the National Blood Service is not running short of blood of your type) will be sent to the NICD where it will be stored and used in current and future research projects. Use of blood will be done anonymously, meaning no researchers will know your name, although they will know some demographic information about you including your age, sex and race.

Tests to be done include HIV testing and other immunological and/or genetic tests. You will not find out the results of these tests. Results will be used for research purposes only and will not be given to the South African Blood Transfusion Service or to your doctor.

There is no advantage for you from participating in this study except for advancing scientific knowledge about HIV/AIDS. The only disadvantage of participating the risk of bruising and/or discomfort during blood donation. You may refuse to participate, in which case your blood will be used by the South African National Blood Transfusion Service in the routine way.

We are aware that your blood is a precious resource and very valuable to patients who are in need of blood transfusion. We feel that HIV research is an equally important and urgent endeavor. We are desperately seeking large volumes of blood for our research which we are struggling to obtain. We therefore ask for your help. This is a once-off commitment. The next time you donate, your blood will not be used for research purposes but will be processed by the South African National Blood Transfusion Service as usual.

If you have any questions, please contact

Dr Melinda Suchard (011) 386-6369
AIDS Research Unit 083 671 3909
NICD

Greg Khoury (011) 386-6399
AIDS Research Unit 082 467 8732
NICD

APPENDIX J: Consent Form for Blood Donors at Blood Bank

I understand the above information. I give consent for a sample of my blood, or the unit of blood I donate, to be stored and used for laboratory testing. These tests may include HIV and genetic tests. I understand that I will not find out the results of these tests.

Name:

Signed:

……..

Date……

Place……..

Person conducting Informed consent:

Name:

Signed:……

Date……

Place…….

6. REFERENCES

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