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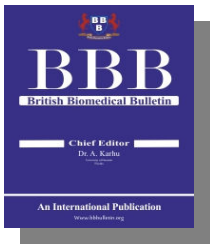

Original

HAART Restore the $CD4^+CD25^+$ Regulatory T Cells (Tregs) Frequency Among HIV/AIDS Patients.

H. Durgadevi[1], M. Suthandhira[1], O. R Krishnarajasekar[2], C. Chandrasekar[2], K. Raja[2], B. Rayvathy[2], V. Gopinath[2], Sowmya Swaminathan[3], Luke Elizabeth Hanna[3], S. Anbalagan[3], J. Sathyavathi[4], N. Rohan[1], R. Gopinath[1] and Elanchezhiyan Manickan*[1]

[1]*Department of Microbiology, Dr. ALM PG IBMS, University of Madras, Taramani,Chennai, India*
[2]*Govt. Hospital of Thoracic Medicine, Tambaram Sanatorium, Chennai, India*
[3] *National Institute for Research in Tuberculosis (ICMR), Chetpet, Chennai, India*
[4]*Biostatistician, Chennai, India*

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Corresponding author: Professor, Department of Microbiology, DR. ALM. PG. IBMS, University of Madras, Taramani campus, Chennai-600 113, India.
E-mail address: emanickan@yahoo.com

A B S T R A C T

Aims: HIV infection is a secondary immunodeficiency disorder associated with loss ofCD4+Tcells and immune suppression. Tregs are immune suppressive cells and involved in peripheral tolerance. Tregs express$CD4^+CD25^+$ on the surface and $Foxp3^+$ internally. Relative frequency of Tregs during HIV/AIDS is not fully known. $CD4^+CD25^+Foxp3^+$Tcells (Tregs) play a vital role in peripheral tolerance but its role during HIV/AIDS is not fully known. Hence this study was conducted with the aim to characterize the Tregs frequency among HIV patients and healthy controls.
Study design: Cross sectional study
Place and Duration of study: Department of Microbiology, University of Madras, chennai and Government Hospital Of Thoracic Medicine, Chennai between October 2012 to December 2013.
Methodology: 108 individuals comprising of 71 HIV positive patients (44 patients undergoing HAART and 27 without HAART) and 37 were HIV negative healthy controls were enrolled in this study. PBMCs were isolated and stained with CD4/FITC, CD25/APC, FoxP3/PE antibodies (BD Pharmingen) and analyzed for Tregs frequency by flow cytometry (BD FACSCalibur).
Results: Our study showed that healthy controls had a Tregs frequency of 4.1%. When Tregs frequency analysed among HIV/AIDS patients who are yet to receive HAART we found a significant reduction in Tregs population i.e. 1.2%. Encouragingly this deep plunge in Tregs population was found to be restored and surged upon HAART upto 12.6%. There was no difference when compared between gender or age or absolute CD4 count.
Conclusion: HAART is known to decrease viral load and improve the patient's CD4 cells count. Here we report that HAART has improved not only the CD4 cells but also the Tregs frequency.

This study add one more dimention to Tregs in infectious diseases especially HIV/AIDS.

Introduction

HIV infections are continue to be ubiquitous and virtually there is no place in the globe we completely eradicated the HIV infections. Though HIV was discovered more than 3 decades ago it is unfortunate that there are no drugs discovered which can offer a complete cure. Thirty year of research has yielded some remarkable drugs that can alter the course of HIV/AIDS and enhance the post HIV infected life. These developments have changed the way the people used to think and apparantly the taboo and stigma is loosing the grip. HIV is a retroviridae member containing two identical positive sense RNA genome embedded with reverse transcriptase. The genome is encircled with capsid and the whole complex is further protected by an envelope. The envelope contains several glycoproteins (gp) of which gp120 does the major role of attaching to the host cells[1]. HIV gp120 has the unique property of binding to CD4 molecules of heper T cells and a few antigen presenting cells[2]. After this primary interaction the attachment of virus is further assisted by host chemokine receptors namely CCR-5[3] or CXCR-4[3] and these chemokine receptors offer a perfect docking site. This binding is further strengthened by HIV gp41[4] and lead to viral penetration and uncoating. Then the RNA genome enter the CD4 cell cell membrane and the viral reverse transcriptase enzyme converts the RNA into DNA in the cytoplasm[5] Now the DNA cross the toughnulear membrane and integrate with the host genome with the help of a viral enzyme, integrase[6]. While the virus exist in the integrated for it is called a provirus. After this event the virus slowly shuts down the host functions and utilizes the host cell machinary for further replication[7]. During transcription the virus produces mRNA which gets translated into a long polyprotein[7]. By post translation modifi-cations 15 viral proteins are made and each protein helps in the futher replication of HIV[8].

The CD4 molecule crowned Helper T cells had several immune functions. Prior to antigen encounter they are called naïve cells and after they encounter an antigen they develop into effector cells and memory cells. As the name implies, majority of the CD4 cells become effector cells which seek infected target cells and attack while a small protion is programed to become memory cells which assist the host if it encounter the same antigen again in the future. Recently there was another flock of helper T cells was uncovered. They are $CD4^{+}$, $CD25^{+}$ and $FoxP3^{+14}$cells and their major function is peripheral tolerance[9]. They constitute about 5-10% of the T cells and more studies on this cell type showed presence of other markers such as as CTLA4 15, CD45 RO^{hi16} and on Tregs. They produce immune suppressive cytokines such as IL-10 and TGF-β[17,5] These cells monitor the self reactive cells at the periphery and an imbalance of the frequency of these cells could lead to autoimmune diseases[10].

Owing to its immuno suppressive property presence of these cells in large number can affect the prognosis of viral diseases. It is known that majority of viral diseases are not immune suppresive diseases but they are rather occur by over-powering the immune response by outnumbering or deceiving it. In contrast, HIV infection is notorious in which immune system is

sequentially and categorically paralysed and eventually completely collapsed[13]. Thus it causes morbidity and mortality by immune suppression. Tregs on the contrary suppress chronic activation of immune cells by HIV but can inadvertently suppress HIV specific immune responses. HIV is an immunodeficiency virus and Tregs are immunosuppressive cells. In the light of above information (i.e. immunosuppressive cell's role in immunosuppressive disease) Tregs has been key interest to viral immunologists. Previous studies had shown that Tregs can inhibit HIV infection and replication[11]. Other studies showed that HIV infections can increase Tregs frequency[12]. To have a clear idea about the frequency of Tregs among HIV infected individuals (who under ART and not under ART) was analysed. Relative frequency of Tregs was compared between HIV infected patients and controls.

Materials and Methods

Study population

A total number of 71 HIV infected patients were from out-patient unit and in-patient ward of Government Hospital of Thoracic Medicine, Tambaram Sanatorium, Chennai and 37 healthy controls were included in the study. This study was approved and ethical clearance was obtained from Institutional Human Ethical Committee (No.2012/150). They belong to various parts of Tamilnadu. HIV-infected patients were divided into two groups namely HAART negative (cases enrolled for therapy and they were yet to received HAART at the time of sample collection) and HAART positive (cases who already started HAART therapy). A comparable number of healthy volunteers were included in this study as control group. Information on the current clinical status of the patients was collected from all the patients and concerned clinicians. 5 ml of venous blood from peripheral vein in K3 EDTA vacutanier was collected from all the enrolled individuals and used for the Tregs study.

Confirmation of HIV infection

A Rapid Trispot Test was done to confirm their positivity to HIV1 and 2[37]. Initial screening for $CD3^+$cell counts and $CD4^+$cell counts were done to confirm their CD4 status. These tests were performed as per the manufactures instructions (BD Biosciences).

Tregs frequency analysis

Percentage of Tregs among HIV positive and negative individuals were enumerated by flow cytomentry (BD Biosciences). For this analysis Tregs enumeration kit developed by BD Biosciences (Cat. No. 560753) was used. Tregs enumeration kit consisted of two cell surface markers namely CD4 (anti human CD4-FITC labelled) and CD25 (anti human CD25-APC labelled) and one intracellular marker namely FoxP3 (anti human FoxP3-PE labelled). Appropriate isotype controls were included.10^6 cells/ml were used for the staining and protocol used for staining was as per the manufacturer. Data were acquired on a FACS Caliber flow cytometer and analyzed using the Cell Quest software (BD biosciences). Results are expressed as percentages of $CD4^+$ $CD25^+$ $FOXP3^+$ cells out of the total PBMC.

Statistical Analysis

Data were analyzed for statistical significance using student's *t-test*.

Results

Regulatory T cells (Tregs) are recently identified repertoire of T cells and their main role is induction of peripheral tolerance. Tregs accomplish this by

synthesis and release of immunosuppressive cytokine such as TGF-β. One of the important questions is that what is the fate of this cell during HIV infection since Tregs are also CD4s and secondly what is its role during HIV infection i.e. induction of more immunosuppression? In order to study this it is important to understand the frequency of Tregs among HIV patients in comparision with healthy human controls. To study this we collected venous blood from patients suffering from 71 HIV/AIDS and 37 healthy control individuals. Among HIV patients, 27 samples were collected from HIV patients who were not yet treated because they were just enrolling (HAART negative) for treatment. 44 serum samples were collected from HIV patients who were already on HAATR therapy (HAART positive). From these blood samples PBMCs were isolated as described in the materials and methods and aliquot for antibody staining. The cells were stained with Treg specific antibodies i.e. anti CD4-FITC, CD25-APC and FoxP3-PE antibodies and the cells were analyzed for the frequency of Tregs using a BD FACS Calibur Flow cytometer and the results are shown in the Table-1 and Fig.9. When the CD4 cell percentage was evaluated it was found that the healthy controls (n=37) had a mean CD4 value of 37.2 ± 5.2% which is a normal range. However, the magnitude of damage caused by HIV is much evidenced among the HIV (HAART negative) group which is 13.3 ± 9.8% i.e. about 65% reduction in CD4s. Patients who had been taking ART (HAART positive group) had a swing back to 18.0 ± 8.6%, thanks to life saving HAART therapy (Fig.2).

Next we evaluated the $CD4^{+}CD25^{+}$ cells in the different groups. A normal range of 4.1 ± 1.1% of Tregs were found among healthy controls (Table-1). However, we found a significant reduction in Tregs among the newly enrolled HIV patients who were yet to initiate HAART treatment. These patients had only 1.2 ± 0.7% of CD25+ T cells and this reduction in values are statistically significant ($p<0.001$, Student's *t* test) in comparison to healthy controls. As discussed above for CD4s, CD25 cells also restored back to 12.6 ± 6.2% and again this value is statistically significant ($p<0.001$) when compared to untreated HIV population. These data clearly showed a drastic plunge in Tregs population during HIV infection and the frequency is rebound to a way above normal level by HAART treatment. Though there was a similar trend line noticed with percentage of FoxP3, these values did not correlate with HIV infection (healthy controls = 0.3 ± 0.4 versus HIV without ART=0.3 ± 0.4 ($p=0.7$)) though there was a minor increase of FoxP3 level among HIV with ART therapy. Hence, we did not include FoxP3 data for the rest of the study. Thus, our study based on CD4 and CD25 positivity suggested that Tregs also suffer a worst hit by the HIV and fortunately they could be rescued and restored by HAART therapy.

Previous literature had shown that Tregs increases during several viral diseases caused by respiratory syncytial virus (RSV), West Nile virus, influenza-A virus etc. It has also been shown to be in high frequency during autoimmune diseases especially among women. To test this hypothesis, the Treg frequency was estimated among the males and females of our study group and the results are illustrated in Table-2. As shown in the table there was a marginal increase of CD4 cells among females and no such predilection was observed when we analyzed the CD25 + cells. Overall, Patients without ART had lower Tregs which was in increasing trend with treatment. Thus, our study showed that there was no significant difference between females and males in terms of Treg frequency during HIV/AIDS.

In the recent past the cut-off CD4 count to initiate HAART therapy was < 200 per mm^3. Recently WHO, NACO and NARI has recommended initiating ART when the CD4 count is as low as 350 and below per mm^3. Knowing the profound suppressive profile exhibited by Treg, we wanted to evaluate the frequency of Tregs at the CD4 count cut-off point and the results are illustrated in the Table-3. As shown in the table there was an increase in mean percentage of CD4 cells among patient having >350 cells per mm^3 however in terms of CD25+ cells no such correlation could be made. As noted before, a low Treg percentage was noticed with HAART negative group in comparison to HAART treated cases. Our results suggested that there was no difference in Tregs frequency irrespective of their general total CD4 count.

It has been reported that cardiovascular diseases (CVD) are a major non infectious cause of death in people with HIV/ AIDS and patients with age group 40 and more are at high risk of atherosclerosis and CVD. It has been postulated that Tregs in general protective to atherosclerosis and diminution of Tregs makes the patients vulnerable to atherosclerosis and CVD. To check the frequency of Tregs in relation to age, we divided population into age group below 40 and above 40 years. Compared to healthy controls, the Treg frequency has diminished among HIV patients without HAART therapy. However, there was no difference between age group below 40 years and above 40 (Table-4).

The current study revealed that HIV/AIDS patients had significantly diminished Treg frequency. These percentages restored back to above normal levels upon HAART at the time point when we tested. It is not known that when exactly the Treg frequency will reach back to the normal level. For such studies follow up samples are required and our study is restricted to single samples only. Second problem with our study is that there was a weaker staining with FoxP3 antibody. Repetition of this antibody staining would be an important supporting data. In addition to the percentages (mean) of each group, we also evaluated the mean fluorescence intensity (MFI) of each group and we did not find any correlation among the various groups tested (data not shown). From the available data it could be concluded that HIV infection perhaps targets CD4+ and CD25+ Tregs in humans. It also appears that these cells rebound to near nomallevels upon treatment with HAART. The observed difference in the study appears to be not gender, age or CD4 count dependent. To the best of our knowledge this is the first report showing the frequency of Tregs among HIV patients and controls among Chennai population.

Discussion

Humans are blessed with ultra-precision guided immune system and immune cells by their constant immune surveillance recognize the danger posed by the microbes and orchestrates a wide array of responses and fights against the invaded pathogens and eradicate them successfully. This fight is not a just one time event but it occurs almost all the time until the life of the host. In all those encounters the immune system is to be the winner otherwise we succumb to diseases. Some diseases affects humans almost rarely while the others almost all the time but irrespective of the nature of the threat the immune system has methods handle them and eliminate them efficiently. Of the microorganisms the viruses are considered to be the smallest of all but the diseases caused by them are mightier and intimidating. Fortunately many viral diseases are self-healing and get cleared spontaneously. But some of them are

known to cause chronic diseases[18]. The viruses that cause the chronic diseases are more bothersome since it causes the immune reactivity indefinitely that can lead to secondary and/or tertiary complication. These complications could be malignancy or immune exhaustion or immune deterioration and/or death. To cause such consequences viruses uses several immune evasive tricks and to counteract these events immune system responds by several means and measures. Understanding this virus (parasite)-host relationship is primordial in developing antiviral strategies and with this notion the current study was conducted to understand the one of the immune events that occurs in response to HIV infection[19-21]. One of the important set of $CD4^+CD25^+$ cells also known as regulatory T cells (Tregs) are involved in prevention of self-antigen recognition of immune cells and thus maintain the peripheral tolerance[22]. This is accomplished by their immunosuppressive molecules (e.g. cytokines). HIV is a chronic disease which targets immune cells especially cells expressing the molecule CD4. Besides antigen presenting cells, CD4 molecules are found throughout the T helper cells and as the name implies these cells are helper cell to all the cells of the immune system. CD4 T helper cells are like power houses and infection and destabilization of these cells lead to permanent collapse of the immune system. Thus HIV is an immunosuppressive disease and Tregs are known for their immunosuppressive property[23]. The question is what Tregs role in HIV is? To answer that big question, the first thing to do is to find out the percentage of Tregs in the peripheral blood of HIV patients and that is exactly what we have done in this study. We compared the percentage of Tregs among HIV infected but yet to undergo treatment and the others who were already on HAART therapy and these values were compared with apparently healthy individuals. Our study disclosed that HIV patients who are not on any therapy had minimum number of Tregs and we noticed a restoration of its frequency upon HAART therapy. It appears that drug treatment in conjunction with the existence of Tregs worked together and prevented the CD8 killing of HIV infected CD4 cells[24]. Rebounding of CD4 cells will take control over the situation. This will lead to gradual reduction of plasma viral load and elimination of all the circulating HIV eventually. With the available data this is what our hypothesis is and previously this type of information is not available from Southeast Asian Indian population.

Tregs frequency had been studied extensively from various populations ranging from Caucasians to African population. Some studies showed that Tregs were indeed increased in frequency upon HIV infection while the others suggested the opposite. Eller, MA. *et al.,* 2011 conducted[25] a study on CD4 cell chronic activation during HIV infection and they found that there was a negative correlation of Tregs (*FoxP3*+) and CD4 T cells chronic activation in clinical HIV infection. They found the percentage of CD4+CD25+ (Tregs) were much lesser among HIV positive individual in comparison to healthy controls ($p<0.001$). In our study we showed here that CD4+CD25+ cells in healthy individuals was 4.1% and the frequency decreased to 1.2% ($p<0.001$) during HIV infection. In addition they claimed that there was no correlation between Treg frequency and CD T cell count. Our results completely support Eller's results. In a study conducted by Sachdeva M *et al.,*[26] 2010 they found the percentage of CD4 cells among the controls was 30% and it reduced to 16% among HIV positive individuals. In their study they also showed that the Treg frequency in HIV viraemic patients was 0.16 and among healthy controls it was 2.0 percent. In our

study also we found an almost same trend. Cesar, MRR, *et al.,* 2012[27] has reported the same and our study corroborated the above reports.

While some more reports suggest a direct decrease in Tregs during HIV infections[29-31] Kyra Oswald-Richter., *et al.,* 2004[28] others showed there was an absolute CD4 count decrease. Other investigators distinguished Treg percentage positivity and absolute Treg count by Treg isolation and showed that there was an increase of Treg percentage positivity but the actual absolute Treg number had declined[32-34]. Julian Schulze zur Wiesch, 2011 [35]has shown that healthy control had a Treg frequency of 3.5% and it increased to 11.5% among HIV progressors. When these patients were treated with HAART the Treg frequency has swing back to 7.5% and has not gone up to the control population's frequency (3.5%). This observation was evident in our study also. We had a Treg percentage of 4.1% among healthy controls. This frequency was decreased to 1.2% among untreated patients however the frequency was swinging back to 12.6%. As we already described this swing back discrepancy may be due to the time taken for these cells to realign to control level.

Presicce*et al.,* 2011[36], had shown that Tregs frequency has increased significantly at 4 weeks of study but the frequency decline to normal number at 45 weeks. Thus the increase of percentage of Tregs may be transient. Though those studies showed decreased absolute Treg numbers they found an increase of Tregs frequency. The reason for this dicrepancy in unknown however it could be that in those studies they took follow up samples to compare the Tregs frequency. But we had only one sample per patient. Thus a paired sample from our patient may ravel whether patient also had an increase. Another thing which also to be considered will be the HIV strains that infected these various populations. Our samples are mostly HIV-1 Clade C origin (data not shown) however the strains in the reports by other investigators could be other clades or HIV-2. Those information need to be analyzed further.

Uniqueness about our study is that we have also analyzed the differences in Treg frequency between the genders, age of the patient and total CD4 count. In our study we did not find much difference between different groups. However the study population in our study was less to make a meaningful conclusion. Similar study with more number of patients would through more light on any such predilections. Our study showed that Treg frequency declines significantly during HIV infections. However upon HAART treatment the frequency was getting restored. Restoration was not only seen among the CD4 cells frequency but also among $CD4^{+}CD25^{+}$ cells as well. Our study did not distinguish whether our Tregs population was natural Tregs (nTregs) or induced Tregs (iTregs). It is speculated that it was a combination of both. From this study it could be reasonably assumed that we have to develop methodologies to prevent depletion of Tregs frequency since they are important regulatory cells during HIV infections and self-tolerant cells on the whole[37].

Conclusion

In this study we found that Tregs are in reduced concentration among HIV patients. However, the number is restored by HAART treatment. In addition we found no correlation with gender, age or host CD4 count with Treg frequency during HIV infections. This is probably the first study among Chennai population about the frequency of Tregs among HIV patients and healthy controls. Our study is a pilot study

in nature and further studies are thus required to confirm our finding. In addition there are many other markers for Treg such as CD127, CD45RO, etc. needs to be included for a better understanding. Our findings are important in the context of anti HIV immunotherapy and prevention of Treg depletion during early HIV infection may change the course of infection hopefully towards protection side. Immunotherapy has a hope to find a permanent cure for HIV and a significant breakthrough is the "Berlin Patient" Timothy Ray Brown's incident. 1% of Caucasians have a CCR5 gene mutation and they are resistant to HIV infections. Dr. Gero Huetterv of Fred Hutchinson Cancer Research Center, Seattle, WA, USA had transplanted stem cells to the Brown who was HIV infected for 18 years and now he is free of HIV and he is no longer taking anti HIV pills[38]. This is one of the rarest incidences in the medical history. But consistent and focused further research in this area can change the rarest things to routine. This study throws light on the importance of Tregs management and such management would offer better treatment protocols for HIV infections.

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Table 1. Percentage of CD4+ CD25+FoxP3+ Tregs cells among HIV/AIDS patients and healthy controls

S. No	Groups	Subgroups	Percentage of cells		
			$CD4^+$ cells	$CD25^+$ cells	$FoxP3^+$ cells
1	HIV	HAART Negative	13.3 ± 9.8^d	1.2 ± 0.7^a	0.3±0.4
		HAART positive	18 ± 8.6^e	12.6 ± 6.2^b	0.8±0.9
2	Controls		37.2 ± 5.2^f	4.1 ± 1.1^c	0.3±0.4

Venous blood samples from HIV positive patients and healthy controls were collected and stained with monoclonal antibodies against CD4, CD25 and FoxP3 molecules and quantified by Flow cytometry. Table shows the percentage ± Standard deviation (SD) of CD4+Tregs. HIV without ART cases was just enrolling for therapy and they have not received HAART at the time of sample collection. HAART positive (with ART) are cases that already started HAART therapy. Out of 71 HIV cases, 27 patients were HAART negative (without ART) and 44 were with ART and 37 were healthy controls. (Student's *t* test-a vs b-$p<0.001$, a vs c-$p<0.001$, b vs c-$p<0.001$; d vs e-$p=0.037$, e vs f-$p<0.001$, d vs f-$p<0.001$).

Table 2. Percentage positivity of Tregs among different sexes of HIV positive and healthy control population

S. No	Groups	Subgroups	Sex	Percentage of the cells (Mean ± SD)	
				$CD4^+$ Cells	$CD25^+$ cells
1	HIV (n=71)	HAART Negative (n=27)	Male (n = 15)	11.9 ± 7.1	1.3 ± 0.8
			Female (n=12)	15.1 ± 12.5	1.2 ± 0.6
		HAART Positive (n-44)	Male (n=25)	17.6 ± 8.7	12.2 ± 6.1
			Female (n=19)	18.9 ± 8.5	13.0 ± 6.4
2	Controls (n=37)		Male (n = 15)	36.6 ± 5.5	4.6 ± 1.0
			Female (n=12)	37.7 ± 4.9	3.7 ± 1.3

Blood samples from HIV infected patients and control population was collected as described above. Samples were divided based on their gender and the table shows the Mean ± SD of Tregs among HIV patient who are undergoing HAART therapy (HAART POSITIVE) and patients without it (HAART NEGATIVE). Similarly the healthy control were further divided and analysed.

Table 3. Percentage of Tregs in relation to total CD4 count

Groups	Subgroups	Sex	Total CD4 count	Percentage of Tregs (Mean ± SD)	
				$CD4^+$ cells	$CD25^+$ cells
HIV (n=71)	HAART Negative(n=27)	Male (n=15)	<350	8±2.8	1.7±1.1
			>350	15.1±8.3	1±0.3
		Female (n=12)	<350	7.7±2.8	0.9±0.2
			>350	17.6±13.6	1.2±0.6
	HAART Positive (n=44)	Male (n=25)	<350	13.5±9.3	11.3±6.6
			>350	22.3±4.6	12.5±7.0
		Female (n=19)	<350	20.6±14.4	19.8±4.7
			>350	18.3±6.7	11.2±5.5
Controls (n=37)		Male (n=20)		36.6±5.5	4.6±1.0
		Female (n=17)		37.7±4.9	3.7±1.3

Blood samples from HIV infected patients and control population was collected as described above. Samples were divided based on the gender which was further segregated into Total CD4 count. CD4 count of 350 and below is the cut-off for the initiation of HAART therapy hence this count was correlated with Treg frequency. Since CD25 is a strong marker for Treg in the following experiments CD4 with CD25 was considered as Tregs. Control population was not divided based on CD4 count.

Table 4. Percentage of Tregs in relation to age of the patients and controls

Groups	Subgroups	Sex	Age group (in Years)	Percentage of Tregs (Mean ± SD)	
				CD4+ cells	CD25+ cells
HIV (n=71)	HAART Negative(n=27)	Male (n=15)	<40	11.4±15.2	1.6±1.1
			>40	12.3±14.2	1.0±0.2
		Female (n=12)	<40	16.5±13.3	1.1±0.5
			>40	8.3±1.9	1.5±0.9
	HAART Positive (n=44)	Male (n=25)	<40	15.7±8.6	13.9±6.0
			>40	19.6±8.8	10.4±6.0
		Female (n=19)	<40	18.5±7.2	13.8±7.1
			>40	19.6±12.1	10.5±2.8
Controls (n=37)		Male (n=20)	<40	36.1±5.4	4.8±0.9
			>40	37.2±5.9	4.3±1.0
		Female (n=17)	<40	37.3±6.1	4.1±1.4
			>40	38.4±3.0	3.0±0.8

Blood samples from HIV infected patients and control population was collected as described above. Samples were divided based on the gender which were further segregated based on the age of the patients. We considered an age of 40 and below as cut-off since they form the sexually active population and hence the population was divided into <40 years and >40 years.

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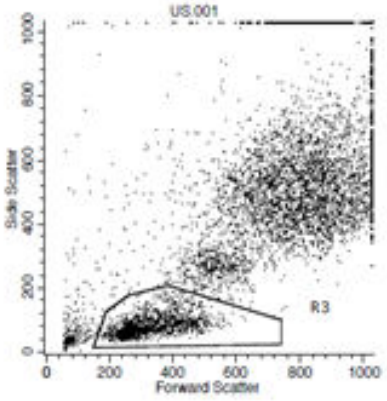

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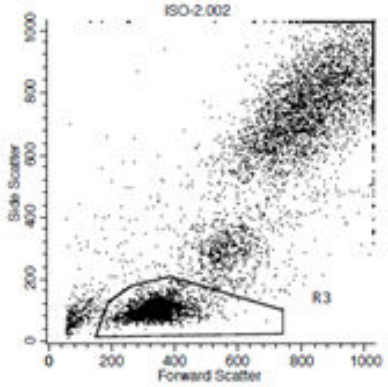

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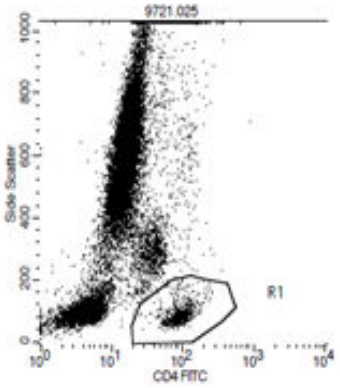

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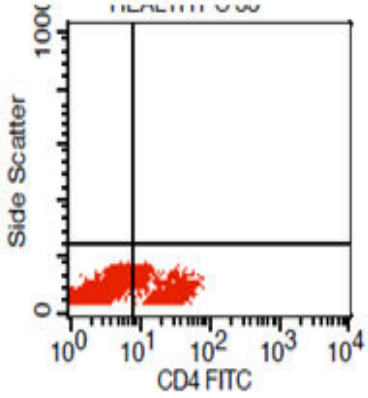

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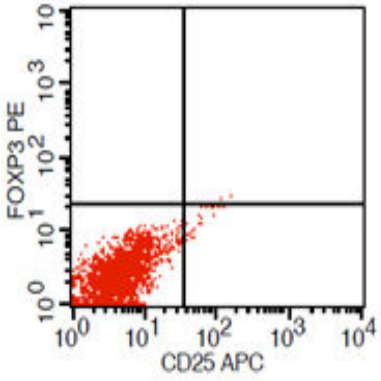

(e)

Figure 1. Representative figure for CD4+CD25 T Reg in peripheral blood samples by analysis of surface CD25 on CD4 cells. (a) unstained control. (b) Isotype control. (d) CD 4 gated. (e) CD-25 and Foxp3

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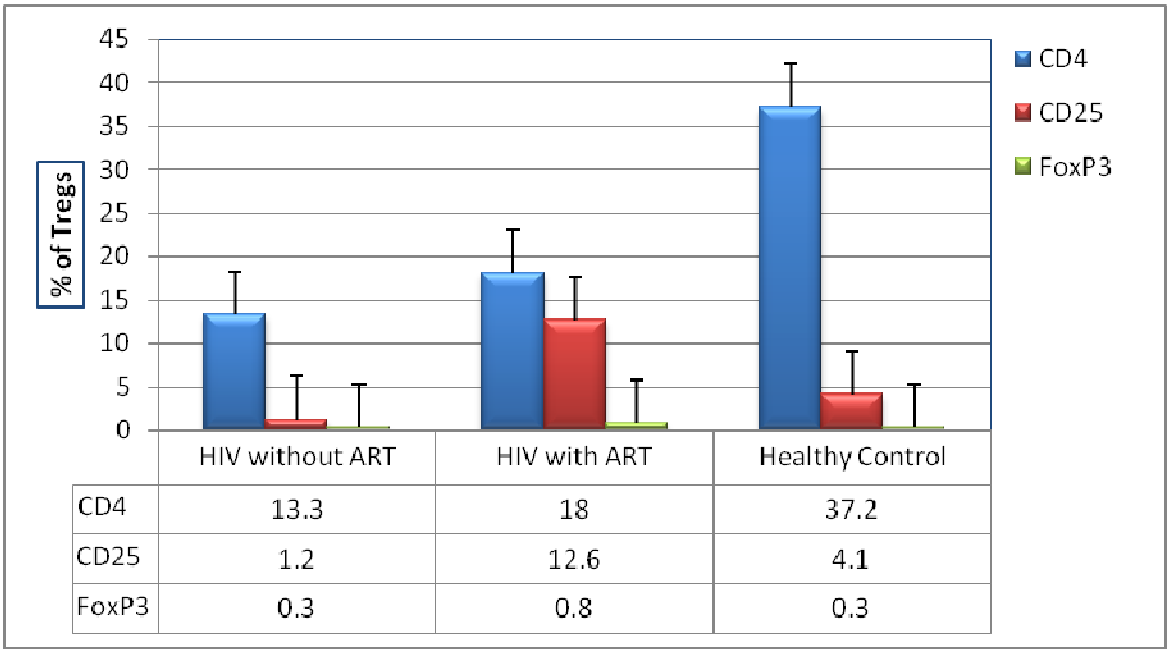

	HIV without ART	HIV with ART	Healthy Control
CD4	13.3	18	37.2
CD25	1.2	12.6	4.1
FoxP3	0.3	0.8	0.3

Figure 2. Frequency of CD4+ CD25+FoxP3+ Tregs among HIV/AIDS patients and healthy controls. Venous blood samples collected from HIV positive patients and healthy controls were collected and stained with monoclonal antibodies against CD4, CD25 and FoxP3 molecules and quantified by Flow cytometry. Figure shows the percentage ± Standard deviation (SD) of CD4+Tregs. In the graph X axis shows the various groups tested in the study and Y axis shows the percentage of Tregs