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i. Title Page

Title: High frequency of memory stem cells with a distinct gene signature in HIV patients with treatment interruption

Running Title: Memory stem cells and HIV-infection

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List of Abbreviations

Tscm, Memory stem cells; ART, anti-retroviral therapy; TLE, Tenofovir, Lamivudine, Efavirenz; ATV, Atazanavir; 3TC, Lamividune; NVP, Nevirapine; qRT-PCR, Qunatitative real time polymerase chain reaction;

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ii. Abstract

Reservoirs of HIV remain a major obstacle to the complete eradication of virus despite regular anti-retroviral therapy (ART). Memory stem cells (Tscm), one of the major reservoirs are relatively less studied owing to their presence in lower numbers and inaccessible anatomical locations. We have evaluated the molecular characteristics of Tscms in patients with ART interruption (n=15) versus patients on uninterrupted ART (n=12) using flowcytometry. RNA sequencing was done in the sorted Tscms to study the differential gene expression. Patients with ART interruption had significantly lower baseline CD4+T-cell counts and high viral loads as compared to patients on ART. The former group had significantly higher frequency of CD4+ and CD8+Tscms with a higher expression of PD-1 on CD8+Tscms. The transcriptome profile of Tscm was significantly different amongst the patient groups. The main pathways were cellular and metabolic pathways, cellular development pathways, cell differentiation and negative regulation of cellular migratory pathways. An increased yet dysfunctional CD8+ memory stem cells describes HIV-1 infected patients with break in ART and a distinct transcriptional signature of CD4+ Tscm as compared to those of patients on ART. A more detailed understanding of biology and dynamics of Tscm in future studies is warranted. Strategies to improve the functionality of the CD8+ Tscm will help these patients to tackle the outburst of viral replication that occur after the cessation of therapy.

Key words HIV, memory stem cells, Tscm, anti-retroviral therapy, transcriptome, gene-signature

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Conflicts of Interest

The authors declare no conflicts of interest

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iv. Main Text

Introduction

With the widespread use of antiretroviral therapy (ART), the life expectancy of HIV-infected patients has gone up and number of AIDS related deaths has gone down [1]. However, the therapy remains life-long because interruption in ART leads to virus rebound even after a successful suppression of viremia. This is due to the persistence of virus in certain pockets or reservoirs in the infected individuals, where the HIV remains hidden from the effects of ART, despite the virus remaining undetectable for long periods of time. Besides many other cells such as myeloid cells, dendritic cells, follicular dendritic cells [2, 3], a unique population of resting CD4+ T cells has recently been shown to be involved in the maintenance of stable reservoirs of HIV in the patients on ART [4]. With the identification of memory T cells with stem cell properties in 2011, increasing attention is being focused on this subpopulation termed as the stem cell-like memory T-cells or Tscm, because of their enhanced self-renewal capacities and multipotency to generate all memory and effector T cell subsets *in vitro* [5, 6]. Recently, it has been reported that this subset serves as a stable reservoir for HIV and harbor high per-cell levels of HIV-1 DNA and make increasing contributions to the total viral reservoir over time [7]. These cells represent the earliest developmental stage of memory T cells; differentiate into large numbers of effector T cells, while maintaining their own pool size through homeostatic self-renewal. It has been demonstrated that Tscm can be infected *in vitro* by CCR5-tropic HIV-1 strains and that the relatively low frequency of infection correlates with a relatively low level of CCR5 expression as compared with other subtypes. Phenotypically, Tscm are defined in humans as CD45RA+CD45RO+CD62L+ CCR7+CD27+CD28+CD127+CD95+CD122+ cells. These are found predominantly in peripheral blood and secondary lymphoid tissues, but are rare in mucosal tissues.

The mechanisms involved in the maintenance of memory in CD4+ T cells might be operative in the establishment of HIV reservoirs. Asymmetric division of latently infected cells upon sporadic antigen encounter may replenish the reservoir [8]. Homeostatic proliferation driven by cytokines like IL-7 and IL-15 may also drive the maintenance of HIV reservoir [9]. The negative immune regulatory pathways such as those modulated by negative receptors, such as PD-1 interaction with its ligand PD-L1, may also lead to persistence [10]. It has been observed that PD-

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1^{high} cells from patients preferentially harbor HIV- integrated DNA when compared to their PD-1^{low} counterparts, indicating that these cells constitute a preferential reservoir for the virus [11].

ART discontinuation has deleterious health effects with poor clinical outcomes signified by viral outburst and suppressed CD4 T-cell numbers in such patients [12]. The effect of such an ART non-adherence on memory stem cells has not been studied before. Therefore, it becomes imperative to understand how and how much this phenomenon affects the memory stem cell population. In this context, we have studied the patients with a break in ART and compared them with patients who were on regular ART.

Methods

Study Groups

HIV-1 infected subjects visiting the HIV Diagnostic and Disease Monitoring Laboratory in the Department of Immunopathology and Anti-Retroviral Therapy (ART) clinic at the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh were recruited. The study protocol was approved by the Institute's ethics committee. Written informed consent was obtained from each one of the subjects. Twenty-five milliliters of whole blood were collected in heparin coated vacutainer tubes. Individuals confirmed positive for HIV-1 infection by Strategy III as per National AIDS control organization (NACO) guidelines (NACO guidelines on HIV testing, 2007) (n=27; 23 males and 4 females) were recruited in the study. These patients were monitored periodically for CD4+ T cell counts and plasma viral load. **Table 1** shows the demographic and clinical profiles of all these patients. These patients were divided into 2 groups based on their treatment continuation. The ART regimens consisted either of a combination of Tenofovir, Lamivudine, Efavirenz (TLE) or Atazanavir; 3TC: Lamividune; NVP: Nevirapine (ATV).

Group 1 consisted of 15 patients (age 35.80 ± 1.3 years) taking uninterrupted ART for a minimum of 1 year, with a median (range) CD4+ T-cell count of 492 (134-1479) cells/mm^3. The CD4 count these patients increased from 310 ± 34 cells/mm^3 to 587±83 cells/mm^3 (an increase 248±37 cell/mm^3) over a period of 6 months of ART. Plasma viral load was less than 150 copies per ml. Group 2 included 12 patients (age 30 ± 1.5 years) with a self-reported interruption in ART for

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about 2 years, had a median (range) CD4+ T-cell count of 217.5 (77-496) cells/mm^3 at the time of reporting. On average, these patient took ART for 3 years before discontinuing their therapy. They were not taking any ART at the time of recruitment. The median (range) viral load was 11706 (149-897247) copies/ml in this group. Patients with Tuberculosis and other chronic infections (Hepatitis C and B) were excluded from the study.

CD4+ T cell counts

Whole blood CD4+ T cell counts were determined using BD Trucount tubes™ (BD Biosciences) by Flowcytometry.

Plasma Viral load

HIV-1 plasma viral load was quantitated by real time polymerase chain reaction (qRT-PCR) using Roche platform (Roche, USA) as per the manufacturer's guidelines. Results were expressed as HIV-1 RNA copies per ml of plasma.

Phenotypic analysis using flow cytometry

A standardized protocol using Ficoll-hypaque density gradient centrifugation for isolating PBMCs was used and the cells were stained with various fluorochrome-conjugated monoclonal antibodies from BD Biosciences, San José CA, USA. The antibody conjugates used for phenotype analysis were CD4-phycoerythrin [PE]-Cy5, anti-CD95-allophycocyanin [APC], anti-CD3 APC-H7, anti-CD45RA PE-CF594, anti-CD197 (CCR7)- PE anti-CD62 L PE-Cy7, anti-CD122-Brilliant violet [BV421], anti-CD279 (PD-1)-fluorescein isothiocyanate [FITC]. Fluorescence minus one (FMO) control were used as gating controls. Precisely, one million PBMCs from each subject were stained with antibodies for different cell surface markers in dark for 30 minutes at room temperature. The stained cells were suspended in equal volumes of wash buffer and 1% paraformaldehyde solution. Cells were acquired on flowcytometer (FACS ARIA, Becton Dickinson, USA) and data analyzed using Flowjo™ software (BD Life Sciences, San José, CA, USA).

Tscm cell sorting by flowcytometry

All the settings for cell acquisition were done using Comp Beads (the compensation bead particle set from BD Biosciences) and the drop delay settings were done using the BD Accudrop™ reagent. The lymphocyte population was gated to exclude doublets and further the CD3+CD4+ cells were

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gated to include CD45RA+CCR7+ memory T-cells. The memory cells were further gated down to include only CD62L+ central memory cells, and further the CD95+CD122+ cells were selected as memory stem cells (Tscm).

RNA extraction, sequencing and analysis

RNA from sorted cells was extracted using the Trizol (Invitrogen, USA) reagent as per manufacturer's instructions. The purity of RNA was assessed initially on a nanodrop by measuring absorbance at 260 and 280 nm. The quality and integrity were assessed by capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies. Spain). The RNA sequencing and analysis was done from MedGenome Labs Ltd (Bengaluru, India). The transcriptome of a total of 6 human samples was analysed. Since the RNA yield was less, a modified SMART-seq stranded kit protocol (Clontech, now Takara Bio USA, Inc) was used to prepare the libraries for total RNA sequencing. Prepared libraries were sequenced on Illumina HiSeqX to generate 60M, 2x150bp reads per sample.

Bioinformatics analysis:

Quality check for raw data: The following parameters was checked from fastq file: Base quality score distribution, Sequence quality score distribution, Average base content per read, GC distribution in the reads, PCR amplification issue, check for overrepresented sequences and Adapter trimming. Based on the quality report of fastq files we trimmed sequence read where necessary to only retain high quality sequence for further analysis. In addition, the low-quality sequence reads are excluded from the analysis. The adapter trimming was performed using Trimmomatic (v-0.36).

Contamination removal: For the RNA-Seq analysis, the unwanted sequences were removed, especially non-polyA tailed RNAs from the sample (assuming that poly-A tailed RNAs are sequenced). The unwanted sequences included - mitochondrial genome sequences, ribosomal RNAs, transfer RNAs, adapter sequences and others. Contamination removal was performed using Bowtie2 (2.2.4).

Read alignment: The paired-end reads were aligned to the reference human genome (hg19). Alignment was performed using HISAT2 (2.1.0).

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Expression estimation: The aligned reads were used for estimating expression of the genes. The raw read counts were estimated using FeatureCount (1.5.2). Read count data were normalized using DESeq2.

Differential gene

expression analysis: The raw read counts were normalized using DESeq2.The ratio of normalized read counts for treated over control was taken as the fold change. Genes were first filtered based on the pValue (<= 0.05). A distribution of these log2 (foldchange) values were found to be normally distributed. Those genes which were found to have -1 <= log2(foldchange) >= 1 were considered as statistically significant.

GO Annotation and Pathway analysis: GO annotation and Reactome pathway information for differentially expressed genes were done using Panther database.

Statistical Analysis: Data are expressed as mean values ± standard error. The statistical significance of differences was determined by the Mann-Whitney U test for comparison between 2 different groups, Kruskal–Wallis test followed by Dunn's multiple comparison test for comparisons between different. Spearman's r test was used to determine correlations between two variables. Column statistics, including medians, means, standard error and frequencies were tabulated for the raw data separately for all the study groups. A p value of less than 0.05 was considered statistically significant. All data was analyzed using Graph Pad Prism software (version 5.0, Graph Pad Software Inc., La Jolla, CA, USA) and the comparative graphs between different groups were plotted.

Results

1. High frequency of memory stem cells in patients with break in ART

The frequency of total CD4+ T helper cells, CD8+ T cytotoxic cells and CD4+ memory stem cells (Tscm) were analyzed in the two patient groups, comprising group 1 having patients who were taking ART without any interruption and group 2 with patients who had interruption in ART. **Figure 1** depicts the scheme for gating memory stem cells. We observed a significant difference in the percentage as well as absolute count of CD4+ helper T cells (**Figure 2a**) between the two group of patients ($p<0.05$). The group 1 had significantly higher percentage of CD4+ T-cells (mean

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± SE: 21 ± 3.7) as compared to group 2 (mean ± SE: 11 ± 1.3). The absolute CD4+ T-cell count was also statistically significant ($p<0.001$) between the 2 groups, CD4+ T-cell count (mean ± SE) in group 1 being 587 ± 83.5 and in group 2, it was 249 ± 37. The difference in the frequency of CD8+ T cells was not statistically significant between the 2 patient groups (data not shown). The frequency of CD122+CD95+ Tscm cells was significantly ($p<0.05$) higher (19±6 %) in group 2 patients as compared to group 1 (3.7 ± 1.1 %) **(Figure 2b).** There was a negative correlation in the frequencies of Tscm and CD4+ T-cells in the two groups ($r = -0.7$, $p = 0.02$ in group 1; **Figure 2c**) and ($r = -0.65$, $p = 0.015$ in group 2; **Figure 2d**). These results indicate the generation of a higher number of circulating memory stem cells as the patients discontinue their therapy and a decrease in this with reconstitution of CD4 T cell numbers. Considering their stem cell like characteristics, it might also be possible that these cells are relatively depleted less than other subsets after treatment interruption. In addition, there was no significant difference in the number of other T cell subsets (naïve, central memory, effector memory and effectors) between the 2 patient groups. Both the patient groups harbor more number of central memory CD4+ T-cells as compared to other T cell subsets. On the other hand, both the groups had higher numbers of CD8+ effector memory and effector cells as compared to central memory population (**Supplementary Figure**).

2. Higher frequency of PD-1+CD8+ memory stem cells in patients with break in ART: Since CD8+ memory stem cells are efficient in suppressing the viral infected cells, we sought to determine the frequency of CD8+ Tscm in both the patient groups. In addition, we also evaluated the frequency of PD-1+CD4+ and PD-1+CD8+ T-cells in patients with break in ART. **Figure 3** shows a representative picture for gating PD-1 from CD4+ (b) and CD8+ T-cells (c) and from CD4+ (d) and CD8+ Tscm (e). Gating was done as per the fluorescence minus one control (a). The frequency of CD195+CD122+ cells were measured in CD45RA+CCR7+CD62L+ cells from the CD3+CD4-ve cells that would represent the CD8+Tscm. The frequency of this population was significantly higher in patients with break in ART as compared to patients undertaking regular ART (**Figure 4a**). The percentage of PD-1+CD8+ T-cells (50 ± 4.6) was significantly higher than the PD-1+CD4+ T-cells (28 ± 5) ($p<0.05$) (**Figure 4b**). In addition, the frequency of PD-1+CD8+ Tscm (24 ± 3) was also higher than the PD-1+CD4+Tscm in the group 2 patients (13 ± 2) ($p<0.05$) (**Figure 4c**). These cells had a positive correlation with viral load, although statistically non-significant. The patients on ART did not show any differences in the PD-1 expression on CD4+

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T-cells and CD4+Tscm as compared to CD8+ T-cells and CD8+Tscm. These results indicate that although the frequency of circulating CD8+ memory stem cells were higher in patients with a break in ART, these cells owing to higher expression of PD-1 may be functionally exhausted and hence not able to clear the viral infected cells.

3. CD4 memory stem cells from patients on ART show differential transcriptional profile compared to patients on break in ART

To further evaluate whether it is just the frequency difference of memory stem cells between these patient cohorts or there are differences at the transcriptional levels also, we sorted the memory stem cells from both the patient groups and did the mRNA sequence analysis. CD4 memory stem cells were sorted from 3 patients from each group. For this, approximately, 18 to 20 million PBMCs were sorted to obtain a yield of 80,000 to 100,000 CD4 memory stem cells. The purity of sorted cells was approximately 96-97 % (**Figure 5a**). We used 100 ng of RNA from each sample. All samples had a RNA integrity number more than 7. RNA extracted from these cells was subjected to next generation sequencing. Data generation for each sample was in the range of 6GB to 13GB and the average % Q30 was nearly 90%.

The hierarchical clustering of the data of all the samples is shown in **Figure 5b** indicating that the samples of each group clustered separately. Based on the thresholds set for fold-change and p value, (p-adj <=0.05 and log2FC >=1), the differential gene expression comparing gene expression between group 1 versus group 2 was done. A total of 664 genes were significantly upregulated and 6524 genes downregulated. The volcano plot also displays the results of a differential gene expression between the two groups (**Figure 6**). Each point in the scatter plot represents a gene; the axes display significance versus fold-change estimated by the differential expression analysis. Green points indicate significantly up and red points indicate significantly down-regulated genes.

On the basis of gene expression**, Figure 7a** displays top 20 upregulated and top 20 downregulated genes (p value). We have plotted the top biological process for upregulated and downregulated genes for each comparison **(Figure 7b).** The top enriched biological pathways for up-regulated genes belong to cytoplasmic translation, peptide biosynthetic process, peptide metabolic process, amide biosynthetic process, system process and negative regulation of leukocyte migration. The top enriched biological pathways for down-regulated genes belong to multicellular organism process, anatomical structure development, system development,

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multicellular organism development, anatomical structure morphogenesis, animal organ development, cell differentiation, cellular developmental process and tissue development.

Discussion

Studies have demonstrated that suboptimal adherence or non-adherence to ART could be a significant contributing factor to the chronic residual inflammation, coagulopathy and immune activation observed in patients living with HIV, even if it is sufficient to achieve and sustain plasma viral suppression [13]. An elusive cure of HIV would require that cells that contribute to infectious virus must be completely eliminated. Therefore, in recent times the attention is being focused on understanding the reservoirs of HIV and the mechanism of their establishment [14]. Resting memory T cells particularly with stem cell properties have been regarded as the main reservoirs of HIV, although other reservoirs also exist such as peripheral T follicular helper cells, macrophages, astrocytes and microglia in central nervous system [15]. These cell types are capable of integrating a replication competent HIV and can therefore support latency for a long time. Research is ongoing in understanding these reservoirs. The memory stem cells were originally defined as a CD122pos/CD95high population by Buzon et al 7, however, we observed a weaker CD122 staining in our analysis. Cessation of therapy at some point occurs almost inevitably in the life of a HIV infected patient for one reason or the another. Hence, studying the immune system particularly the reservoirs of HIV become quite pertinent in this cohort of patients.

In this study, we tried to understand the phenomenon by looking at the differences in the frequency of not only the main subsets of T-lymphocytes but also the population of memory like stem cells among the CD4 and CD8 cell subsets. We observed that the patients who had a disruption in the ART have a higher frequency of both CD4 and CD8 memory stem (Tscm) cells that are not just numerically different but also transcriptionally different than the memory stem cells of patients who had adherence to ART without any interruption. Our data revealed some interesting facts as it showed that the CD8+ memory stem cells that have a significant role in viral suppression might be functionally defunct because of a higher expression of PD-1, a classical exhaustion marker, on both total CD8+ T-cells as well as CD8+ memory stem cells. Owing to their property of long *in vivo* life-span and self-renewal, CD4+ T memory stem cells (Tscm) have been proposed as an important site of viral latency. The HIV has been indicated to actually exploit these stem cell like properties to promote its own persistence in these cells [7]. It has been reported in

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earlier studies that duration of ART correlated directly with the relative contribution of memory stem cells to the overall reservoirs of HIV in patients undertaking ART [16]. A higher number of CD4+ memory stem cells in patients who have discontinued therapy is expected in the circulation and eventually may contribute to the active virus in blood of such patients. In another report, CD4+ memory stem cells were found to positively correlate with disease progression in chronically infected HIV patients. In this study, prolonged ART led to a reconstitution of CD4+ Tscm in parallel with CD4 T-cell numbers and decreased T cell immune activation [17]. This is in contrast to our findings of an overall negative correlation of CD4+ Tscm with CD4+ T-cell counts in all patients. This finding indicates a role of these cells in HIV pathogenesis, because their presence in more numbers would mean differentiation of these cells into a greater number of memory cell types, which ultimately would lead to establishment of more viral reservoirs and virus dissemination. In addition, this observation that frequencies of CD4+ T-cells and memory stem cells had a negative correlation in all patient groups indicate that this finding may be generalized to all HIV patients. An HIV patient with low CD4 T-cell count irrespective of therapy status may have a higher number of circulating memory stem cells although this finding needs to be validated in more of HIV patients with different therapy status and CD4+ T-cell counts. In support of our results, a recent study reported an expansion of CD4+ memory stem cells during acute HIV-1 Infection, which was linked to rapid disease progression [18]. These authors attributed this expansion of Tscm to an upregulation of Fas on the surface of naïve T cells, although unclear, this may induce Fas-mediated killing of CD4+ T-cells and their contribution of bystander cell death.

Similar to circulating CD4+ Tscm, HIV-infected patients with an interruption in the ART had a strikingly increased frequency of CD8+ Tscm as compared to patients on ART in our index study. The CD8 Tscm are thought to be involved in suppressing viral infection and are associated with an improved prognosis during chronic HIV-infection [19, 20]. It has also been reported that potent ART preserves the number of CD8+ Tscm [21]. It is important that the cells should be functional rather than just being merely abundant. All chronic viral infections are characterized by a state of CD8+ T-cell exhaustion that is associated with expression of the programmed cell death 1 (PD-1) inhibitory receptor [22]. We observed that these patients had an increased expression of PD-1 on them which will make them functionally exhausted. This also explains the negative correlation of CD8 Tscm with CD4+ T-cell counts in the patients with break in therapy. This would also imply

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that these CD8 memory stem cells expressing PD-1 will be the ones that will proliferate after the blockade of the interaction this molecule with its ligand.

To further elucidate the molecular mechanisms, we tried to decipher the differences at the mRNA levels. We evaluated the differential mRNA abundance in the memory stem cells sorted from both the patient groups. The gene among most differentially expressed genes belonged to long non-coding RNAs (lncRNAs) that can regulate the expression levels of messenger RNAs (mRNAs) by decreasing the amount of miRNAs interacting with mRNAs and now been identified as key players in the cellular differentiation processes and tissue homeostasis [23]. More recently these are also observed to be involved in maintenance of immunological memory [24]. Another gene found to be significantly upregulated in the group 2 patients is NOG. This is mainly involved in the differentiation of mesenchymal stem cells, thus indicating similar pathways being regulated in the memory stem cells as well [25,26].

Significant difference in the expression profiles was observed among the genes regulating the cytoplasmic translation, peptide biosynthetic process, peptide metabolic process and translation process, all of these significantly upregulated in patients with interruption in ART. On the other hand, the genes regulating the multicellular organismal process, anatomical structure development, system development and multicellular organism development were found to be down-regulated in these patients. Differences in the cytoplasmic translation would lead to differences in the array of proteins formed in the cytoplasm.

A major limitation of the study is the small sample size of study subjects, which can be attributed to the fact that the patients with a break in ART are difficult to catch and recruit. Despite this limitation, we recruited a comparable number of patients in both the study groups. A longitudinal analysis of such patients after ART initiation and immune reconstitution would shed more light on the dynamics of memory stem cells. To the best of our knowledge, such an evaluation of memory stem cells in patients with break in ART and comparison with patients on ART has not been reported till date. The strength of the study lies in a uniform criterion for recruitment of patients in both the study groups.

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Conclusion

HIV-infected patients with a break in ART are most vulnerable to the effect of viral outburst and hence deserve more attention to tackle the ongoing spurt of aberrant immunological changes. In our study, these patients were observed to have an increased expansion of both CD4 and CD8 memory stem cells in their circulation that had a negative correlation with CD4+ T-cell numbers. However, such an increased armory of CD8+ memory stem cells is functionally exhausted and unable to keep the virus replication in check. Gene regulation and activation of different biological pathways occur in the memory stem cells of these patient's vis a vis patient on regular ART suggesting changes that affect at the transcriptional level during this break period. A more detailed understanding of biology and dynamics of these memory stem cells, particularly in non-adhering patients is warranted in future so as to create new strategies to improve the functionality of these memory stem cells.

Authors Contributions

Meenakshi Sachdeva obtained the funding, performed the experiments, analyzed and interpreted the data and wrote the manuscript. Aman Sharma provided the study patients, reviewed the manuscript. Sunil Arora conceptualized and designed the study, finally reviewed and approved the article.

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Figure Legends

Figure 1: Representative gating strategy for CD4+ memory stem cells: The lymphocytes were gated on the basis of size and scatter properties from the singlet population after excluding doublets. From the lymphocytes, the CD3+CD4+ cells were further gated to choose CD45RA+CCR7+ cells, further to gate CD62L+ cells (central memory). From this population, the CD95+CD122+ cells or the Tscm were finally gated out. The gating of all these stained populations was done on the basis of unstained populations. The gating of CD95 and CD122 was done on the basis of fluorescence minus one (FMO) control.

Figure 2: Lower frequency of CD4+ T-cells and higher frequency of CD4+ Tscm in patients with break in ART. Flow cytometry was used to determine the frequency of cell populations in both the patient groups. (a) Frequency of CD4+ T-cells measured as percentage (left axis) and absolute cell count (right axis) was significantly lower in Group 2 patients as compared to Group 1 patients. (b) Frequency of CD4+ Tscm was significantly higher in Group 2 patients as compared to Group 1 patients. Spearman correlation was used to determine the correlation between the frequency of CD4+ T-cells and frequency of CD4+ Tscm. Negative correlation of CD4+ T-cells and CD4+ Tscm in (c) patients on ART (d) patients with break in ART. * $p< 0.05$, *** $p<0.001$, Non-parametric Mann-Whitney test was used to compare groups, r: spearman correlation coefficient, Group 1: patients on ART, Group 2: patients with break in ART.

Figure 3: Representative dot plot showing gating strategy for PD-1. (a) On the basis of FMO control, the gating of PD-1 was done in all subsets. The PD-1+ cells were gated from (b) CD3+CD4+ cells and (c) CD3+CD4-ve cells. The PD-1+ cells were also gated from the (d) CD3+CD4+ Tscm and (e) CD3+CD4-ve Tscm to evaluate the frequency of PD-1 + cells in these cell subsets. FMO: Fluorescence minus one

Figure 4: Higher frequency of CD8+Tscm and PD-1+CD8+ and PD-1+ CD8+ Tscm in patients with break in ART. Cumulative data in patients with break in ART showed higher frequencies of (a) CD8+Tscm as compared to CD4+ Tscm. (b) PD-1+CD8+ T-cells as compared to PD-1+CD4+ T-cells and (c) PD-1+ CD8+ Tscm as compared to PD-1+CD4+ Tscm cells in patients with break in ART. * $p<0.05$; ** $p<0.01$, Mann-Whitney test to compare the group of patients, Group 1: patients on ART, Group 2: patients with break in ART.

Figure 5: Flow cytometry was used to purify the CD4+ Tscm from both the patient groups. The purity of sorted CD4+Tscm was determined. (a) Representative picture to show the purity of CD4+ Tscm post sorting. The sorted cells were more than 95% pure. RNA was extracted from the sorted cells for sequencing by NGS. (b). Hierarchical clustering of the data depicted samples belonging to Group1 (Green) were clustered together while 3 samples of Group 2 (Orange) were clustered together indicating genetic similarity between these samples within a group.

Figure 6: Differential gene expression between the 2 groups of patients. Volcano plot for the comparison between patient groups displaying difference in log2–fold and statistical significance (p value*) of each gene calculated based on differential gene expression analysis. Every point in the plot represents a gene. Green points indicate significantly up-regulated genes and red points indicate significantly down-regulated genes.

Figure 7: a). Heat map plot displaying top 20 upregulated and top 20 downregulated genes (p value) on a gradient scale based on expression. b). The figure shows top enriched biological pathways for up-regulated genes (top) and down-regulated genes (bottom), (based on p value) of Group 1 vs Group 2 comparison.

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Figures

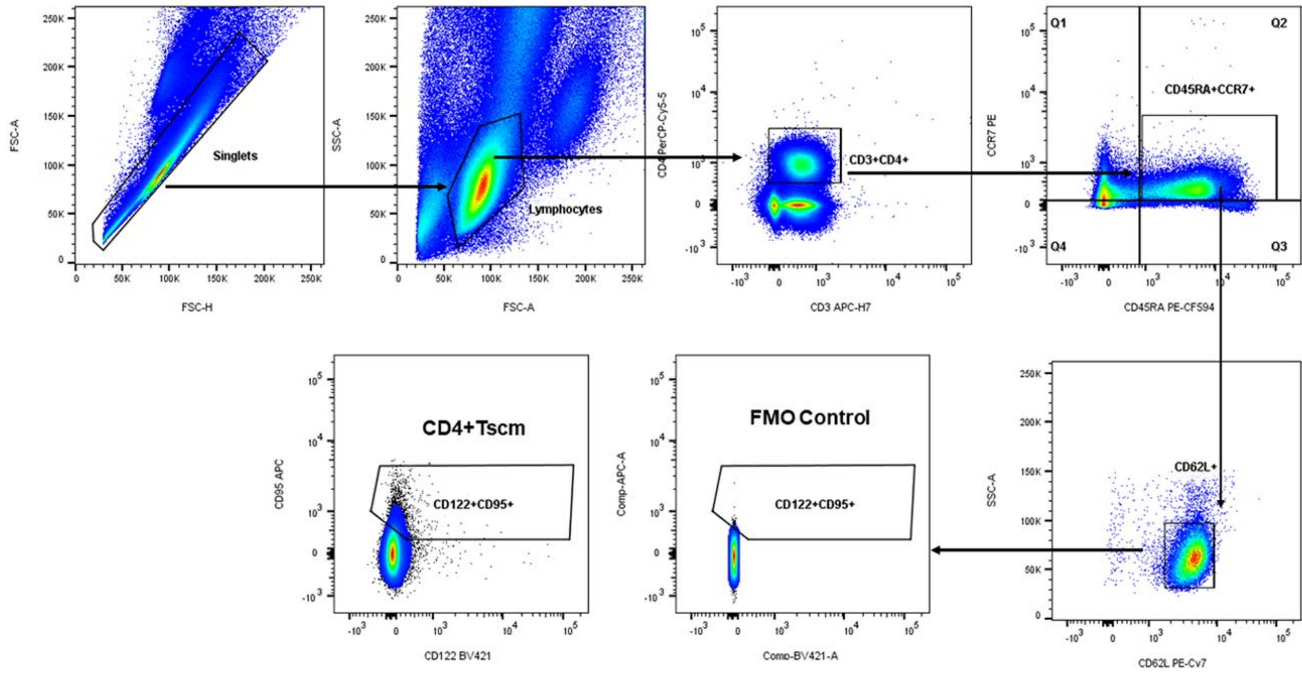

Figure 1

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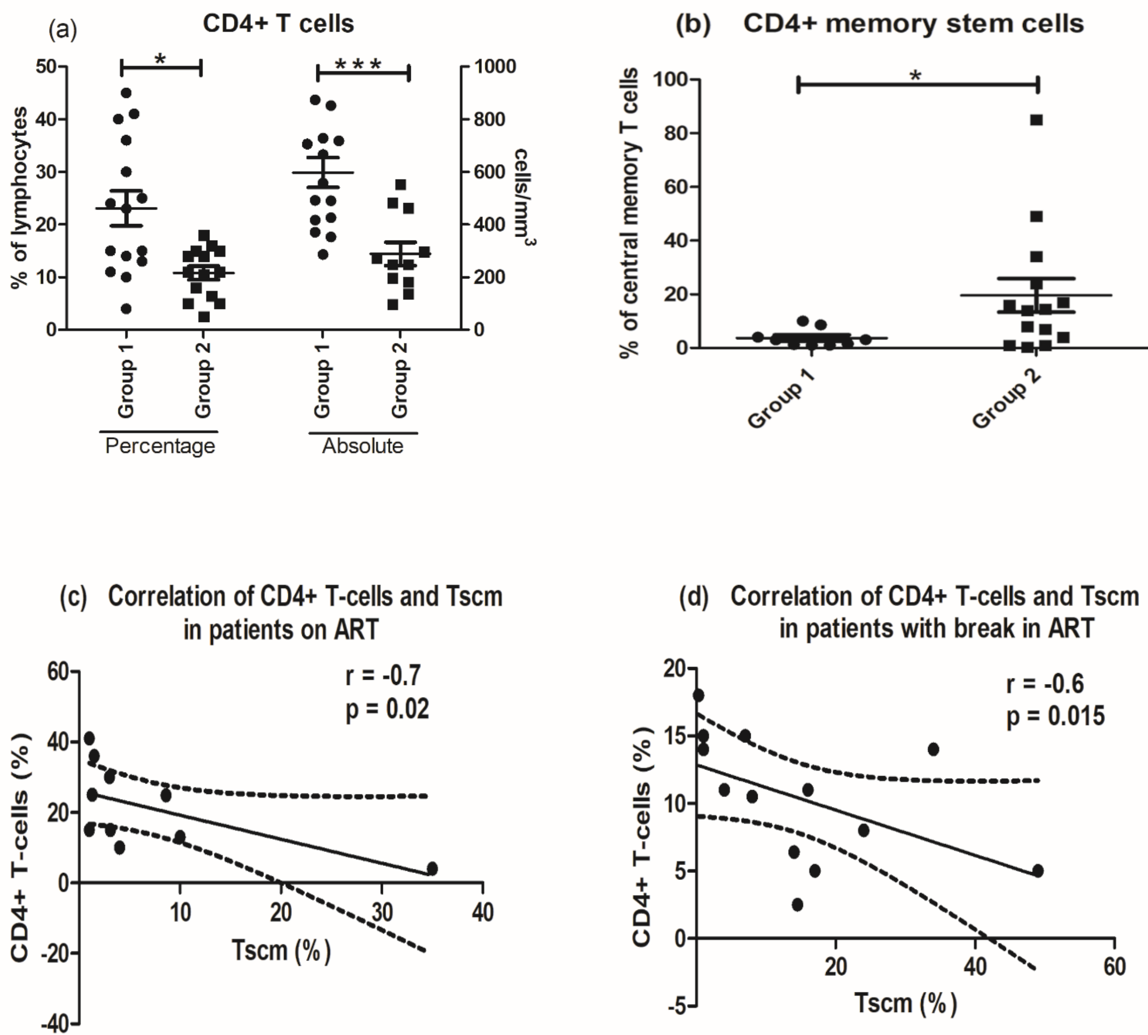

Figure 2

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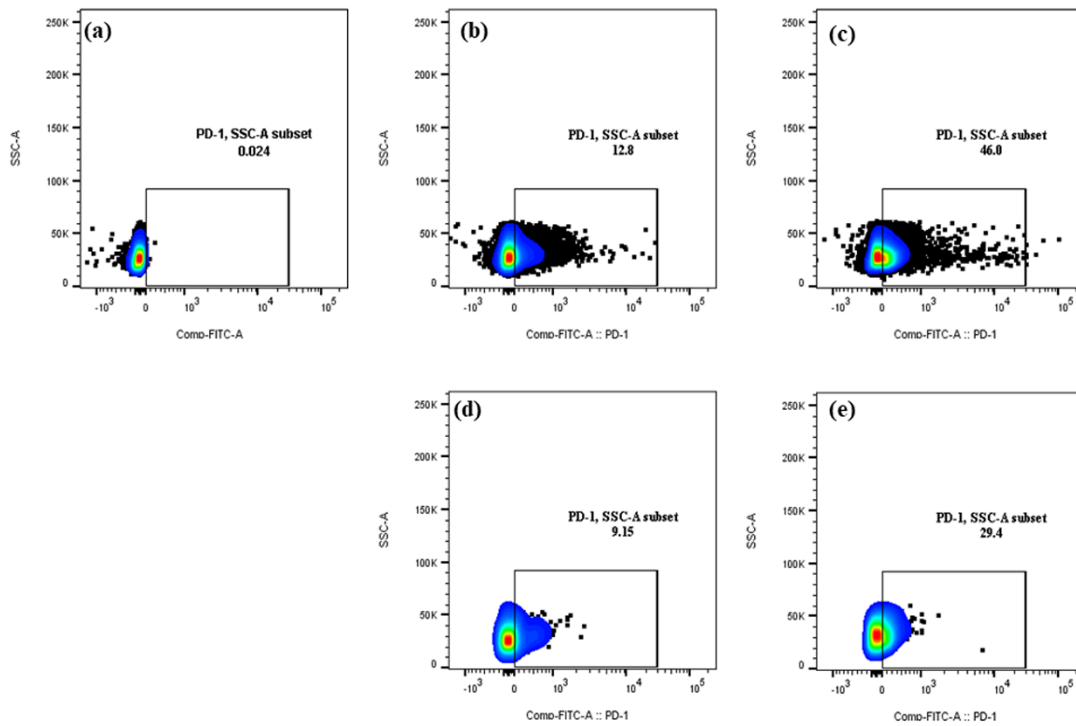

Figure 3

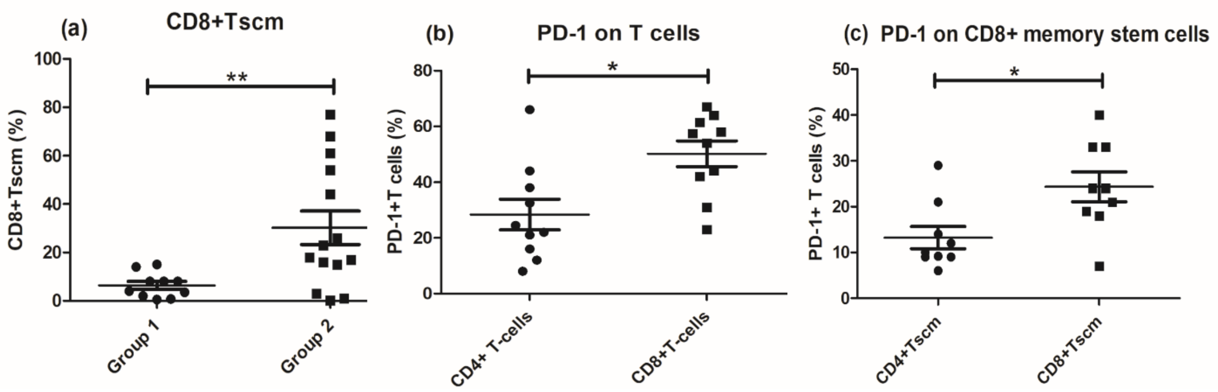

Figure 4

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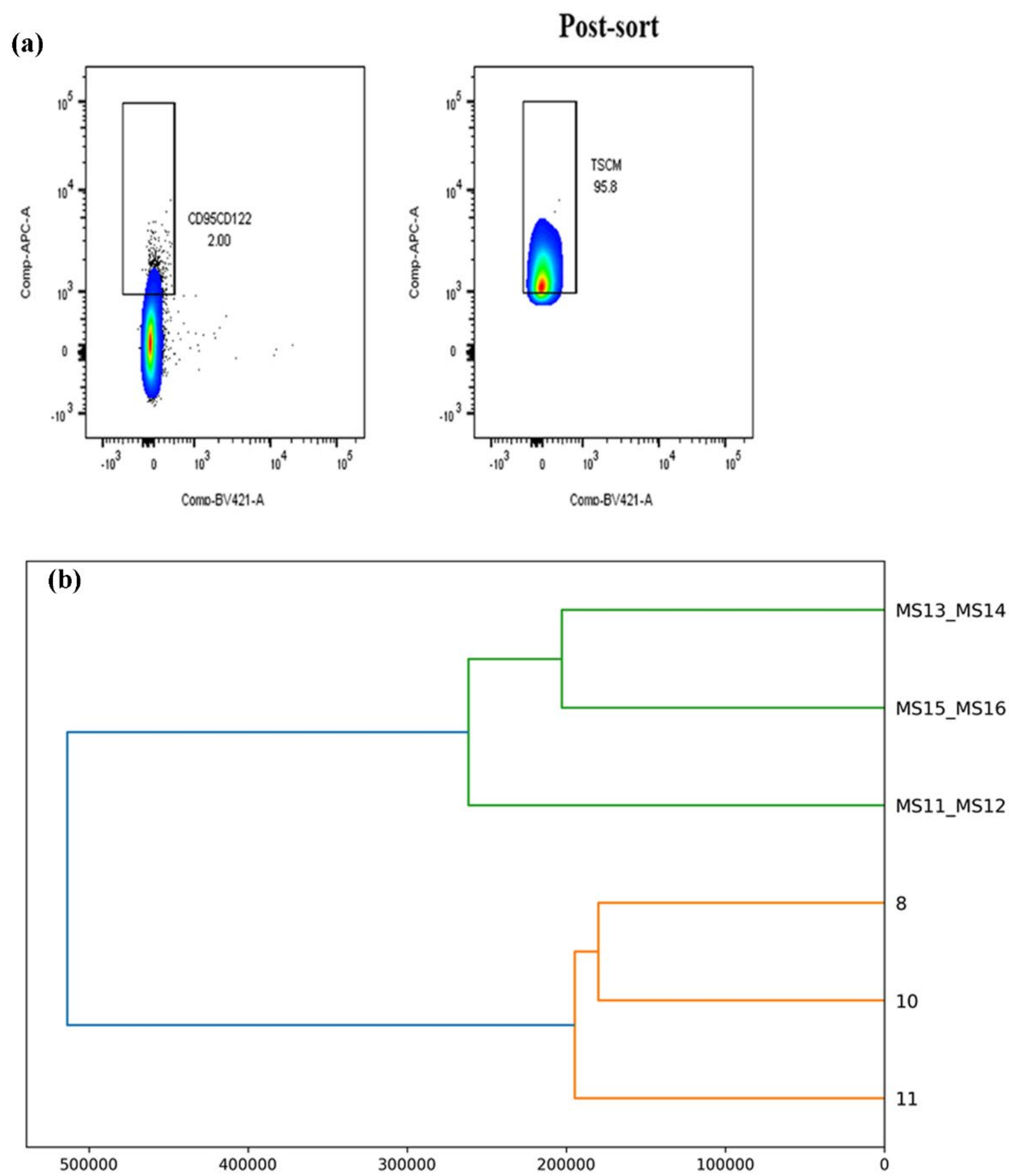

Figure 5

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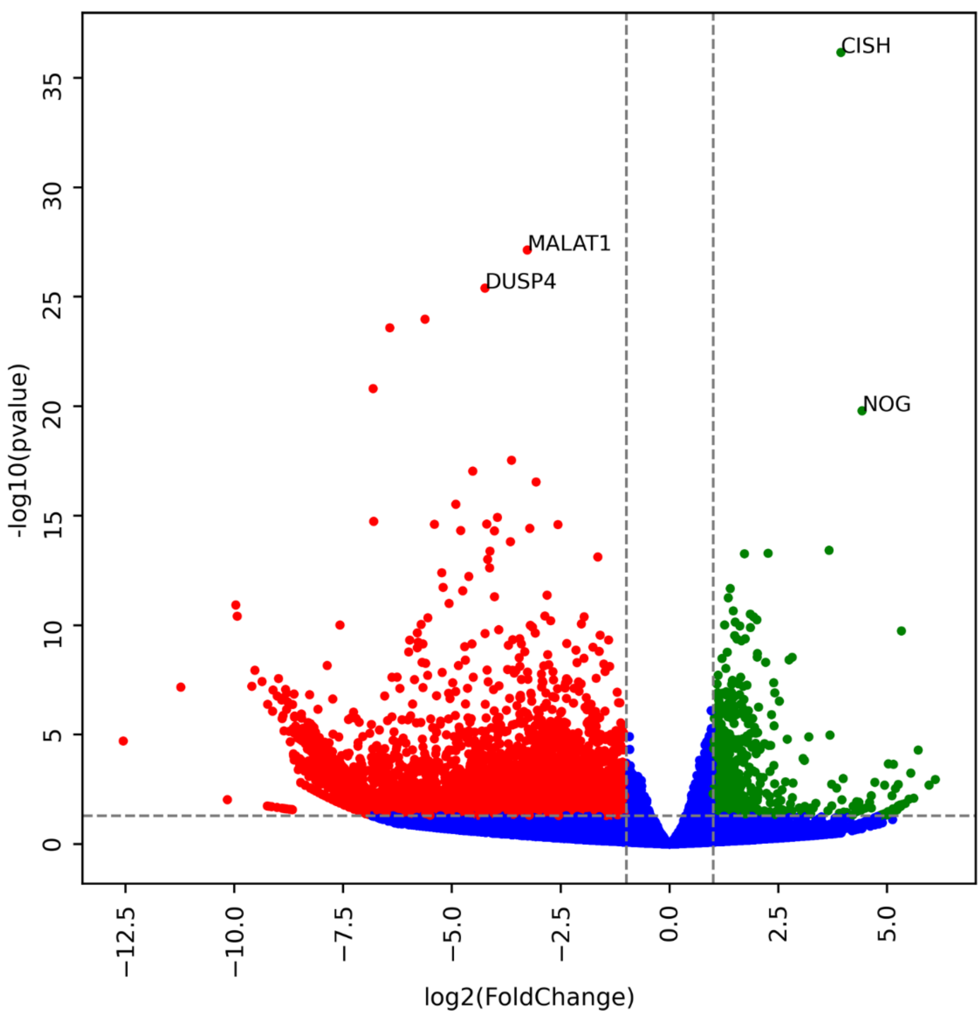

Figure 6

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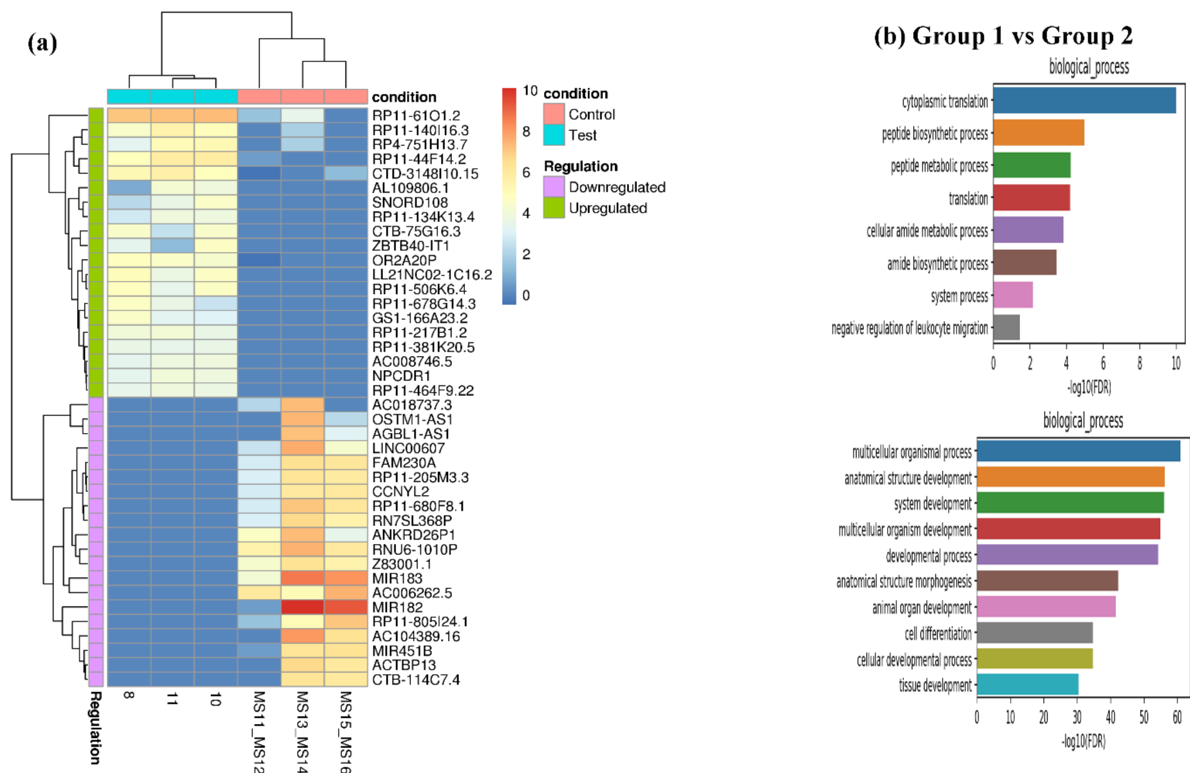
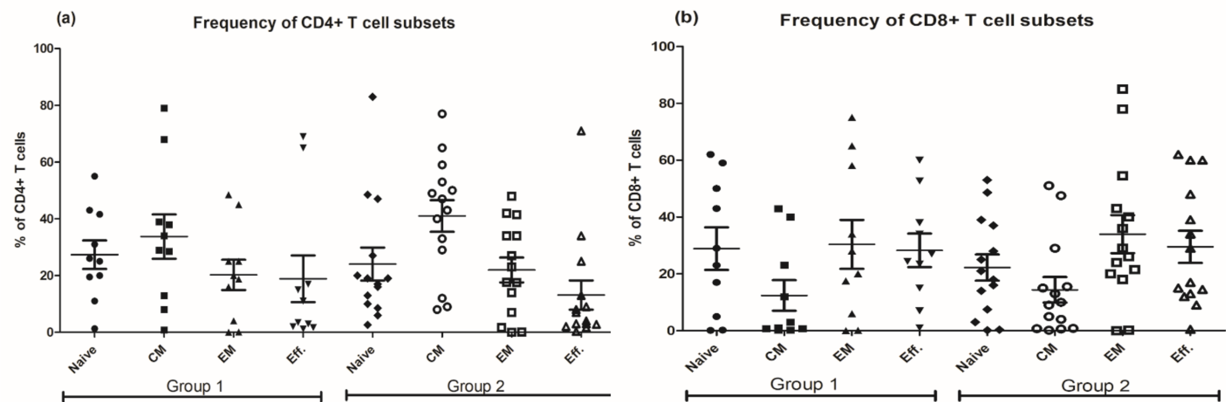


Figure 7



Supplementary Figure

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