UNIVERSITY OF CALIFORNIA SAN DIEGO

An Atlas of Immune Cell Exhaustion in HIV-Infected Individuals Revealed by Single-Cell Transcriptomics

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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Professor Michael David

2020

The Thesis of Hui Hui is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California San Diego

2020

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ABSTRACT OF THE THESIS

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Professor, Matthew Daugherty, Co-chair

Chronic infection with HIV (human immunodeficiency virus) impairs immune cell function, and leads to immune cell exhaustion, and thus results in incapability of controlling virus replication. But the development and maintenance of immune cell exhaustion remain unclear to researchers still. Hereby this project uses single-cell RNA sequencing technique to unravel the gene expression landscape, and to study the effect of HIV infection on immune cell exhaustion. Peripheral blood mononuclear cells samples from six patients were used for sequencing, in which three were low viral load (15758 cell counts in total), and the other three were high viral

load (12852 cell counts in total). Two healthy donor samples were used as control, with a total number of cells add up to 15121. Nine major immune cell clusters and eight T cell subtypes were identified based on their unique gene signatures. Among the T cell subclusters, exhausted memory CD8+ T cells and CD4+ T cells, and interferon high CD8+ T cells were only found in HIV-infected donor samples. An inhibitory receptor gene KLRG1 was found to be differentially expressed in HIV-infected donors, which was further identified to be a potential exhaustion marker. Experiments showed that there was an exhausted CD8+ T cell population expressing KLRG1, TIGIT, and T-bet (dim) EOMES (high) markers. Ex-vivo antibody blockade of KLRG1 restored the function of exhausted T cells, indicating that KLRG1 plays an important role in T cell exhaustion, which could be a potential immunotherapy target to treat chronic HIV infection. Also, analysis of integrated healthy and HIV-infected donor samples further revealed B cell and NK cell dysfunction induced by HIV infection. This project studied gene expression patterns of immune cell exhaustion as a result of HIV infection, providing potential immune cell exhaustion markers, which is useful in studying exhaustion mechanisms, and even developing new cure therapies.

Chapter 1: Introduction

1.1 Single Cell Gene Expression RNA Sequencing

Single-cell RNA sequencing, as the name implies, is a collection of RNA sequencing technology that allows RNA content of individual cells to be sequenced (Regev et al., 2017)[1]. Some of the methods focus on mRNA coming from 3' or 5' ends (Islam et al., 2014; Macosko et al., 2015)[2, 3], some mainly address mRNA structure and splicing question by sequencing nearly full length sequences (Hashimshony et al., 2012; Ramsköld et al., 2012)[4, 5]. One important technique involved in scRNA is the single cell isolation method. Strategies for single-cell isolation include manual cell picking (Eberwine et al., 1992; Van Gelder et al., 1990)[6, 7], utilizing microfluidic devices (Shalek et al., 2014; Treutlein et al., 2014)[8, 9], and FACS-based sorting (Ramsköld et al., 2012; Shalek et al., 2013)[5, 10]. Most recently, droplet-based and microwell based isolation approaches have been used widely, as they have large throughout, and allow rapid processing of large numbers of cells simultaneously. Samples used for scRNA seq are typically fresh dissociated tissue, and sometimes fixed cells (Nichterwitz et al., 2016; Thomsen et al., 2016)[11, 12]. Nuclei isolated from frozen or fixed tissue can also be used in some protocols, which provides the possibility of using archival materials for a wider range of research purposes. As scRNA sequenciing technology is still developing, RNA isolated from live cells can be analyzed as well, allowing the transcriptomic signature of the cells in their natural microenvironment to be studied (Lovatt et al., 2014)[13].

Table 1.1: Summary table of single-cell isolation methods by the year of 2013 (Shapiro et al. 2013)[14].

Method	Unbiased (randomized) or biased (targeted)?	Throughput	Cost	Manual or automatic isolation process?
Micromanipulation	Unbiased	Low-throughput	Low	Mainly manual
Fluorescence-activated cell sorting	Either biased or unbiased	High-throughput	High	Automatic
Laser-capture microdissection	Unbiased	Low-throughput	High	Manual
Microfluidics	Unbiased	High-throughput	High	Automatic

Single-cell RNA sequencing can be used in a variety of research fields: analyzing the genomes and transcriptomes of individual cells, revealing transcriptomic variability among cells, studying functional states of heterogenous cells, characterization of earliest differentiation events in cells, and so on (Shapiro et al., 2013)[14]. scRNA sequencing together with other sequencing technologies can also shed light on genomic, transcriptomic, and epigenomic states of single cells simultaneously, mapping disease development in pseudotime, and monitoring immunotherapy response (Shapiro et al., 2013)[14].

A global initiative called the Human Cell Atlas Project is working to utilize fast developing high-throughput single-cell RNA profiling to determine all human cell types using unique gene expression patterns (Regev et al., 2017)[1]. This project would make a profound influence on our understanding of biology, as it brings a new level of resolution to what we study everyday (Regev et al., 2017)[1].

1.2 Human Immunodeficiency Virus (HIV) and Immune Exhaustion

The human immunodeficiency virus (HIV) belongs to the genus Lentivirus in the family of Retroviridae, subfamily Orthoretrovirinae (German Advisory Committee Blood, 2016; Luciw et al., 1998)[15, 16]. It is classified into type 1 and type 2, or HIV-1 and HIV-2, based on genetic characteristics and viral antigen differences. HIV is known to be introduced to human population

around 1920s to 1940s. HIV-1 is generally thought to be evolved from immunodeficiency viruses from Central African chimpanzees, while HIV-2 is believed to come from West African sooty mangabeys (German Advisory Committee Blood, 2016; Gao et al., 1999; Sharp et al., 2011; Faria et al., 2014)[15, 17-19].

There are more than 76 million people in total been infected with human immunodeficiency virus (HIV) since it was first recognized in the 1980s. Nowadays, there are about 37 million people currently having HIV infection, but only 21 million people have access to antiretroviral therapy (UNAIDS.org; http://www.unaids.org/en/resources/fact-sheet). The development of cART (combination antiretroviral therapy) greatly helps controlling HIV viremia, and significantly reduces the mortality of HIV-infected patients. However, patients are not completely cured—withdrawal of cART treatment leads to a rebound of HIV viremia, meaning that patient's immune system is still unable to control viral replication. (Chun et al., 2010; Palella et al., 1998)[20, 21] The main reason for this is that HIV induces host immune system dysfunction in the long run. Continuous exposure to HIV viral antigens, leads to chronic activation of immune cells, progressive loss of immune cell functions, and in the end, causes immune cell exhaustion (Cheng et al., 2017; Haas et al., 2011; Jones et al., 2008; Lederman et al., 2013; Zhen et al., 2017)[22-26].

Immune cell undergoing exhaustion loses effector functions and is no long able to control virus. For example, exhausted CD8 T cells lose cytotoxic effector function, and thus are unable to eradicate HIV-infected cells (Wherry, 2011)[27]. In addition, exhausted CD8 T cells fail to differentiate from effector cells into memory cells, which can be rapidly reactivated upon encounter with antigen (Wherry, 2011; Wherry and Kurachi, 2015)[27, 28]. CD4 T cells also lose their effector functions under the influence of chronic HIV infection, resulting in failure to produce cytokines, like IL-2 and IL-21, which sustain HIV-specific CD8 T cells (Elsaesser et al., 2009; Porichis et al., 2011; Wang et al., 2017)[29-31]. What's worse, CD4 T cell depletion and

exhaustion leads to dysfunction of CD8 T cells, issuing in disease progression (Wherry, 2011; Wherry and Kurachi, 2015)[27, 28].

Sustained high expression of inhibitory receptors such as CTLA-4, PD-1, CD160, TIM-3, and TIGIT is a signature for T cell exhaustion. Expression of several such inhibitory receptors was found by previous studies (Chew et al., 2016; Day et al., 2006; Trautmann et al., 2006; Jones et al., 2008; Kaufmann et al., 2007; Peretz et al., 2012; Petrovas et al., 2006)[24, 32-37] to be associated positively with plasma viral load and HIV disease progression. One thing worth noticing from literatures is that, neutralizing antibody-mediated blockade of these inhibitory receptors can reverse T cell exhaustion by augmenting effector production, and HIV-specific CD4+, CD8+ T cells proliferation (Chew et al., 2016; Jones et al., 2008; Kaufmann et al., 2007; Peretz et al., 2012; Trautmann et al., 2006)[24, 32, 34-36]. For example, blockade of PD-1 in SIV (simian immunodeficiency virus)-infected macaques result in SIV-specific CD8 T cells expansion, memory B cell proliferation, reduced viremia, and prolonged life span (Dyavar Shetty et al., 2012; Velu et al., 2009)[38, 39]. Also, treatment with PD-L1 blocking antibody in humanized mice with chronic HIV infection decreases viremia and increases CD4 T cell population (Palmer et al., 2013)[40]. Literature even shows that, anti-PD-1 treatment in a HIV-infected patient with non-small-cell lung cancer results in observation of decreased HIV viremia, and restoration of HIV-specific CD8 T cell functions (Guihot et al., 2018)[41]. There is a clinical trial ongoing to evaluate PD-1 blockade on its safety and efficiency in HIV-infected patients with insufficient CD4 T cells (NCT03367754).

Not only T cells undergo exhaustion, B and NK cells can also be exhausted when exposed to chronic HIV infection (Costanzo et al., 2018; Mavilio et al., 2005; Moir et al, 2008)[42-44]. Although it is promising to develop cure strategy that focuses on reversing immune cell exhaustion, little is known about the mechanisms involved at transcriptomic level. Thus, analysis of immune exhaustion gene expression pattern helps understanding HIV-induced

immune exhaustion mechanisms, and is crucial for curing and preventing HIV infections, and potentially other similar viral infections.

1.3 Single Cell Application in Virology Research

As a powerful tool to analyze gene transcriptomic profiles of individual cells, single-cell RNA sequencing technology has been widely used in immune and virology research. With the advantage of measuring gene expression levels by cells, scRNA sequencing is able to reveal the heterogeneity of cells involved in biological processes (Svensson et al., 2018)[45]. Thus, it is quite useful in unraveling immune cell identities by gene signatures, no matter how large or complex the dataset is (Giladi and Amit, 2018; Wagner et al., 2016)[46, 47]. Before scRNA sequencing, bulk RNA sequencing can only provide averaged values from complex, and potentially heterogeneous populations (Papalexi and Satija, 2018)[48]. Thus, before scRNA sequencing becomes available, the complexity and dynamic states of each individual cell can hardly be analyzed. This major obstacle makes it impossible to reveal host immune response to HIV infection by bulk RNA sequencing. Not to mention the difficulty of identifying rare or novel cell populations bulk RNA sequencing puts before us (Villani et al., 2017)[49]. However, scRNA sequencing is already able to discern cell types, and even discover new cell types like human blood dendritic cells (DCs), monocytes, and progenitors, giving us a revised taxonomy of blood cells (Villani et al., 2017)[49]. Thus, we believe scRNA sequencing can make it possible to analyze the gene expression landscape in HIV infected donor samples, and even discover the effect of HIV infection on certain cell types or subtypes.

scRNA sequencing technology has been widely used in virology research as well. For example, a unique transcriptional profile was discovered in reactivated latent CD4 T cells in HIV-infected patients, which allows cell division without activating cell death pathways normally triggered by HIV replication (Cohn et al., 2018)[50]. In another study on HIV latency,

transcriptional heterogeneity induced by HIV latency and reactivation was analyzed using single-cell RNA sequencing (Golumbeanu et al., 2018)[51]. Other literatures unraveled gene expression dynamics in viral infections like Zika and dengue using scRNA sequencing techniques (Zanini et al., 2018)[52]. Researchers also used scRNA sequencing to analyze transcriptional landscapes and heterogeneity during cytomegalovirus latency (Shnayder et al., 2018)[53], and influenza virus infection (Russell et al., 2018)[54]. What's more, related new technologies like scATAC sequencing was used together with other methods like mass cytometry to reveal the epigenetic atlas of T cell exhaustion caused by cancers and chronic viral infections (Bengsch et al., 2018; Sen et al., 2016)[55, 56] Been used to investigate viral diversity, latency and reactivation of certain virus, heterogeneity of infection states, and virus-host interactions, scRNA sequencing definitely has the potential to provide unique insights into immune exhaustion caused by HIV infection.

1.4 Aim of Study

This project aims to utilize high throughput sequencing technology single-cell RNA sequencing to discover the landscape of HIV infection induced immune cell exhaustion by comparing HIV-infected patient PBMC samples with healthy donor samples. We hope single-cell RNA sequencing could make it possible to discover rare cell types, and analyze their gene signatures, and the HIV infection effect on them. Furthermore, this project aims to discover novel marker genes unique to known, or new cell types. In the end, we hope our finding will help understanding T cell exhaustion mechanisms, and even provide promising targets for future immunotherapy drug development.

Chapter 2: Results

2.1 An Atlas of PBMCs in Healthy and HIV-Infected Donors

In order to study immune cell exhaustion in HIV-Infected individuals, PBMCs from healthy donors and HIV-Infected patients were isolated and used to perform single-cell RNA sequencing. In patient samples, three low viral load samples and three high viral load samples were included. A total of 12,852 cells were sequenced from high viral load samples, and a total of 15,758 cells were detected in low viral load samples. Thus, around 4000 to 5500 cells were from each patient sample. In contrast, a total of 15,121 cells were found in two healthy control samples. Summary of donor information, including medication, is in table 2.1.

An atlas of HIV-Infected donors compared with healthy donors was thus obtained. Through single-cell RNA sequencing analysis using scripts and R packages, distinct clusters were produced, and were identified and assigned with cell types using cell type specific genes. The absolute $CD4^+$ T cell counts in the clinical blood samples and the numbers estimated from the scRNA-seq analysis showed a strong correlation, indicating that the scRNA-seq datasets accurately reflect the cell clusters present in the original blood samples.

Table 2.1: Summary table of HIV-infected individuals and healthy donors.

PID	Age	Gender	Ethnicity	Duration of HIV infection, years	Duration of viral suppression*	ART regimen	Plasma HIV RNA (copies/mL)	Stage of infection	CD4+ count (cells/μL)	Elite controllers
529	59	Male	Non-Hispanic	0.3	Intermittent suppression	GENVOYA	585,100	Chronic	203	No
717	56	Male	African American	27	Intermittent suppression	DESCOVY + TRUVADA+PREZISTA+PREZCOBIX+NORVIR	185,072	Chronic	299	No
168	36	Male	African American	3.5	Never suppressed	-	259,111	Chronic	37	No
876	33	Male	White	7.6	Fully suppressed	Triumeq	<20	Chronic	806	No
630	58	Male	other race	22	Fully suppressed	ODEFSEY + TIVICAY	<20	Chronic	603	No
471	60	Male	other race	11	Fully suppressed	JULUCA	<20	Chronic	638	No
HD1	22	Female	Non-Hispanic	-	-	-	-	-		-
HD2	23	Male	White	-	-	-	-	-		-

* Duration of viral suppression: fully suppressed (plasma HIV viral load < 50 copies/mL for >6 months), never suppressed (No ART treatment), intermittent suppression (ART treatment and plasma HIV viral load >50 copies/mL)

2.1.1 Well-Separated Clusters are Produced by Clustering Analysis

Seurat clustering analysis clusters each sample into distinct clusters. Usually 20 – 30 original clusters were found in each dataset. Further cell type identification resulted in nine major cell type clusters in samples: CD4+ T cells, CD8+ T cells, natural killer cells (NK), B cells, CD14+ monocytes, CD16+ monocytes, conventional dendritic cells (cDC), plasmacytoid dendritic cells (pDC), and megakaryocytes (Mk). Visualization method tSNE (t-distributed stochastic neighbor embedding) shows cell type clusters are well-separated.

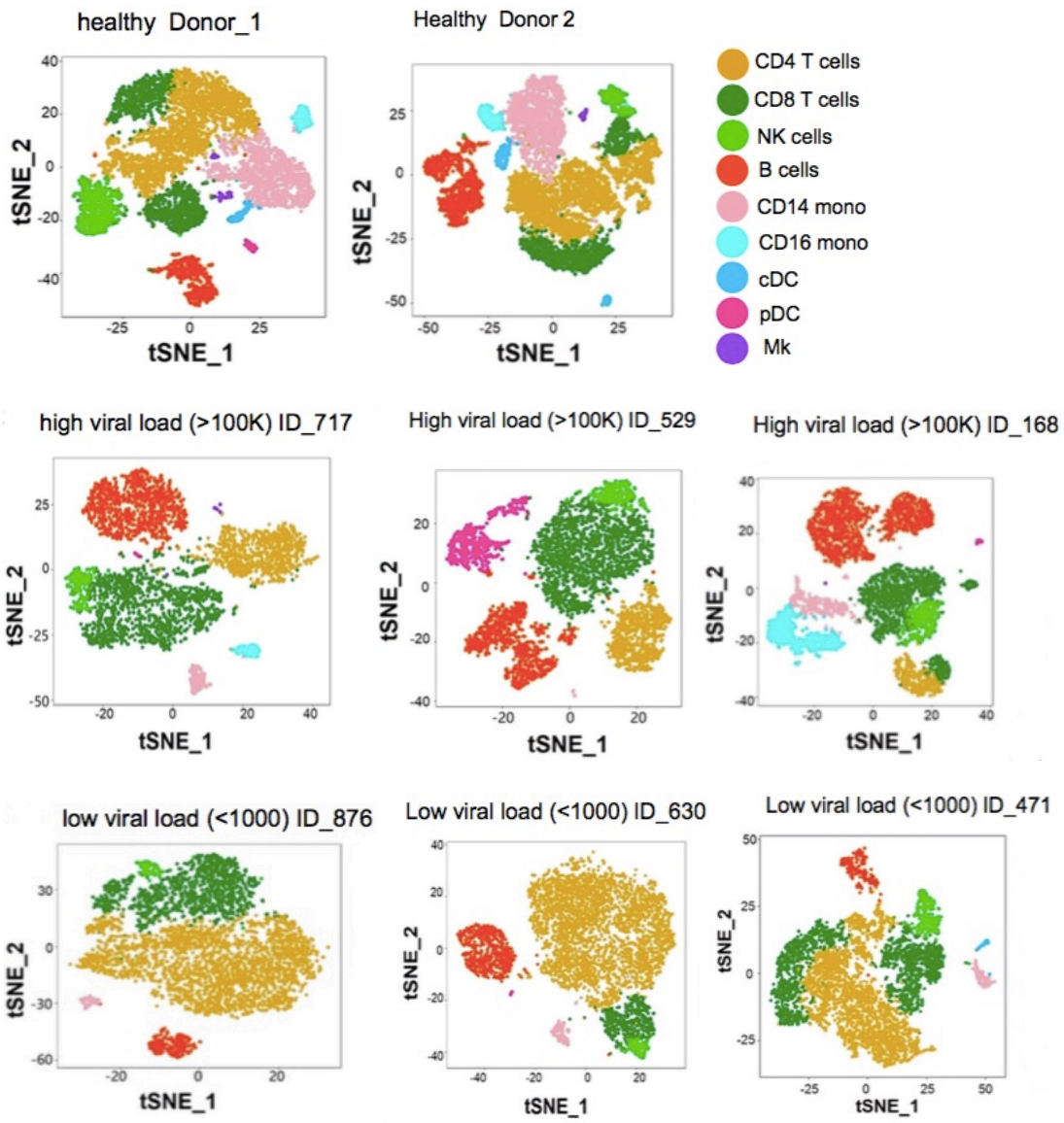
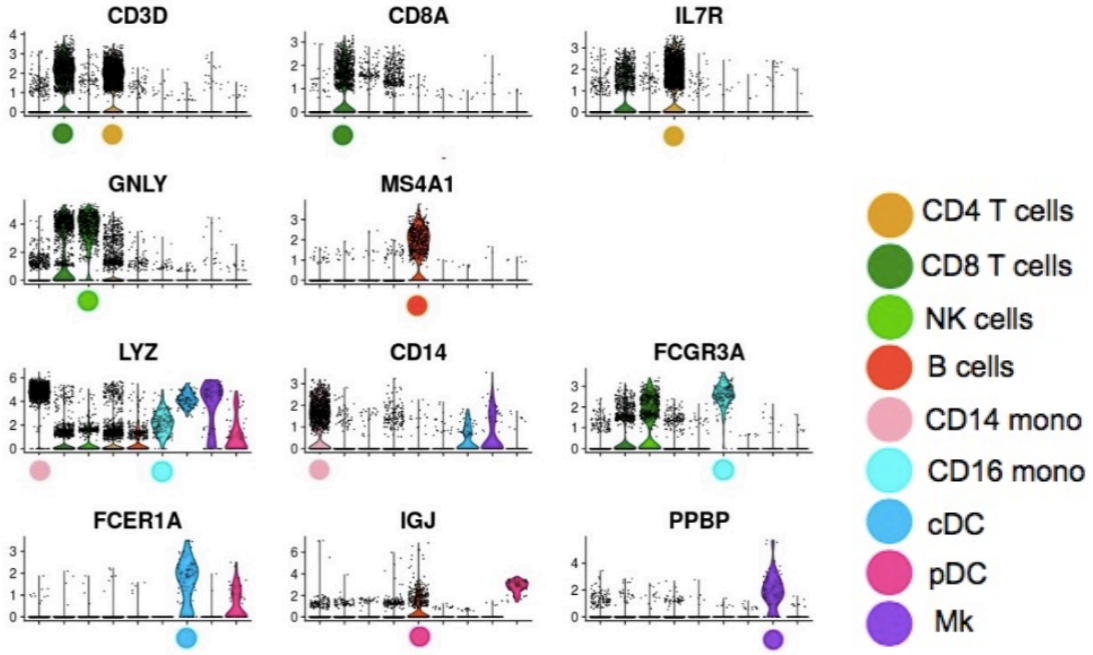


Figure 2.1.1: Cell distribution plot of eight samples using t-distributed stochastic neighbor embedding (tSNE) projection. Sample name and condition is labeled above each plot. Major cell clusters were identified based on gene markers and shown for each sample, using the same color and naming scheme as indicated in the legend on the upper right corner: CD4 T cells, CD8 T cells, NK cells (natural killer cells), B cells, CD14 mono (CD14+ monocytes), CD16 mono (CD16+ monocytes), cDC (conventional dendritic cells), pDC (plasmacytoid dendritic cells), and Mk (megakaryocytes).

2.1.2 Major Cell Types are Identified in Both Patient and Healthy Donor Samples using Established Cell Type Specific Genes

These nine major cell clusters were identified in healthy donor samples and in HIV-Infected individual samples. Gene expression of canonical biomarkers for cell types were used to classify them: CD4+ T cells: CD3D+, CD8A-, IL7Rhi; CD8+ T cells: CD3D+, CD8A+; NK cells: CD3D-, CD8A-, IL7R-, GNLYhi; B cells: MS4A1+; CD14+ mono: LYZhi, CD14hi; CD16+ mono: LYZhi, FCGR3Ahi; cDC: LYZhi, CD14hi; pDC: LYZhi, IGJhi; Mk: PPBP+.

Graph 2.1.2: Violin plots of healthy donor 1 (HD_1) dataset showing marker genes expression across cell types indicated by color on the right hand side. Each dot represents a cell, and the shape of the violin indicates expression distribution within the cluster.



2.1.3 CD8 and CD4 T Cell Ratios are Significantly Changed in HIV-Infected Patients

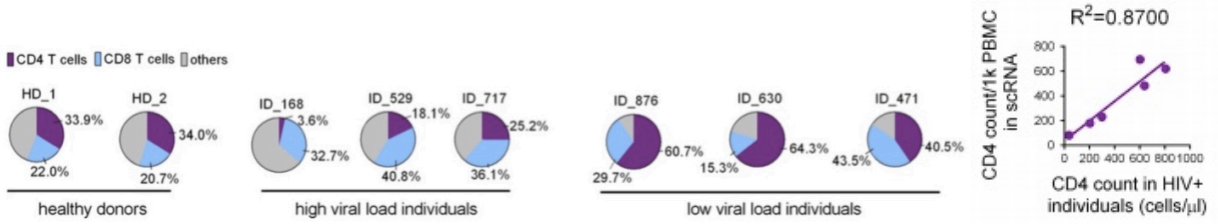
The healthy donor PBMC samples showed expected proportions of CD4+ and CD8+ T cells: roughly 50% sequenced cells are T cells; within T cell populations, CD4+ T cells and CD8+ T cells had a ratio of 2:1 (Chen et al., 2018)[57]. T cell populations in HIV-infected samples, however, were drastically changed.

The percentage of CD4+ T cells in high viral load samples (3.6%, 18.1%, and 25.2%, respectively) were considerably lower than that in healthy control samples (33.9%, and 34.0%, respectively). But at the same time, CD8+ T cells percentage (32.7%, 40.8%, and 36.1%, respectively) were significantly higher than that in healthy donor samples (22.0%, and 20.7%, respectively).

On the contrary, higher-than-normal percentage of CD4+ T cells was observed in three low viral load samples (60.7%, 64.3%, and 40.5%). %).

Importantly, the absolute $CD4^+$ T cell counts in the clinical blood samples and the numbers estimated from the scRNA-seq analysis showed a strong correlation (R^2=0.87), indicating that the scRNA-seq datasets accurately reflect the cell clusters present in the original blood samples.

Graph 2.1.3: On the left is the pie charts showing the percentage of CD4 T cells, CD8 T cells, and other cell types within samples. Sample ID for each dataset is labeled above each pie chart. On the right is the linear regression analysis showing the correlation between CD4+ T cell counts calculated from scRNA analysis (cells/1000 PBMCs) vs flow cytometry (cells/μl) of PBMCs from HIV-infected donors.



2.1.4 Percentage of Specifc Cell Types are Significantly Changed by HIV Infection

Although T cell ratio changes resulted from HIV infection was certainly interesting and worth further research on, I can't help to notice that percentage of other cell types were also changed by HIV infection, and even viral load.

B cell population percentages are significantly higher in high viral load patients compared with healthy controls. CD14+ mono percentage, however, is lower in all HIV-Infected samples (both high and low viral loads), comparing to that of healthy donors. Low viral load datasets showed way lower CD16+ mono percentage in all three donors, compared to both healthy and high viral load individuals. cDC, on the other hand, was only found in healthy controls, but not in HIV-infected patients.

Table 2.1.4: Summary table of sample PID, plasma HIV RNA concentration (indicating viral load), cell counts in total and in cell type clusters, and percentage of each cell type within each sample.

PID	Plasma HIV RNA	CD4+ T cell		CD8+ T cell		B cell		NK cell		CD14+ monocytes		CD16+ monocytes		cDC		pDC		Mk		Total
	(copies/mL)	counts	ratio	counts	ratio	counts	ratio	counts	ratio	counts	ratio	counts	ratio	counts	ratio	counts	ratio	counts	ratio	counts
HD1	-	2299	33.9%	1495	22.0%	588	8.7%	672	9.9%	1345	19.8%	153	2.3%	96	1.4%	62	0.9%	76	1.1%	6786
HD2	-	2835	34.0%	1721	20.6%	1255	15.1%	322	3.9%	1678	20.1%	222	2.7%	243	2.9%	0	0.0%	59	0.7%	8335
529	585,100	789	18.1%	1780	40.8%	966	22.1%	271	6.2%	18	0.4%	0	0.0%	0	0.0%	544	12.5%	0	0.0%	4368
717	185,072	1038	25.2%	1486	36.1%	1107	26.9%	200	4.9%	153	3.7%	100	2.4%	0	0.0%	17	0.4%	18	0.4%	4119
168	259,111	157	3.6%	1429	32.7%	1469	33.7%	329	7.5%	375	8.6%	566	13.0%	0	0.0%	33	0.8%	7	0.2%	4365
876	<20	2882	60.7%	1410	29.7%	271	5.7%	99	2.1%	82	1.7%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	4746
630	<20	3534	64.3%	841	15.3%	779	14.2%	156	2.8%	163	3.0%	0	0.0%	0	0.0%	21	0.4%	0	0.0%	5494
471	<20	2228	40.4%	2399	43.5%	328	6.0%	334	6.1%	165	3.0%	54	1.0%	0	0.0%	0	0.0%	0	0.0%	5508

2.1.5 The Composition and Proportion of T Cell Subtypes (Exhausted and IFNhi Populations) are also Markedly Altered by HIV Infection

In order to study the intriguing pattern of percentage change in T cell populations, subsetting and clustering on the T cell populations were performed on each sample. Signature genes indicating the cell subtype and state were used to further identify subsets: naïve CD4+ T cells (CD4-Tn): CD8A-, CCR7+, IL7Rhi; effector memory CD4+ T cells (CD4-Tem): CD8A-, IL7Rhi, CCR7-, GZMA+ (Gattinoni et al., 2011)[58]; precursor memory cells (CD4-Tpm): CD8A-, IL7Rhi, CCR7low, LTBhi; naive CD8+ T cells (CD8-Tn): CD8A+, CCR7hi; effector memory CD8+ T cells (CD8-Tem): CD8A, IL7R-, CCR7-, GZMA+, NKG7+. The putative CD4-Tpm cluster having unique gene signiture was found in some of the samples. It showed a similar pattern to that of CD4-Tn cells (TCF7, FOXP1, etc.), however, effector funtion-associated genes (e.g. GZMA, CCR5, NKG7) were found lowly expressed in this cluster. This may suggest the cells in this cluster were undergoing a transitional state between naïve and effector memory (Gattinoni et al., 2011; Youngblood et al., 2017)[58, 59]. Moreover, high expression of LTB gene (TNF family molecule lymphotoxin beta) was found in this cluster, which matches the description of a CD4-Tpm cytotoxic cell cluster in literature (patil et al., 2018)[60].

Exhaustion cells and cells with high expression of IFN-stimulated genes should also be expected in patient samples. The following marker genes were used to identify said T cell populations: exhausted memory CD8+ T cells (CD8-Tex): CD160hi, TIGIThi (Chew et al., 2016; Peretz et al., 2012)[32, 36]; Exhausted memory CD4+ T cells (CD4-Tex): TIGIThi, CTLA4 (Kaufmann et al., 2007)[35]; CD8+ Tem cells with upregulation of IFN-stimulated genes (CD8-Tem-IFNhi): OASLhi, ISG15hi, IFIT2hi, and IFIT3hi. Expression of the used marker genes for CD8-Tem-IFNhi showed an expansion of the host antiviral response.

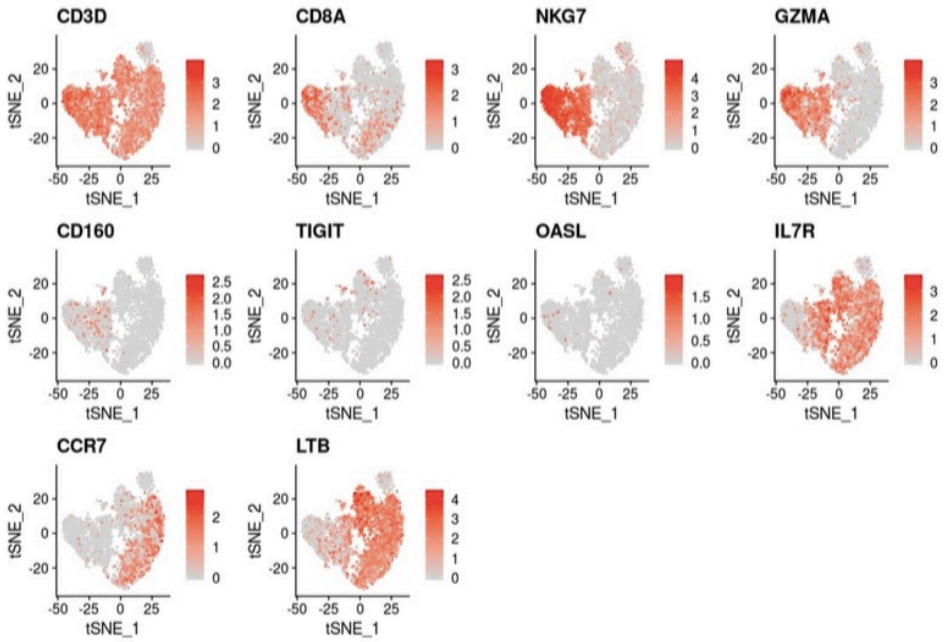
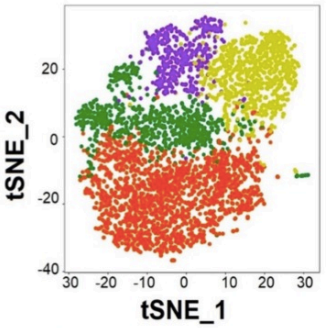


Figure 2.1.5: Feature plot showing expression level of marker genes used to discern cell types in tSNE plot of HD_1. Expression level was indicated by color scale: red: high expression, and grey: low expression.

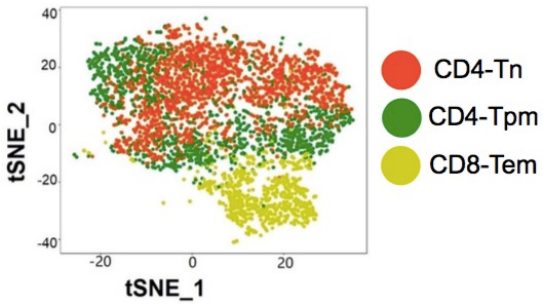
After clustering and cluster cell type assignment, The T cell subtypes in each dataset and the relative proportion of them were shown even clearer on tSNE plots. It is quite obvious that high viral load samples contained smaller populations of CD4-Tem and CD8-Tn cells, but CD8-Tex, CD4-Tex, and CD8-Tem-IFNhi populations can be discerned instead. Tex gene signatures were found to be at percentages of 18.1%, 10.1%, and 33.9%, respectively in three high viral load samples. On the other hand, low viral load donor samples showed a reduction in CD4-Tem and CD8-Tn populations as well but had CD8-Tem-IFNhi cluster instead. However, no distinct CD4-Tex and CD8-Tex cells were found.

Figure 2.1.5.2: Cell distribution plot of T cell subsets for eight samples using t-distributed stochastic neighbor embedding (tSNE) projection. Sample name and condition is labeled above each plot. T cell subtypes were identified based on gene markers and shown for each sample, using the same color and naming scheme as indicated in each figure legend: CD4-Tn: naïve CD4+ T cells; CD4-Tem: effector memory CD4+ T cells; CD4-Tpm: precursor memory cells; CD8-Tn: naive CD8+ T cells; CD8-Tem: effector memory CD8+ T cells; CD8-Tex: exhausted memory CD8+ T cells; CD4-Tex: exhausted memory CD4+ T cells; CD8-Tem-IFNhi: CD8+ Tem cells with upregulation of IFN-stimulated genes.

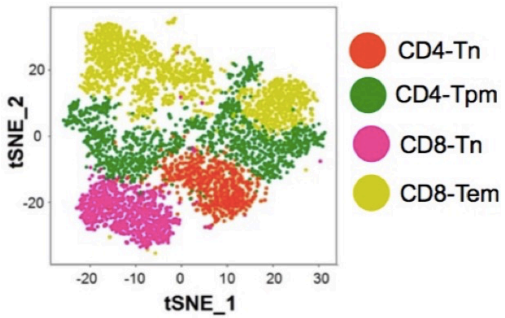
low viral load (<1000) ID_876



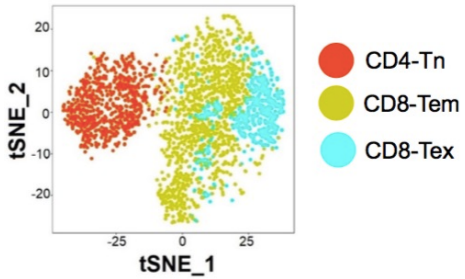
Low viral load (<1000) ID_630



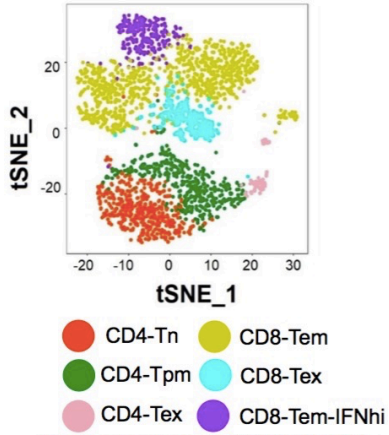
Low viral load (<1000) ID_471



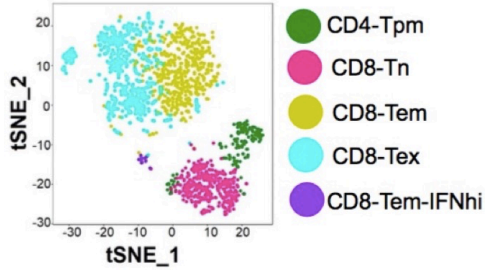
High viral load (>100K) ID_529



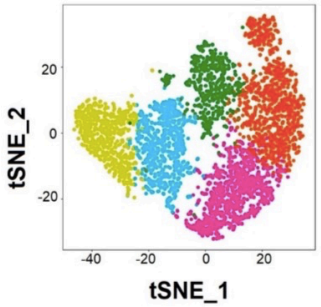
high viral load (>100K) ID_717



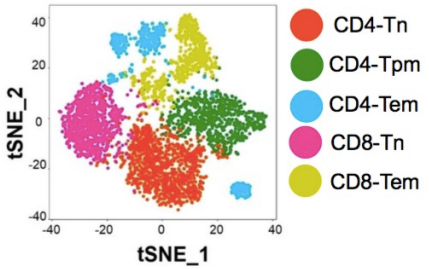
High viral load (>100K) ID_168



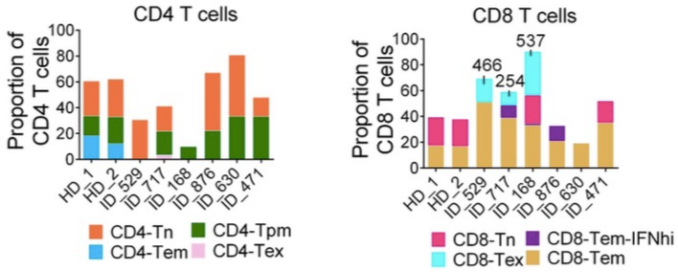
healthy Donor _1



Healthy Donor_2



Graph 2.1.5: Proportion of CD4 T and CD8 T subclusters within each sample.



2.1.6 Pseudotime Reveals Split Trajectory of CD8 Exhaustion Cells and CD8 High Interferon Effective Memory Cells

Next, pseudotime analysis was used to study the developmental process from CD8-Tem to CD8-Tex. CD8 T cell populations were analyzed, and organized into a pseudotime trajectory, with the root state set to be CD8-Tem cells. It is obvious that CD8-Tem cells branched into CD8-Tem-IFNhi cells and CD8-Tex cells. This pattern suggests a bifurcating trajectory of CD8 differentiation under HIV infection. Exhausted CD8+ T cells were at the end of pseudotime trajectory, indicating a terminal differentiation state, just as expected. As CD8-Tex and CD8-Tem-IFNhi divided, highly different gene expression patterns must had been playing a role in defining them.

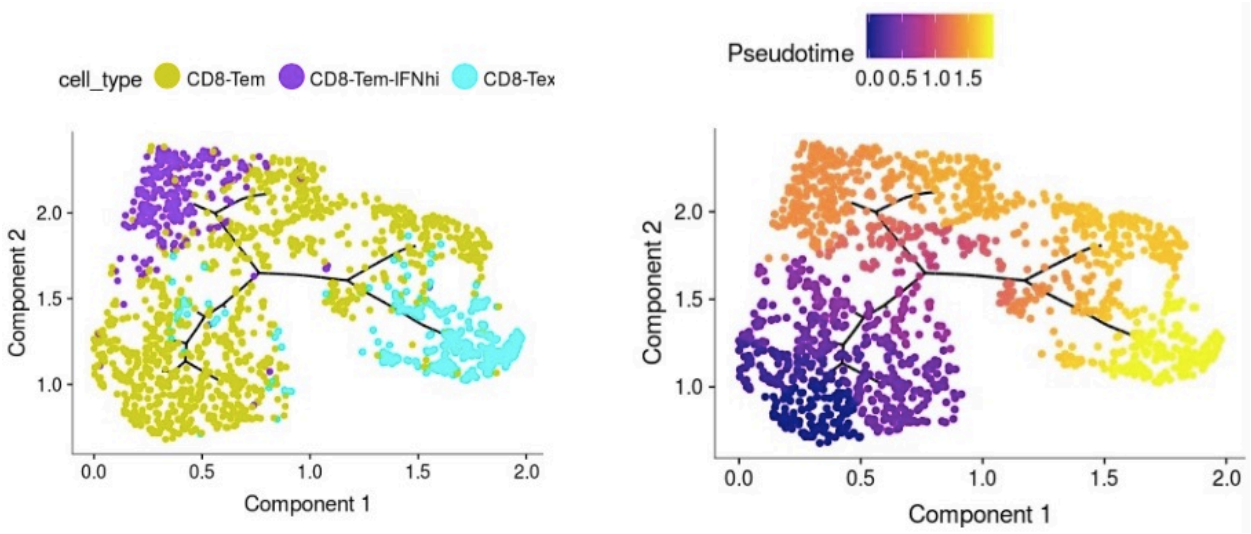
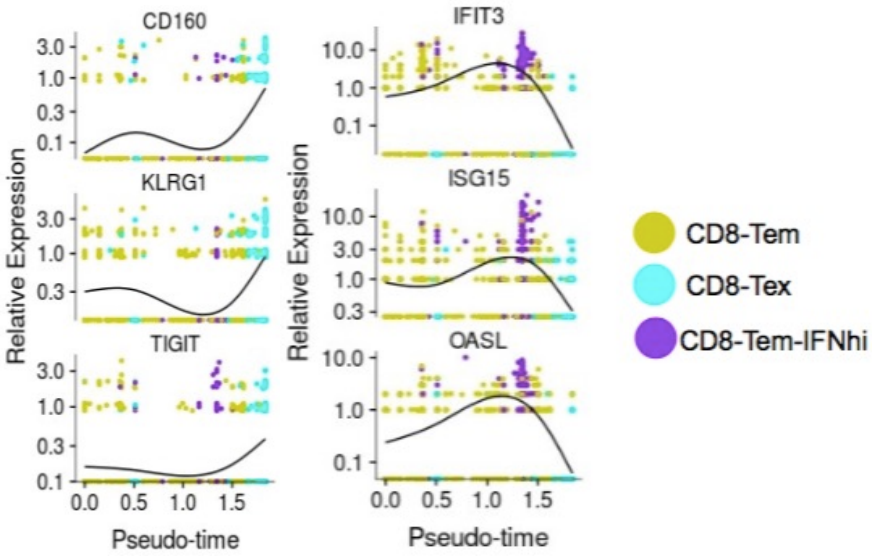


Figure 2.1.6: Trajectory plots resulted from pseudotime analysis showing two major developmental branches for CD8-Tem in HIV infected patients. The root state of pseudotime analysis was set to be CD8-Tem cells. The trajectory plots were colored by cell types (left) as indicated on the figure legend above, and by pseudotime (right), with pseudotime scale indicated on the figure legend above as well.

2.1.7 Differentially Expressed Genes across Pseudotime Timeline

It can be discerned that known exhaustion marker genes CD160, TIGIT, along with our newly identified possible exhaustion-related gene KLRG1 had high relative expression level in exhaustion sub-branch cells, which were positioned at the end of the pseudotime line. At the same time, CD8-Tem-IFNhi cells showed high expression of representative genes IFIT3, ISG15, and OASL.

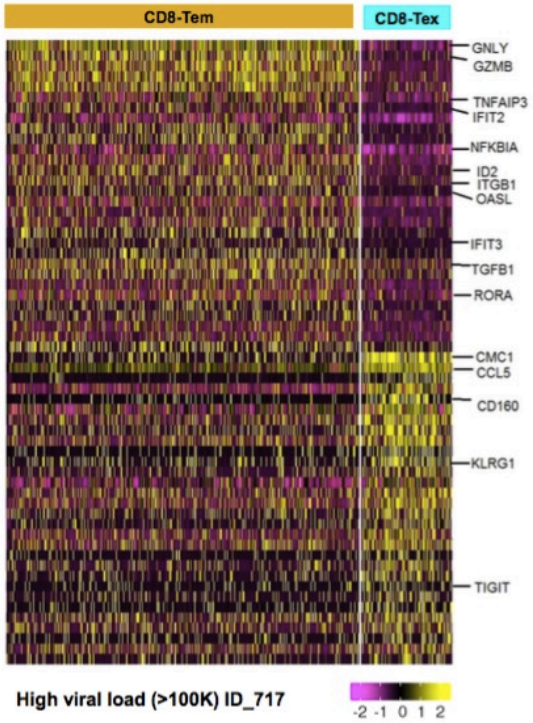
Graph 2.1.7: Relative expression level for representative genes across pseudotime time line, colored by T cell subtypes. Genes in the first column were found to be enriched in CD8-Tex population, while genes in the second column were found to be enriched in CD8-Tem-IFNhi cells.



2.2 Identification of Novel Genes Associated with T Cell Exhaustion

In order to further study the gene signature of exhausted T cells in HIV-Infected patients, CD8-Tex subclusters were compared against CD8-Tem cells. A total of 39 genes that were altered in at least two high viral load datasets were identified. Among these genes, 24 were up-regulated, including known exhaustion markers TIGIT and CD160 (Chew et al., 2016; Peretz et al., 2012)[32, 36]. These two genes encoding for inhibitory receptors have also been discovered to be T cell exhaustion markers in tumor-bearing and pathogen-infected animals (Chew et al., 2016; Fraietta et al., 2016; Peretz et al., 2012; Tirosh et al., 2016; Wherry et al., 2007; Yamamoto et al., 2011; Zheng et al., 2017)[32, 36, 61-65]. The other 15 genes were down-regulated.

Graph 2.2: Heatmap for HIV-infected patient ID_717 (high VL) exhibiting differentially expressed genes comparing CD8-Tem and CD8-Tex, as labeled above. Some of the highlighted genes were indicated on the right hand side of the heatmap. The color scale below indicates expression level for each gene.



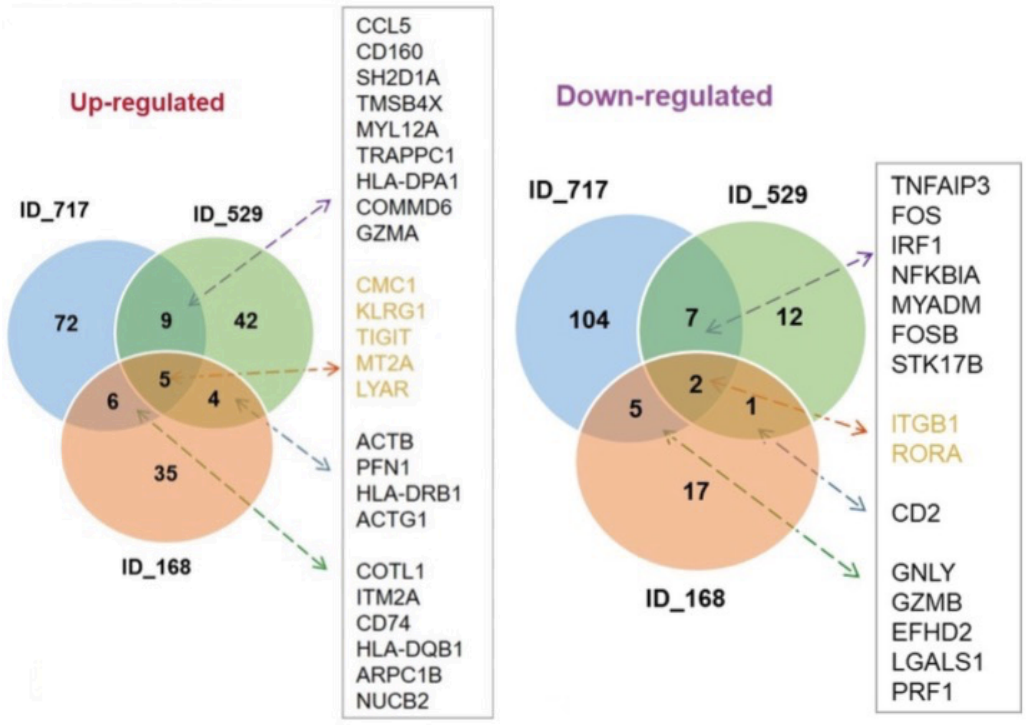
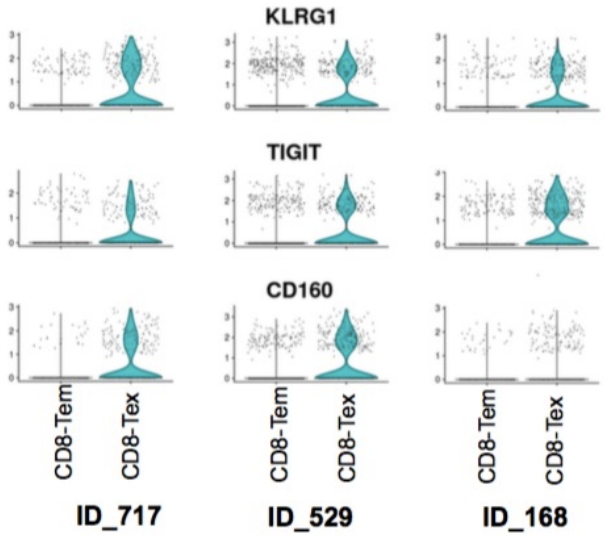


Figure 2.2: Venn diagrams showing conserved up (left) and down (right) regulated genes in CD8-Tex populations in three high viral load patient datasets.

2.2.1 KLRG1 Strongly Up-Regulated and Associated with Exhaustion

Among all the up-regulated genes showed up in the Venn diagram, KLRG1 was the most intriguing one. Like identified exhaustion markers TIGIT and CD160 (Chew et al., 2016; Peretz et al., 2012)[32, 36], KLRG1 is also an inhibitory receptor. KLRG1 is killer cell lectin-like receptor subfamily G member 1, which is known to be a T cell differentiation marker (Joshi et al., 2007)[66]. It contains an immunoreceptor tyrosine-based inhibitory motif in the cytoplasmic domain.

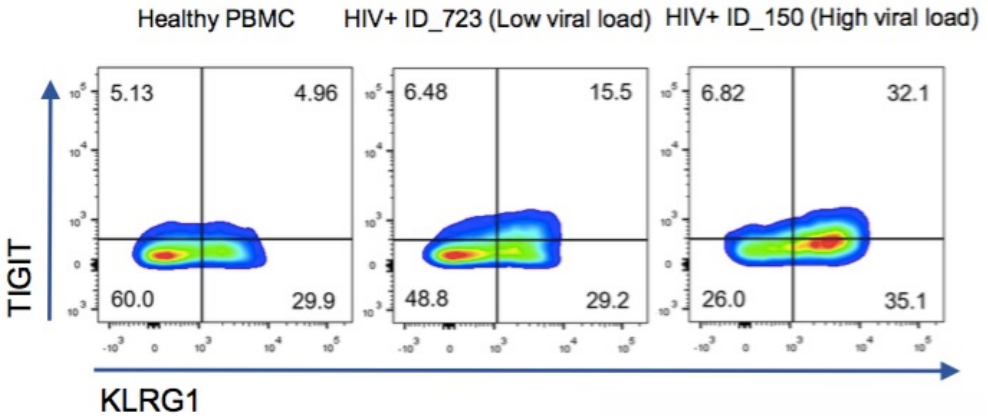
Graph 2.2.1: Violin plots of all three high VL HIV-infected donor datasets showing conserved up regulated genes, including identified exhaustion marker genes CD160 and TIGIT, and also new potential exhaustion related gene KLRG1, comparing CD8-Tem and CD8-Tex populations. Patient ID's and cell types were labeled below plots. Each dot represents a cell, and the shape of the violin indicates expression distribution within the cluster.



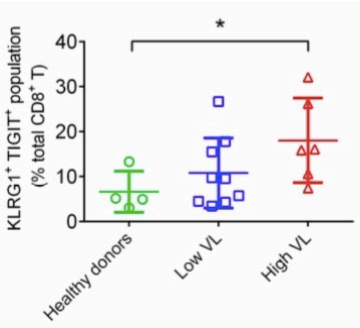
2.2.2 Function Validation of KLRG1

Observing KLRG1 were and co-expressed with CD160 and TIGIT in exhausted T cell clusters, we came to believe that it may be involved in T cell exhaustion. Flow cytometry analysis was done to observe the KLRG1 expression in PBMCs from healthy, low VL, and high VL donors. The frequency of CD8+ T cells expressing KLRG1 and TIGIT increased significantly in HIV-infected donors (low VL: 10.8%, and high VL: 18.1%), compared to only 6.6% in healthy donors. Higher percentage of populations co-expressing KLRG1 and TIGIT was observed in high VL samples compared with low VL, which was expected because PBMCs from higher viral load patients showed more severe exhaustion.

Graph 2.2.2: Flow cytometry data of healthy donors, low VL donor (ID_723), and high VL donor (ID_150), showing percentages of TIGIT and KLRG1 co-expressing CD8 T cells. Numbers in each corner represents the percentage of KLRG1-TIGIT+, KLRG1+TIGIT+, KLRG1-TIGIT-, KLRG1+TIGIT- cell populations.



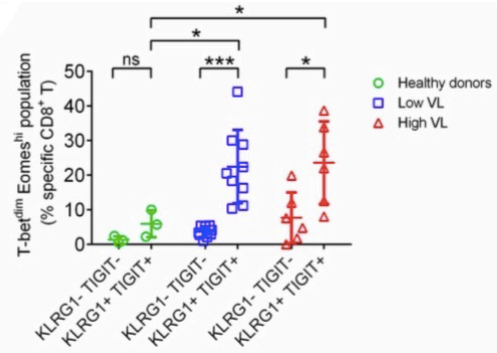
Graph 2.2.2.2: Box plot showing percentages of KLRG1+TIGIT+ population increase with viral load. Data used in this plot was collected from flow cytometry of healthy donors (n=4), low VL donors (n=9), and high VL donors (n=6). Mean ± SD, *p < 0.05, student's t test.



Next, we wanted to discern the exact HIV-induced exhausted KLRG1 population in samples. T-bet and Eomes were proved by previous study (Buggert et al., 2014)[67] to be highly associated with HIV infected exhaustion phenotype in a reciprocal pattern: chronic HIV infection reduces T-bet expression, and up-regulates Eomes. Thus, HIV-infected CD8+ T cell populations expressing dim T-bet and high Eomes functioned poorly (Buggert et al., 2014)[67]. Thus, the ratio of T-bet(dim) Eomes(high) population in CD8+ T cells co-expressing KLRG1 and TIGIT was analyzed. As expected, the percentages of cells showing T-bet(dim) and Eomes(high) were much higher in HIV infected PBMCs (low VL: 22.4%, and high VL: 23.3%), comparing to that of

healthy donors (6.0%). Also, it is clear that in HIV infected groups, much higher percentages of T-bet(dim)Eomes(high) populations were observed in KLRG1+TIGIT+ (potentially exhausted) compared to that of KLRG1-TIGIT- populations. Therefore, a novel exhausted CD8+ T cell population with KLRG1+TIGIT+ T-bet(dim)Eomes(high) gene signature was identified in chronic HIV infected PBMCs.

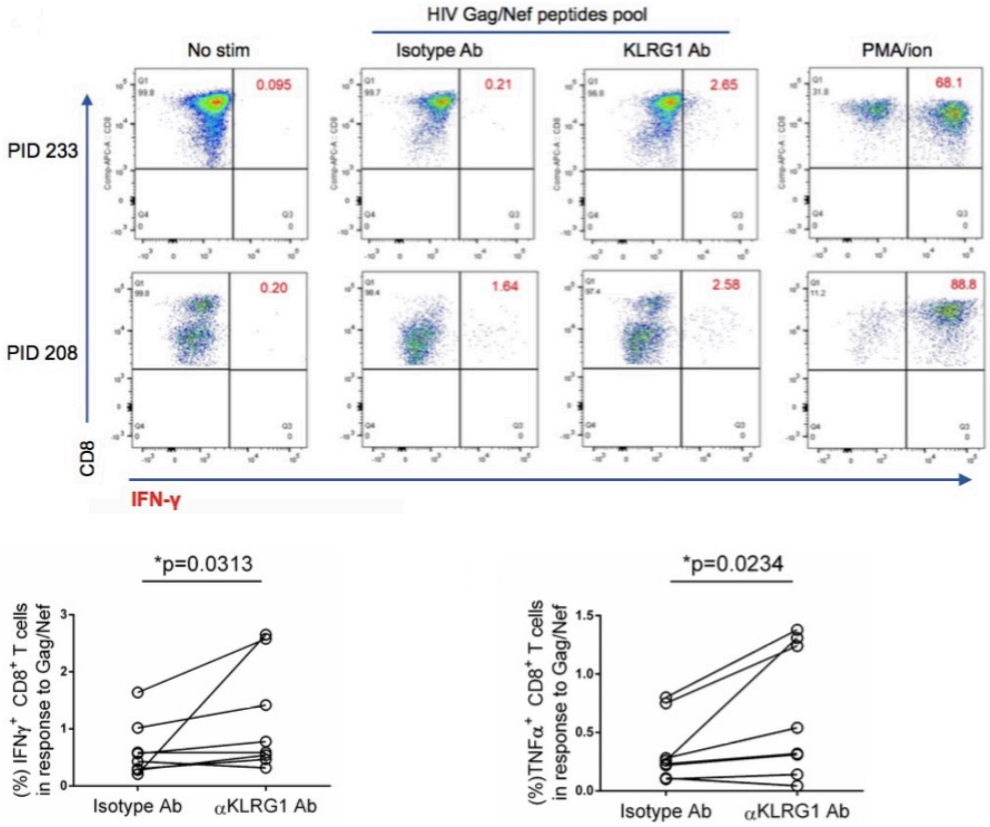
Graph 2.2.2.3: Box plot showing KLRG1+TIGIT+T-bet(dim)Eomes(hi) CD8 T cell population is significantly increased from healthy donors to HIV-infected donors. KLRG1-TIGIT- and KLRG1+TIGIT+ population was extracted from graph 2.2.2.2. The percentages of T-bet(dim)Eomes(hi) in samples were analyzed and drawn. Mean ± SD, *p < 0.05, ***p < 0.001, ns: not significant, student's t test.



To unravel the relationship between KLRG1 and T cell exhaustion, we wanted to determine whether blocking KLRG1 in HIV-specific CD8+ T cells could restore T cell functions. PBMC samples from HIV-infected patients were first stimulated with HIV Gag/Nef peptides pool, and then treated with KLRG1 blocking or isotype antibodies. In the end, percentages of IFN-gamma and TNFalpha expressing cells were measured to determine whether blocking KLRG1 restores HIV-specific T cell function. It turned out that incubation with KLRG1 blocking antibody resulted in a significant increase in the percentages of IFN-gamma expressing cells (from 0.21% to 2.65%, and from 1.64% to 2.58%, respectively). Same thing in TNFalpha expressing HIV-specific T cells: the percentages elevated from 0.26% to 1.31%, and from 0.8% to 1.38%.

Further statistical analysis from eight HIV-infected donor PBMCs showed that KLRG1 blockade significantly restored cytokine responses.

Graph 2.2.2.4: Flow cytometry data and line chart showing blocking KLRG1 restores T cell function. Chronically HIV-infected individuals PBMC samples were stimulated with HIV Gag/Nef peptide pool with the treatment of isotype, and KLRG1 blocking antibodies. Flow was done to evaluate IFN-gamma responses in two HIV-infected patient samples (PID 233 and 208). PBMC with no HIV-1 Gag stimulation with an isotype control was shown here as a negative control. PMA and ionomycin treatment were shown here as a positive control. The percentages of IFN-gamma positive CD8 T cells(n=8) were collected and shown as line charts. Wilcoxon matched-pairs signed ranked test was used to calculate p values.

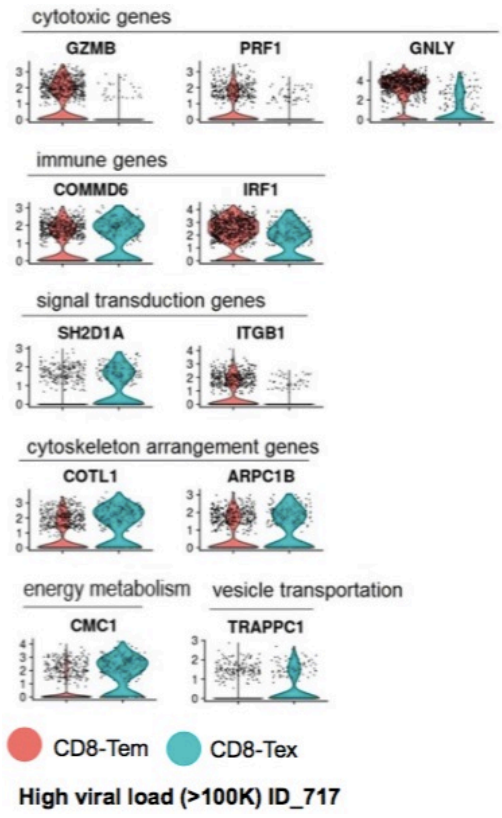


2.2.3 Other Immune Response Genes Associated with T cell Exhaustion

Apart from KLRG1, there are other immune response genes differentially expressed in high viral load donor samples. Up-regulated genes included COMMD6, SH2D1A, COTL1, CMC1, TRAPPC1. Among these genes, COMMD6 is an NF-kB-inhibiting protein, and SH2D1A

encodes SLAM-associated protein (SAP), which is a signaling inhibitor (Wu et al., 2001)[68]. It is proposed in literature (Wu et al., 2001)[68] that SAP plays an important role in antiviral immune response, and T cell differentiation signaling. One thing worth noticing is that, CD8-Tex cells showed up-regulation in cytoskeletal functional genes (TMSB4X, PFN1, ACTG1, COTL1, ARPC1B), in genes involved with energy metabolism (CMC1), and in vesicle transportation genes (TRAPPC1). Down-regulated genes, on the other hand, include genes like IRF1, ITGB1, GZMB, PRF1, and GNLY. Among these genes, IRF1 is the IFN-responsive transcription factor, whose down regulation is consistent with suppression if immune signaling induced by HIV (Day et al., 2006; Maine et al., 2007)[33, 69]. Moreover, GZMB encodes for the cytotoxic T cell effector molecules granzyme B, PRF1 encodes perforin 1, and GNLY encodes granulysin. These novel differentially expressed genes suggests a possible reduction in effector function phenotypes in CD8-Tex cells compared with normal CD8-Tem cells.

Graph 2.2.3: Violin plots of one HIV-infected donor (ID_717) dataset showing differentially expressed genes (including both up and down regulated) comparing CD8-Tem and CD8-Tex populations. Genes were arranged by their associated functions indicated above each panel. Cell types were indicated by color below plots. Each dot represents a cell, and the shape of the violin indicates expression distribution within the cluster.



2.3 B and NK cell Dysfunction Induced by HIV Infection

To further compare the gene expression patterns of healthy donor samples and high viral load patient samples, integration analysis of two healthy datasets and three high VL datasets were integrated.

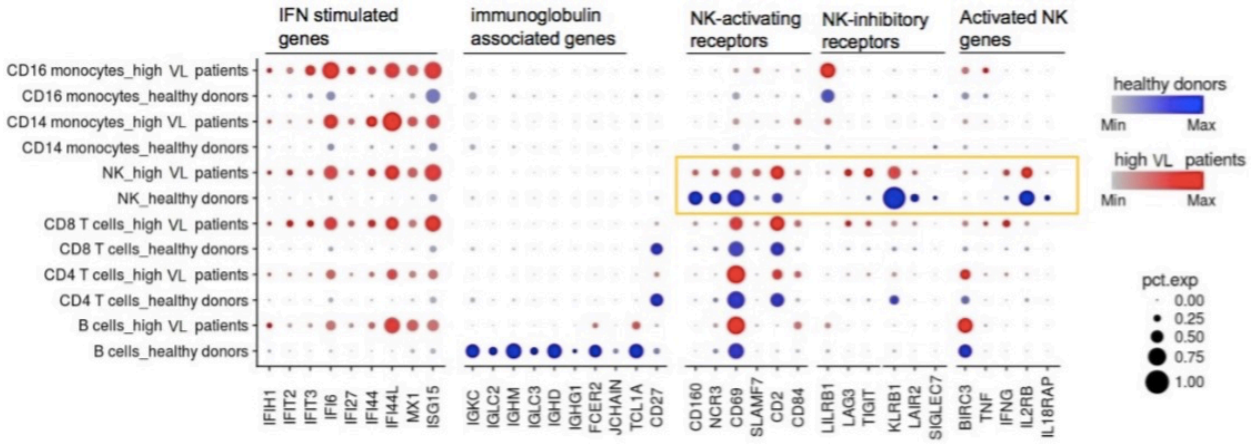
As it is well known, HIV infection hyperactive B cells, and paradoxically suppresses the antibody response at the same time (Le Saout et al., 2014)[70]. It is found in previous sections that B cells had an increased percentage in high viral load HIV-infected samples, compared to healthy control samples (22.1%, 26.9%, 33.7%, versus 8.7%, and 15.1%). However, it is observed in integrated analysis that, expression of IGKC, IGLC2, IGHM, IGLC3, IGHD, IGHG1,

FCER2, and JCHAIN was drastically down-regulated in high VL patients. Along with these immunoglobulin genes, the naïve B cell marker gene TCL1A and the activated B cell marker gene CD27 were also hugely down regulated. On the other hand, the B cell inhibitory receptor gene LILRB1 was up regulated.

NK cell's role in antiviral response to HIV-1 has been gradually revealed in recent study (Scully and Alter, 2016)[71]. It is revealed in our analysis that, the expression of several NK receptor genes was changed by HIV infection (high viral load). As proposed in literature, these activating and inhibitory receptors control cytokine secretion and cytotoxic functions (Vivier et al., 2008)[72]. Activating receptor genes SLAMF7, CD2, and CD84 were up regulated, while the expression of CD160, NCR3, and CD69 were supressed. Also, inhibitory receptor genes LILRB1, LAG3, and TIGIT were up regulated, but KLRB1, LAIR2, and SIGLEC7 were down regulated. Other than activating and inhibitory receptor genes, NK cell activation marker gene BIRC3, TNF, and IFNG (Costanzo et al., 2018)[42] were observed to have increased expression in high HL patient samples, compared to healthy control samples. Although NK cells play a positive role in antiviral response, their functions are impaired by chronic HIV infection (Scully and Alter, 2016)[71]. Consistent with this literature finding, genes involved in encoding cytokine signaling proteins (e.g. IL12RB, and IL18RAP) were found to be down regulated.

Our integration analysis indicates a possible impaired NK cell response in chronic HIV infection.

Graph 2.3: Dot plot demonstrating some of the differentially expressed genes in integrated analysis of healthy and high VL donors. Only six of the major cell types were included. The color intensity indicates expression level, while the size of the dot indicates percentage of cells expressing specific genes.



Chapter 3: Materials and Methods

3.1 Cell Ranger

Cell Ranger is a set of terminal scripts that devised by 10X Genomics to process single-cell RNA sequencing outputs. The analysis pipelines in Cell Ranger perform primary analysis including alignments, feature-barcode matrices generation, clustering, and primary gene expression analysis. In this project, only cellranger count and cellranger aggr from cell ranger pipeline are used.

Cellranger count takes in fastq files, and performs alignment, filtering, barcode and UMI counting, and outputs three matrices files for secondary analysis. The single-cell RNA sequencing dataset in this project was mapped against human hg19 and GRCh38 reference genome using cellranger count.

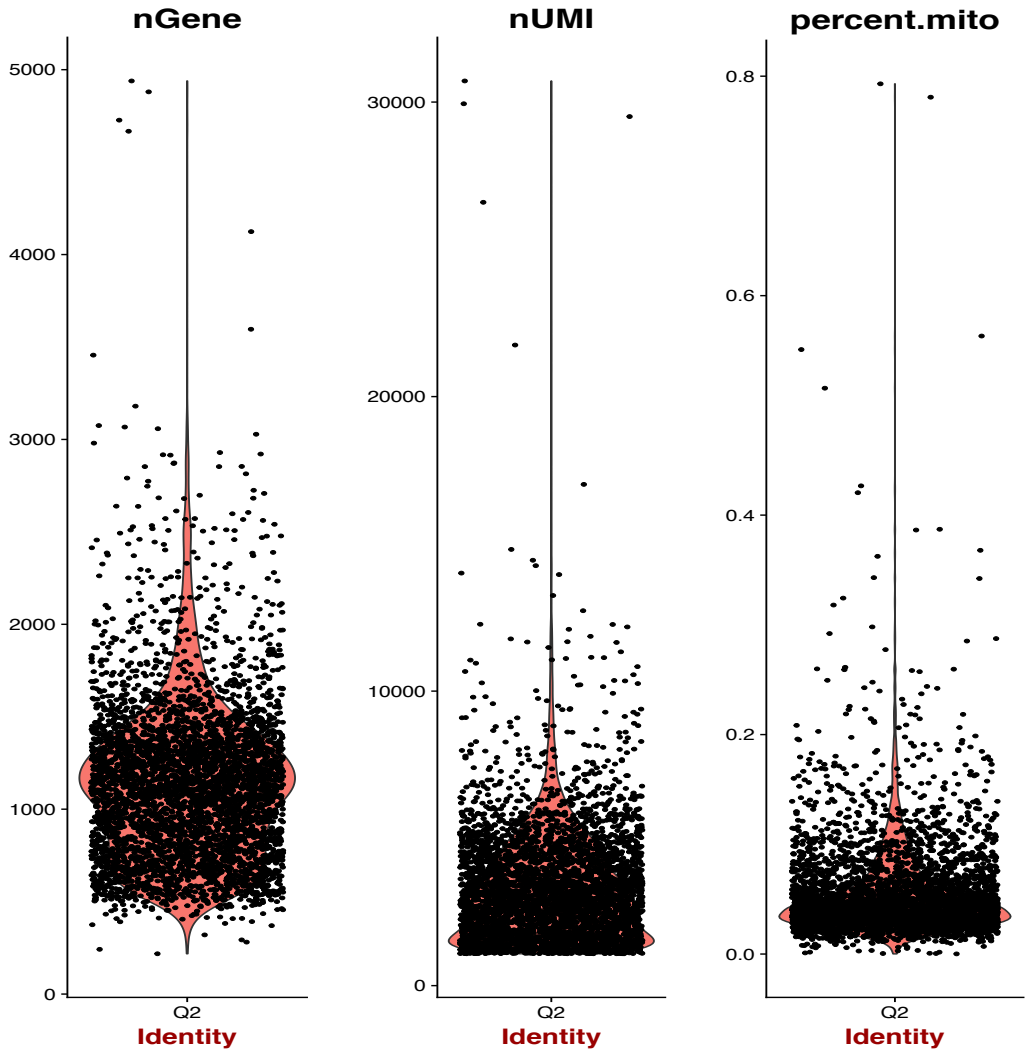
The function of cellranger aggr is simply to aggregate output results from multiple runs of cellranger count. Data resulting from different runs usually have various sequencing depth. It is important to use cellranger aggr to normalize all the runs to the same sequencing depth, and then recompute feature-barcode matrices on the aggregated dataset. All the fastqs from one single run were cellranger counted together first. All the samples that have multiple runs were then aggregated using cellranger aggr. The resulted feature-barcode matrices were used in downstream analysis.

3.2 Seurat scRNA Clustering

Sparse matrices from Cell Ranger pipeline were imported per sample into R package Seurat (v.2.2.0). All the steps mentioned below were performed on each healthy donor and HIV-Infected patient scRNA dataset. First of all, a seurat object is created from the imported matrices, with a number of genes greater or equal to 200 as a minimum cutoff. Before the actual clustering, pre-processing is required for all the datasets.

Firstly, quality control visualizations are used to facilitate cell filtering process. Datasets are filtered based on the violin plots featuring number of genes, UMI counts, and percentage of mitochondrial RNA content. Cells were filtered out if they have nGenes too high or too low, or if they have high expression level of percent.mito. A lower-than-threshold gene counts indicates a badly sequenced cell; a higher-than-threshold gene counts, on the other hand, indicates a possible doublet cell. A high percent.mito indicates a cell undergoing apoptosis or lysis.

Graph 3.2: Violin plot showing QC metrics for one of the HIV-infected donor datasets.



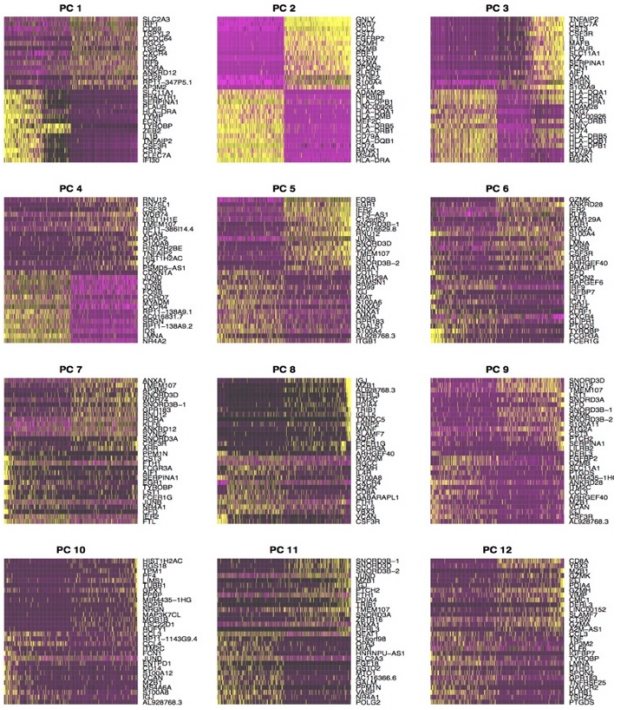
Then, using the Seurat function NormalizeData(), the datasets are each normalized using log normalization method with a scale factor of 10000. LogNormalization normalizes feature counts of each cell by diving each feature count (gene count) by the total counts for that cell, and then multiply it by the scale factor defined.

After normalization, Seurat has a function FindVariableGenes to detect highly variable genes to focus for downstream analysis. Finding variable genes helps controlling the relationship between gene variability and average expression. This function put genes into bins based on calculated average expression and dispersion, and then calculates a z-score for each bin. Although the parameters x.high.cutoff and y.high.cutoff should be adjusted, the dispersion plots of our datasets usually already have these parameters somewhat marking visual outliers, so default parameters from the Seurat Clustering Tutorial are used.

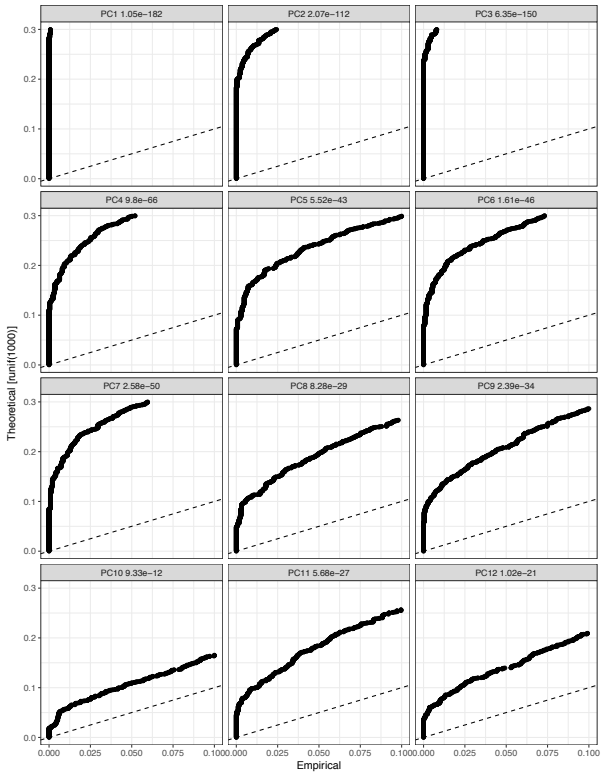
As suggested in the tutorial, the datasets were then scaled to regress out unwanted sources of variation, and batch effects and so on.

After the pre-process pipeline, PCA (principal component analysis) was performed on the scaled data as a linear dimensional reduction method. All the variable genes in previous steps were used as input. Principal component heatmap of first 12 to 15 PC's and JackStrawPlot were used as PC visualizations to facilitate determining significant principal components for each dataset. PC's that are distinctively separated in PC heatmap and are having solid line above dashed line (indicating a strong enrichment of low p-valued genes) in JackStrawPlot were chosen.

Graph 3.2.2: Heatmap showing first 12 PCs of linear dimensional reduction, with cells = 500, for one of the HIV-infected donor datasets.



Graph 3.2.3: JackStrawPlot visualizing the distribution of p values for first 12 PCs for one of the HIV-infected donor datasets.



Finally, after all these steps, the datasets were clustered using Seurat function FindClusters(). K-nearest neighbors algorithm was used as the cluster learning method, and Louvain algorithm was used as the modularity optimization method. The resolution parameter in function FindClusters() are used to adjust number of clusters expected. Changing resolution does not lead to the difference in cell distribution in further visualization steps. The number of dimensions used in FindClusters() were the same as number of PC's in previous step.

Seurat uses tSNE (t-distributed stochastic neighbor embedding) as the cluster visualization method in v.2.2.0. Same number of PC's are used as in cluster finding as suggested.

3.3 Seurat scRNA DEG Analysis

After reasonable number of clusters obtained, the next step was to find differentially expresssed genes in each clusters to define cell types using cluster biomarkers, and to find novel differentially expressed genes across cell types, and across healthy donors and HIV-Infected patient samples. All the steps mentioned below were performed on each healthy donor and HIV-Infected patient scRNA dataset. Seurat FindAllMarkers function was performed for each dataset with default parameters (min.pct = 0.25, and thresh.use = 0.25). This function identifies positive and negative markers for each cluster in a dataset compared to all other cells.

Seurat provides various ways of gene expression visualization. In this project, different visualization methods are used for different purposes. Violin plot and feature plot were particularly useful in defining cluster cell types and subtypes with established biomarkers. Heatmaps were mainly used to find novel differentially expressed genes in each cluster.

3.4 Seurat scRNA T Cell Population Zoom In

In order to further identify signiature genes in various T cell subtypes, T cell clusters were selected using the SubsetData function in supplementary vignettes. All the steps mentioned below were performed on each healthy donor and HIV-Infected patient scRNA dataset. Additional rounds of pre-processing, clustering, and DEG analysis was done on each T cell subset as described in 3.1, 3.2, and 3.3.

Thus, T cell subtypes were identified using specific biomarkers in these subsets. Among all the T cell subtypes, exhausted CD8+ T cells and effector memory CD8+ T cells from three high viral loads patient sample were separately analyzed. Also, up and down regulated differentially expressed genes for each T cell subtype were discovered using the zoom in method.

3.5 Seurat Multiple Dataset Integration Analysis

Seurat v.2.2.0 and v.3.2.0 integration analysis pipeline were used to integrate samples. Three high viral load samples, three low viral load samples, and two healthy donor samples are integrated first. Then, healthy donors and high viral load samples, healthy donors and low viral load samples, and high viral load and low viral load samples were integrated. All the six integration analysis were performed using the following pipelines.

The seurat objects for each sample was normalized using NormalizeData(). Variable genes were found using FindVariableFeatures(). These two steps were the same as in 3.2. Then integration process was performed with functions FindIntegrationAnchors(), and IntegrateData(). After the datasets were merged, functions ScaleData(), RunPCA(), RunTSNE(), FindNeighbors(), and FindClusters() were applied to the integrated dataset as the standard workflow for clustering and visualization, similar to 3.2. tSNE (t-distributed stochastic neighbor embedding) was used as the visualization method, so as to be consistent with previous steps.

3.6 Monocle Pseudotime Trajectories Analysis

Monocle 3 was used as a wrapper package to import processed seurat data metrices of T cell clusters using importCDS function. Size factors and dispersions were pre-calculated using function estimateSizeFactors() and estimateDispersions() to help normalize and perform DEG analysis. The preprocessCDS() function was used to normalize and pre-process the dataset, with parameter num_dim = 10. Cell types were consistent as defined in Seurat. The function reduceDimension() with reduction method UMAP was used to perform dimensionality reduction on the dataset. Then the cells were partitioned to facilitate future trajectory recognition using partitionCells(). The trajectory was then learned using learnGraph(), with RGE_method set to be simplePPT. In the end, the trajectory was visualized using tSNE, with the root of the pseudotime trajectory set to be CD8+ T effector memory cells.

Chapter 4: Discussion and Future Perspective

In our study, we utilized single-cell RNA sequencing technology to analyze the atlas of immune cell exhaustion in HIV-infected individuals. As a result, this project successfully identified significant and common cell type population changes in all HIV-infected samples, and proposed possible function changes of CD4+, CD8+ T cells, B cells, and NK cells based on previous findings. Most significantly, exhaustion markers CD160 and TIGIT, along with our newly identified marker KLRG1 were found highly expressed in exhausted CD8+ T cell populations. Further experiment showed that KLRG1 and TIGIT co-expression discerns new exhausted population, and KLRG1 blockade effectively restores the function of CD8+ T cells, thus suggesting KLRG1 has the potential to be a novel target for immunotherapy for HIV infection. All in all, this project demonstrates how new high throughput sequencing technologies like single-cell RNA sequencing can be utilized to study the gene expression profile under the influence of diseases and viruses like HIV infection. scRNA enables us to characterize rare cell types and zoom in on them to study what is going on in these clusters. Further research on what we learned from bioinformatics analysis provides us with novel target for new cure therapies.

The immune cell atlas revealed by this project and previous literatures (Khaitan and Unutmaz, 2011)[73] together provide a clearer picture of HIV-induced immune deficiency on gene expression level. Although HIV infection and replication leads to chronic proinflammatory signaling , and thus activates the immune system, the depletion of CD4+ T cells results in effector function reduction, and in the end impairs other cell types functions relying on CD4+ T cells, like CD8+ T cells and B cells. Therefore, despite the fact that CD8+ T cells and B cells were activated and expanded encountering HIV infection, these cells were unable to differentiate into effector cells to fight back. Not to mention that T and B cell exhaustion exacerbates the immune deficiency (Catalfamo et al., 2008; Le Saout et al., 2014), which makes

things worse. In conclusion, this project shows the vital role of immune cell exhaustion and chronic inflammation in the pathological process of HIV infection.

This project is currently being prepared for submission for publication of the material. Shaobo Wang, Qiong Zhang, Hui Hui, Kriti Agrawal, Maile Ann Young Karris, and Tariq M. Rana. The dissertation/thesis author was the author of this material.

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