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Sarah Hancock March 18, 2023

Impact of cytokines/chemokines on epigenetic modifications in immune cells isolated from healthy Down syndrome individuals

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Abstract

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Down syndrome (DS) is the most common genetic disorder, affecting individuals' development, neurological function, and immune system. DS is caused by the trisomy of chromosome 21, which encodes 4/6 IFN receptors, causing over signaling of immune cells and thus chronic inflammation. It is proposed that epigenetic modifications of histones have an impact on the immune system signaling and cell functions. The circulating cytokines, immune cell frequencies, and histone modifications of H3K27Me3, H3K4Me3, and H3K9Ac were analyzed in a cohort of DS (n = 51) and non-down syndrome (n=59) individuals from São Paulo, Brazil. The cytokine analysis revealed that DS individuals had lower levels of TGF-β1 and TGF- β2, but higher levels of TGF- β3 and IL-10, and 9 out of the 38 chemokines were significantly elevated. The immune cell phenotypes (monocytes, natural killer cells, $CD4^+$ and $CD8^+$ lymphocytes, and B lymphocytes) were also found to be significantly different between the groups. All three histone modifications were upregulated in DS compared to non-DS individuals in four out of the six immune subsets analyzed. Finally, correlations between H3K27Me3, H3K4Me3, and H3K9Ac modifications to circulating levels of IL-10, FLT3 ligand, TGF-β1, and TGF- β2 were established. These findings suggest that DS has distinct immunological characteristics, which are modulated through cytokine/chemokine expression and epigenetics. The results have implications for understanding the immune dysregulation in DS individuals, which may lead to the development of novel therapeutic interventions.

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Introduction

Down Syndrome

Down syndrome (DS) is a genetic condition which occurs in approximately 1 in 700 live births in the United States [1]. Down syndrome is known to affect the individuals' physical appearance, physical and neurological development, and increases their risk for several clinical conditions and comorbidities such as cardiac pathology, immunodeficiency, auto-immunity, and lymphomas [2-4]. Over the past 60 years life expectancy of DS individuals has increased due to advances in healthcare and research [5]. Furthermore, the prevalence rate of DS individuals in the United States has also increased over the last fifteen years from 13.65 to 15.74 per 10,000 live births [1]. DS individuals are at increased risk for infections and increased risk for severity when infected as compared to the general population. This poorer prognosis has been associated with immune system abnormalities [6, 7].

Down syndrome is caused by partial or complete trisomy of human chromosome 21 (Hsa21) which codes for 225 genes, of which include four out of six interferon receptor (IFNR) subunits: Type 1 IFNR subunit 1 (IFNAR1) and 2 (IFNAR2), Type II Interferon γ Receptor 2 (IFNGR2) and the interleukin (IL) 10 receptor 2 (IL10R2)[8], which encodes for the cytokine IL-10, but also IFN III (IL-29) [9] . This upregulation of interferon receptors on immune cells leads to increased IFN signaling which ultimately affects downstream interferon-stimulated genes (ISG), causing interferonopathies [10]. While upregulation of IFNR is only slightly increased, the collective downstream effects on ISGs produce a larger change in inflammatory cytokines and chemokines, affecting immune system function in both innate and adaptive compartments [11].

Gene expression is modulated by epigenetic modifications. Epigenetic changes, such as DNA methylation and histone modifications (i.e methylation, acetylation), can regulate chromatin

accessibility and consequently downstream gene expression by modulating how the transcriptional machinery binds to gene promoters and promotes transcription and elongation, therefore influencing the immune response. For instance, changes in DNA methylation patterns in immune cells have been associated with altered gene expression patterns and immune dysfunction in various diseases [12]. Moreover, epigenetic modifications are influenced by plasma cytokines, environmental factors, such as diet, stress, and exposure to toxins, which can further impact the immune response. Therefore, understanding how epigenetics modulates the immune system can provide insight into the underlying mechanisms of immune-related disorders and aid in the development of novel therapies. Thus, the major goal of this work was to evaluate how the cytokine and chemokine environment in DS subjects alters the epigenetic modification in innate and adaptive cells as compared to non-DS individuals. *We hypothesized that DS individuals, by presenting a more inflamed immune system [13] would present a significant different cytokine/chemokine profile in the plasma that induces epigenetic modifications in both arms of the immune system associated with activation of gene expression.*

Immune System Overview

The immune system is divided into innate and adaptive responses. Innate immunity is the first line of defense against harmful pathogens and plays a critical role in the recognition and neutralization of foreign microorganisms. Innate immunity is a non-specific defense mechanism that responds to antigens within a short period of time, unlike adaptive immunity which is a long-term memory response that is characterized by the production of antigen-specific antibodies. However, in the past years the so called “trained immunity” has been reported as a form of immune memory in innate cells [14].

The immune response is a cascade of events ranging from sensing of the "foreign" signal by the innate cells to triggering the adaptive immune responses to fight infection in a specific way; when successful this cascade results in the elimination of the "perturbing" signal, reestablishing immune homeostasis [11]. The process begins with the recognition of an antigen by innate immune cells such as monocytes/macrophages and dendritic cells. These innate cells sense environmental alterations and produce cytokines to interact with other immune cells through cytokine/chemokine production and by presenting the antigen on their cell surface to modulate adaptive immune cells, such as T cells and B cells. The adaptive immune cells then undergo activation and proliferation, leading to the development of effector cells that can directly eliminate the antigen or help coordinate a broader immune response. Throughout this process, various signaling molecules and cytokines are released, which can further amplify the immune response and recruit additional immune cells to the site of infection or inflammation [11]. Ultimately, the immune system cascade is a highly coordinated and dynamic process that enables the body to effectively recognize and eliminate a wide range of threats.

Cytokine Profile

Cytokines are small proteins that act as signaling molecules between cells, regulating the innate and adaptive immune responses as well as inflammation. Cytokines can be classified as interleukins (IL), interferons, tumor necrosis factor (TNF), and chemokines, each with unique functions and can either promote or inhibit inflammation. Pro-inflammation cytokines that activate immune cells include IL-1, TNF-α, IL-6, IL-17α, and IL-12 [15-18] while anti-inflammatory cytokines suppress immune function and include TGF- β1, TGF- β2, TGF- β3, and IL-10 [19] (**Supplemental Table 1)**. The balance between pro- and anti-inflammatory cytokines is crucial for maintaining a healthy immune system, as an imbalance can lead to autoimmune diseases, chronic

inflammation, and other disorders. The cytokine profile can vary depending on the type of infection or disease, as well as individual genetics and environmental factors.

Down syndrome individuals have been reported to have higher levels of circulating cytokines compared to controls. TNF-α, IL-1β, IFN-γ were reported to be at a higher level in adults and children, which are known to influence chronic inflammation and autoimmune diseases [20]. Further, greater levels of both pro-inflammatory and anti-inflammatory cytokines, IL-2, IL-6, IL-10, and IL-1Ra respectively, were observed in DS children [21]. This increase in cytokines is hypothesized to impact downstream immune cell function and lead to immune exhaustion.

Chemokines are a subset of cytokines that are responsible for immune cell migration and inflammation (**Supplemental Table 2**). Monocyte chemoattractant proteins (MCP-1and MCP-2) regulate the migration of innate immune cells [22]. MCP-1 have been found to be significantly elevated in diseases such as diabetes mellitus type 1 [23], breast cancer [24], and chronic inflammatory neurodegenerative disorders [25]. Eotaxin also plays an important role in innate cell recruitment, as it signals eosinophils to the site of inflammation. Abnormalities in Eotaxin levels have been associated with rheumatoid arthritis [26, 27] and neuroinflammatory diseases such as multiple sclerosis (MS) and Alzheimer's disease (AD) [28, 29]. Macrophage inflammatory proteins (MIP-1α and MIP-3β) is secreted by macrophages and is related to inflammation regulation and wound healing and the latter to migration of immune cells to lymph nodes, a major player for immune presentation [30]. Interferon-gamma induced protein 10 (IP-10) is secreted by innate cells to activate dendritic cells, natural killer cells, and T cells [31]. IP-10 is associated with autoimmune diseases such as thyroid disease [32]. Increased IP-10, MCP-1, MCP-2, MIP-1α have all been associated with severity of disease of COVID-19 patients, with MCP-1 and IP-10 having the greatest positive correlation [31]. Interferon-inducible T cell alpha (I-TAC) is closely related

to IP-10 and is responsible for recruitment of IL-2 activated T cells [33]. Stromal cell-derived factor 1 alpha (SDF-1α) acts upon lymphocytes, developing neutrophils, and hematopoietic stem cells [34]. It is known to activate the STAT3 pathway and is important for the signal angiogenesis. Increased SDF-1α levels are associated with Ig4 related diseases, rheumatoid arthritis, osteoarthritis as well as influence cancer tumor growth [35, 36]. Fms-like tyrosine kinas 3 ligand (FLT3L) is a growth factor that is responsible for the development of dendritic cells [37]. FLT3L upregulation is known to occur in individuals with GATA2 mutations and acts as an important biomarker for acute myeloid leukemia [38, 39].

Natural Killer Cells

Natural killer (NK) cells are a type of lymphocyte that play a crucial role in innate immunity and defense against infections. NK cells can recognize and eliminate affected cells immediately, without needing multiple infections by detecting abnormalities in cell surface receptors [40]. Target cells fail to express sufficient levels of major histocompatibility complex class I (MHC-I) molecules. Upon recognition of a target cell, NK cells release cytotoxic granules containing perforin and granzymes, leading to the destruction of the target cell. NK cells can also produce cytokines, such as interferon-γ (IFN-γ), that activate macrophages and dendritic cells, to enhance the immune response. Cytokines such as IL-2, IL-12, IL-15, IL-18, and IL-21, have been shown to regulate NK cell maturation and activation. [41]. The dysregulation of NK cell function has been shown to increase susceptibility to infections and cancer, as well as contribute to autoimmune and inflammatory diseases [42]. Multiple studies report higher levels of NK cells in children with DS compared to non-DS children, as well as in adults [43-46]. Research on the effects of interferons on NK cells in adults showed that DS individuals exhibit hypersensitivity [44].

Dendritic Cells

Dendritic cells (DCs) are phagocytic antigen-presenting cells that continuously sample their environment for foreign antigens, such as bacteria, viruses, and tumor cells. Upon encountering an antigen, DCs undergo a process of maturation, which involves upregulation of co-stimulatory molecules, such as CD80 and CD86, and secretion of cytokines, such as IL-12α. DCs present processed peptides from the antigen on their surface via MHC molecules to activate cytotoxic T cells. The absolute number of DCs has been found to be lower in DS children, with the number of plasmacytoid dendritic cells has been reported as normal levels [43]. There is very little literature on DC specific sub-populations with respect to DS.

Monocytes

Monocytes are a type of leukocyte that circulates in the bloodstream. Monocytes originate from hematopoietic stem cells in the bone marrow and are released into the bloodstream, where they circulate for approximately one to three days before being recruited to sites of inflammation or infection. Upon arrival at these sites, monocytes undergo differentiation into macrophages, which are critical for the clearance of foreign particles, such as bacteria and viruses, via phagocytosis. At the arrival site, macrophages secrete both pro-inflammatory cytokines such as TNF-α, IL-8, MIP-1α, and MCP-1, IL-6, and IL-8 and anti-inflammatory cytokines such as IL-10, depending on the immune response [41]. Similarly, to DCs, macrophages activate the downstream T cell response by presenting the antigen on their surface.

Research on monocyte levels in DS children have been observed to be different compared to controls. Studies observed that infants and children had an increased level of intermediate and non-classical monocytes, but one reported a lower level of total monocytes. [43, 47]. Decreased levels of IL-1 and IFN-γ and increased levels of IL-10 production were also observed in infants

[47]. Clearly the number of monocyte cells as well as their functionality of cytokine production is affected in DS.

CD4$^+$ T Cells

T cells differentiate into either $CD4^+$ T cells, also known as helper T-cells, or $CD8^+$ T cells, also known as cytotoxic or killer T cells. Helper T cells are responsible for recognizing the antigen that is presented via the major histocompatibility complex (MHC) Class II protein through their T-cell receptors (TCR). Active helper T cells release cytokines that act upon various immune cell types and mediates the innate immune response. Subpopulations of T helper cells include type-1 T helper (Th1) cells which produce IFN-γ, IL-2, and TNF-β to increase phagocytosis and activate NK cells; and Type-2 T helper (Th2) which releases IL-4, IL-5, IL-10, and IL-13 to activate downstream antibody production [15].

DS individuals and children have been reported to experience T-cell exhaustion, with lower naïve $CD4^+$ T cell populations, but higher central memory $CD4^+$ compared to health controls [16, 17, 21, 48, 49]. Specifically in DS children, the number of helper T cells was significantly lower, however the level of IL-2 was not, suggesting that IL-2 production may not be affected by DS but rather the response mechanism to the cytokine [50]. Pro-inflammatory IL-6 has also been found to be elevated in $CD4^+$ T cells [49].

CD8$^+$ T Cells

$CD8^+$ T cells are vital for the adaptive immune response as they are responsible for recognizing and eliminating infected cells. After recognition of the antigen by their TCR, naïve cytotoxic T-cells undergo clonal expansion and differentiation into effector T cells and memory T cells. Effector T cells have a rapid response to kill infected cells by inducing apoptosis of the target cell, while memory T cells persist as long-term, antigen-specific cells that can proliferate and

secrete cytokines after encountering an antigen for a second time. The activation and differentiation of $CD8^+$ T cells are regulated by several cytokines, including interleukin-2 (IL-2) and IFN-γ. Dysfunction or depletion of $CD8^+$ T cells has been associated with several immunodeficiency disorders, such as HIV infection and primary immunodeficiencies, and can also contribute to the development of autoimmune diseases.

In Down syndrome, $CD8^+$ levels have been reported to be lower in children (ages 0-18) with Down syndrome compared to healthy individuals [48, 51]. However, research has demonstrated that as individuals age, T cell differentiation gradually increases which results in normal to higher $CD8^+$ frequencies [52, 53]. Multiple studies of adult DS populations (over 25 years old) report that $CD8^+$ levels are higher than healthy individuals of the same age [54, 55]. Further, $CD8^+$ T cells of DS individuals have been found to be hyperresponsive to stimuli and overly produce cytokines [55].

B Cells

B cells also play a critical role in adaptive immunity by producing antibodies that recognize and neutralize specific pathogens. B cells are activated by interacting with APCs or by the binding of an antigen directly to the B cell receptor, initiating B cell proliferation and differentiation. Differentiation is regulated by cytokines such as IL-4, IL-6, and TGF-b that act on transcription factors of naïve B cells. Most B cells undergo maturation, located in the bone marrow, into plasma B cells that produce antigen-specific antibodies to fight the infection quickly, whereas other cells differentiate into memory B cells that are programmed to mobilize to produce antibodies upon reinfection. Total B cell populations are also known to be lower in DS individuals, with the greatest difference observed for effector cells and memory B cells, compared to health controls and are hypothesized to produce autoantibodies that contribute to autoimmune disorders [49, 56].

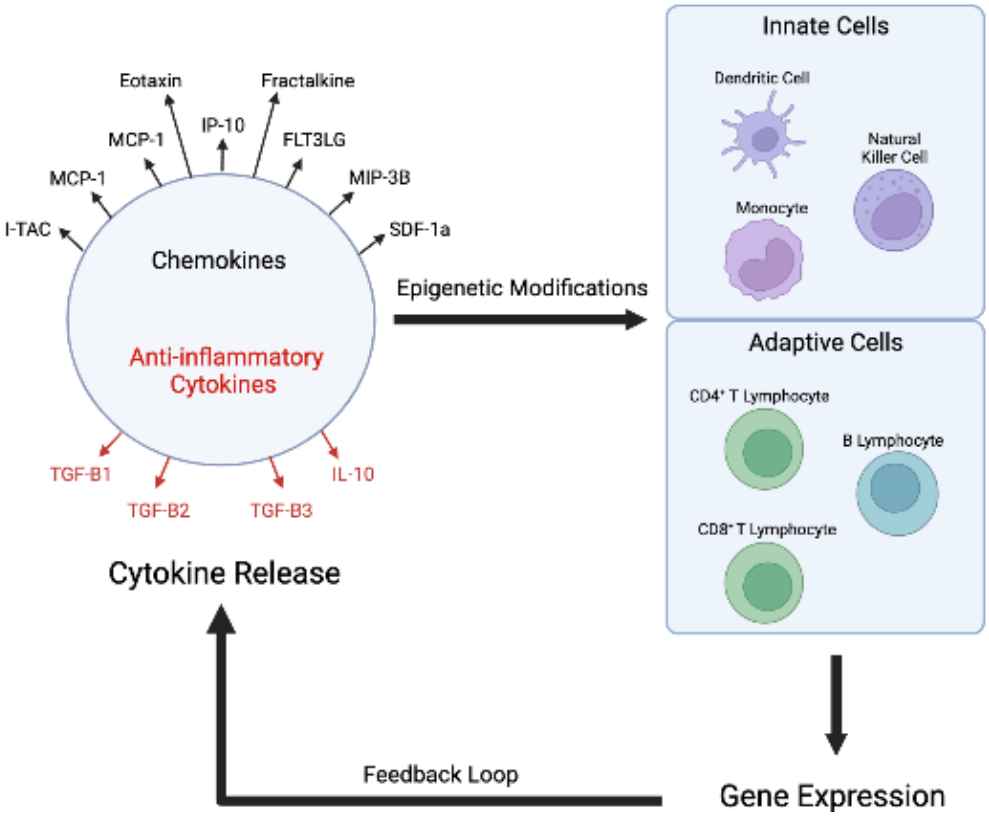

Figure 1. Working hypothesis: cytokine modulation of innate and adaptive immune cells through epigenetics and its downstream effects and feedback loop. Cytokines and chemokines interact on target immune cells. Cytokines may cause epigenetic changes which affect gene expression in innate (i.e dendritic cells, monocytes, and natural killers) and adaptive cells (i.e $CD4^+$, $CD8^+$, and B lymphocytes). Diagram depicts a healthy individual. In individuals with DS, the interactions between cytokines, epigenetics, and immune cells are altered. Created using BioRender.

Epigenetics

Epigenetic modifications are changes in gene expression due to post-translational modifications of DNA, RNA or proteins, such as histones, and are influenced by environmental factors [57]. The most common epigenetic factors are DNA methylation and post-translational histone modifications, and both impact transcription factor accessibility to gene promoters. Genomic DNA is tightly coiled around histone proteins, which are regulated by lysine amino acid residues located in the tail end of the proteins. Histone 3 (H3) has been shown to be under epigenetic control by methylation of lysine 27 (H3K27Me3) and lysine 4 (H3K4Me3) and acetylation of lysine 9 (H3K9Ac) [58]. Research shows that adaptive immune development and

response is linked to epigenetic modifications, specifically $CD8^+$ T cell differentiation is regulated by DNMT1 [59], H3K4me3, and H3K27me3 [60], T cell exhaustion is linked to DNMT3a gene [61], and $CD4^+$ T cell differentiation is linked to H3K4me3 and H3K27Me3 [62]. Previous research has been conducted on the connections between immune system dysregulation and epigenetic modifications in mice models and Down syndrome individuals [63], but the there is still a lack of understanding of this relationship.

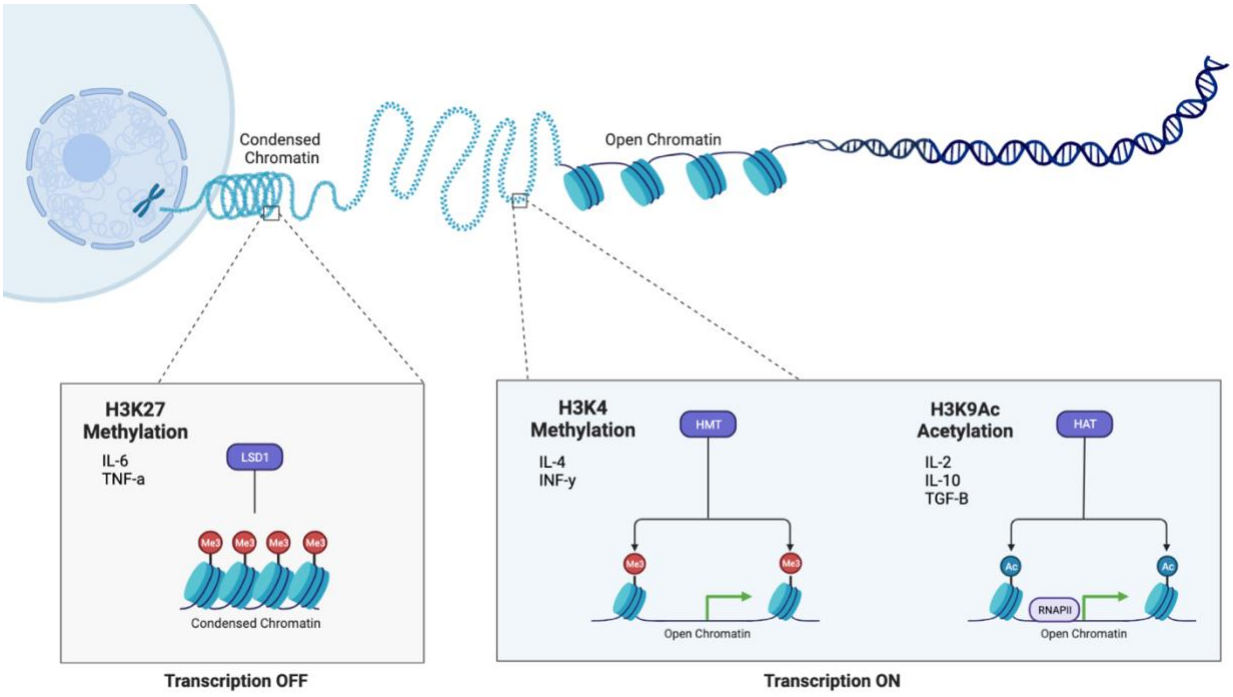

Figure 2. **Histone 3 modifications of K27, K4, and K9 change chromatin accessibility.** Chromatin is wound around histone proteins in a condensed or open form, which determines the accessibility for transcription to occur. Cytokines have been associated with post-translational histone modifications (i.e) methylation by inducing (HMTs) or inhibiting histone methyltransferases (LSD1) or acetyltransferases (HATs) [64-67]. Created using BioRender.

Objectives and Hypothesis

The objective of this project is to evaluate the cytokine/chemokine environment and its consequence to acetylation/methylation of H3 in different immune subsets comparing DS and healthy controls. We hypothesize that the high inflammatory environment in DS is associated with histone modifications promoting active transcription in these subjects in most of the immune subsets. To test this hypothesis cytokines and chemokines were measured in the plasma of a cohort of DS and non-DS individuals by using Meso-scale platform. Additionally, acetylation and methylation on different lysine residues of Histone 3 were evaluated in peripheral mononuclear cells by high density flow cytometry.

Methods

Cohort and Sample Collection

After approval by the Brazilian ethical committee agency (CONEP), DS and non-DS individuals were recruited by the Institute Jo Clement in Sao Paulo, Brazil. A total of 60 DS individuals and age/sex matched non-DS individuals, from the same family or social economic area, signed a consent form and filled out a health survey including their COVID-19 vaccination status, if they had ever tested positive for COVID-19, and their symptoms. Exclusion criteria was the presence of any active infection at the time of collection.

Blood samples (30mL in ACD tubes) were collected from the 120 individuals and processed at the University of Sao Paulo School of Medicine. PBMCs and plasma were isolated and aliquoted immediately upon collection and stored in liquid nitrogen tank or -80C freezers, respectively until shipment to our lab at PATRU, Department of Pathology at Emory University (IRB ID: STUDY00003431).

Thawing of PBMC Samples

PBMC cryovials were transferred from the liquid nitrogen to a 37° C water bath for 1 minute until mostly thawed. Each sample was transferred to a 15mL conical tube containing 9 mL of pre-warmed (37° C) complete RPMI (RPMI-1640 medium, 10% FBS, 1% penicillin, 1% streptomycin, 1% of HEPES) medium. The cells were centrifuged at 1200 rpm for ten minutes, media was discharged in bleach containing bottle, and the pellet was resuspended in 1 mL of complete RPMI containing 0.1% Benzonase Nuclease. This cell suspension was incubated in a 37° C water bath for 15 minutes. Each sample was completed to 15 mL with complete RMPI and centrifuged at 1500 rpm for five minutes. The cells were washed again with 10mL of complete RPMI and then resuspended in 3 mL of complete RPMI for cell counting. The cell number and

viability of the samples was determined using a cell counter (Countess™ Automated Cell Counter, Invitrogen) and the total number of cells per sample was calculated.

Immunofluorescent Cell Staining

An average of 500k cells were used for this flow panel. Cells were transferred into a 96 well V-bottom plate, centrifuged at 1700 rpm for 3 minutes, and the supernatants were discarded using a multichannel pipette. Non-specific Fc-receptor mediated fluorescence blocking control was added: 2.5mg of Fc Block (#564219, BD Pharmigen) as per the manufacturer's instructions, to avoid unspecific binding. Then, the surface antibody mix was added to each sample and incubated for 20 minutes at room temperature in the dark (**Supplemental Table 3**). After surface staining, cells were washed with stain buffer (PBS Corning 1X, 1% FBS), centrifuged at 1700 rpm for 5 minutes, and the supernatant was removed. The cells were permeabilized and fixated by resuspending in 50 µL of Foxp3 Fixation/Permeabilization solution (#00-5523-00 Invitrogen) as per the manufacturer's instructions, and then incubated for 45 minutes at 4° C in the dark. The cells were washed with the Permeabilization Buffer (#00-5523-00 Invitrogen) according to the manufacturer's instructions and centrifuged again. Then the intracellular antibody mix was diluted in perm wash buffer and added to each sample. Intracellular staining was incubated for 45 minutes at 4° C in the dark. The cells were washed with perm wash buffer using the same procedure as before (**Supplemental Table 3**). Then the cells were resuspended in 200 µL of stain buffer for acquisition as below.

Flow Cytometer

Compensation was performed with single-stained controls for each fluorochrome. The samples were acquired using a FACSymphony™ A5 Cell Analyzer (BD Biosciences) and

analyzed by FlowJo software (version 10.8.2). Gating was performed to select for desired cell populations (**Figure 4**).

Cytokine Assay

Mutiplex ELISA (Mesoscale) assay, commercially available by MSD (Meso Scale Discovery Rockville, MD, United States) was used for plasma cytokine evaluation. A cytokine panel containing the following was used: TGF-β1, TGF-β2, TGF-β3, IL-17F, IL-21, IL-22, IL-27, IL-29/IFN-1, IL-9, MCP-2, MCP-3, TRAIL, Eotaxin, IL-13, IL-17α, IL-5, IL-6, IL-8, IP-10, MCP-1, MIP-1α, TNFα, IFN- α2α, IFN-b, IFN-g, IL-10, IL-12p70, IL-33, IL-4, FLT3L, Fractalkine, I-TAC, IL-15, IL-18, IL-7, MIP-3α, MIP-3β, SDF-1α, TNF-β as per manufacturer's instructions (**Supplemental Table1, 2**). Briefly, on the day before, plates were coated with the capture antibody with the assigned linker designating the cytokine was added to each well of a 10-spot U-PLEX plate and incubated overnight at 4°C. After, plates were washed 3 times with PBS containing 0.05% Tween 20 and then the samples and the multi-analyte calibrator standard (after 4-fold serial dilution) were added to their respective wells and incubated for 1 hour at room temperature. After this incubation, plates were washed as previously described and the SULFO-TAG™ conjugated detection antibody were loaded into the plate, and a new round of 1 hour incubation at room temperature was performed. To stop reaction the reading buffer "Read Gold buffer" provided by the kit and, was added and the plates were read by the MESO QuickPlex SQ 120. The results were extrapolated from the standard curve from each specific analyte and plotted in pg/mL, using the DISCOVERY WORKBENCH v4.0 software (Meso Scale Discovery, Rockville, MD, United States)

Statistics

Upon running the mesoscale, statistical outliers were removed by using Grubbs' test, resulting in a cohort of 110 individuals. Donors included non-Down syndrome health controls (non-DS, n = 59) and DS individuals (n=51) (**Table 1**). An unpaired t-test were performed to calculate the statistical differences between the different parameters comparing DS and non-DS samples. A Spearman's correlation plot (**Figure 8**) between cytokines/chemokines and epigenetic markers was performed. A feature selection, to identify the minimal number of features that discriminate both groups was also performed (**Figure 9**) .

	Down Syndrome	**Non-Down Syndrome**
N	51	59
Age (%)		
Under 30	24 (47)	23 (39)
Over 30	27 (53)	36 (61)
Sex (%)		
Female	24 (47)	30 (51)
Male	27 (53)	29 (49)

Table 1. Demographics of DS Cohort. Grubb's test was performed to remove outliers. The cohort included DS (n= 51) and non-DS individuals (n=59). Each individual was classified as under or over 30 years of age (min= 12, max = 56) and by sex.

Results

Cytokine Profile

To visualize the difference between cytokine profiles of non-DS and DS individuals, a principal component analysis (PCA) was performed (**Figure 3**). Cytokines with a larger Principal Component (PC) value in a particular direction contribute more heavily to the multivariate signal in that direction. To determine the significance between cytokine profiles, a two-tailed T test was performed. The results indicate that there is a significant variable ($p = < 0.0001$) contributing to the different clusters in cytokines between DS and non-DS (CI = 0.95) (**Figure 3**).

In order to quantify the differences in cytokine/chemokine expression observed in the PCA, the level each cytokines/chemokine (pg/mL) in each sample (n= 113) was analyzed (**Figure 4**). An unpaired parametric two-tailed T test was used to determine a significant difference ($a = 0.05$) between DS and non-DS groups. We observed that the anti-inflammatory cytokines TGF-β1 ($p= 0.0001$) and TGF- β2 ($p =0.0001$) were significantly lower in DS while levels of TGF- β3 ($p =0.0200$) and IL-10 ($p =0.0729$) were higher (**Figure 4A**). The chemokines MCP-1 ($p=0.0001$), MCP-2 ($p=0.0222$), IP-10 ($p=0.0001$), FT3Lg ($p=0.0001$), Eotaxin ($p=0.0082$), I-TAC ($p=0.0001$), SDF-1α ($p=0.0013$), and MIP-3β ($p=0.0022$) had significantly higher levels in DS compared to non-DS; while Fractalkine was significantly lower ($p=0.0107$) in DS individuals (Figure 4B). The difference of all of the other cytokines (see Cytokine Assay Methods) between the DS and non-DS groups were insignificant.

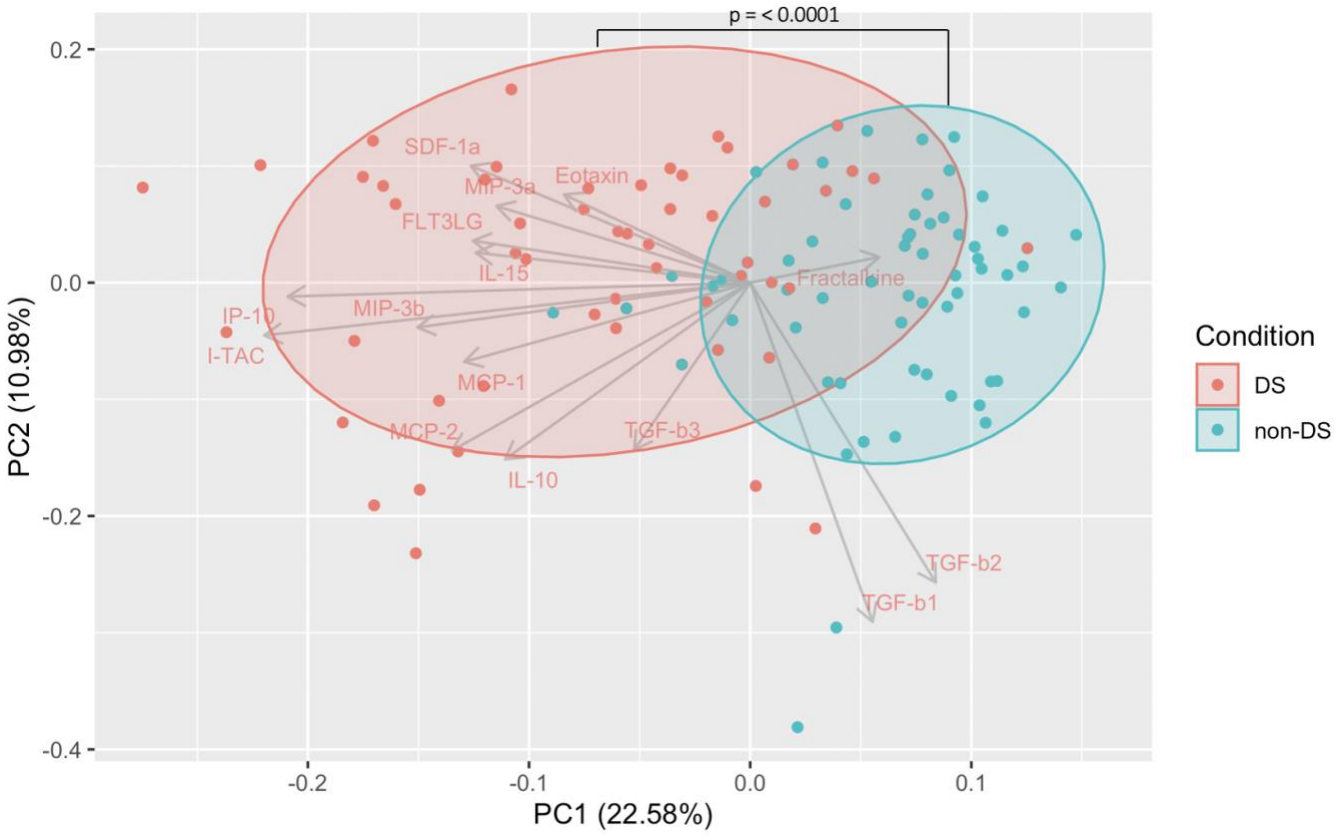

Figure 3. Cytokine profile of DS individuals is significantly different compared to non-DS individuals. A principal component analysis (PCA) of cytokine expression stratified by Down syndrome (DS, n = 51, red dots) and non-Down syndrome individuals (non-DS, n = 59, blue dots) was performed. Each dot represents a sample. The distribution of individuals along PC1 and PC2 is shown; and cytokines directionality is highlighted by the arrows. The PCA was performed including all cytokines and chemokines evaluated. An unpaired two-tailed (p = <0.0001) with a confidence interval of 95% was performed in PC1 and is represented by the ellipses.

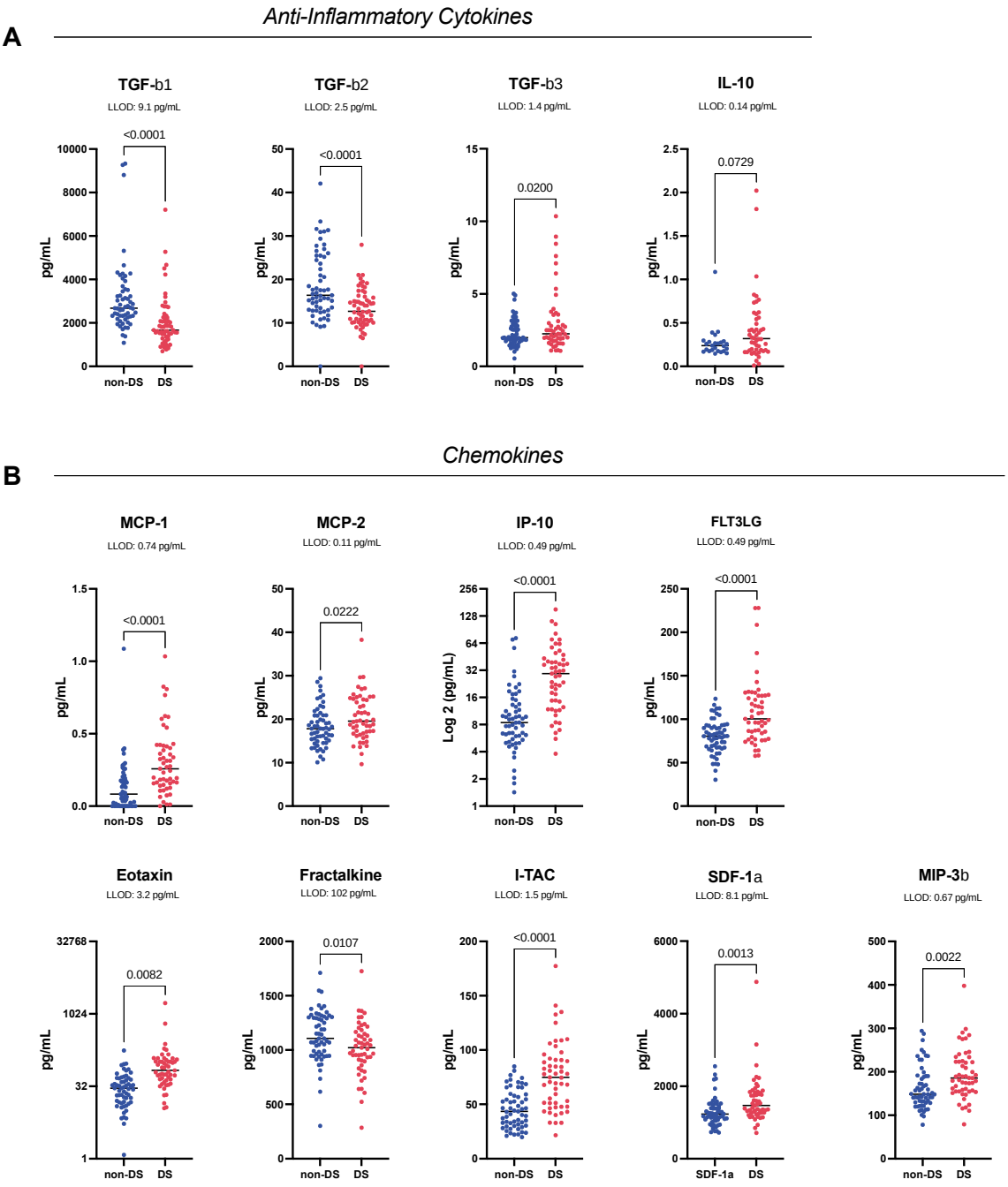

Figure 4. DS cytokine/chemokine profile. Anti-inflammatory cytokines and chemokines levels (pg/mL) were measured for each sample and stratified by DS (n = 51, red) and non-DS (n= 59, blue). An unpaired parametric two-tailed T test was used to determine significance (a <0.05). The p value and lowest level of detection (LLOD) are represented for each. (A) Anti-inflammatory cytokines. (B) Chemokines MCP-1, MCP-2, IP-10, FLT3L, Eotaxin, I-TAC, SDF-1α, and MIP-3β.

Immune Cell Populations

Flow cytometry were utilized to identify immune cell populations and their epigenetic modifications. The gating strategy was implemented to define different immune cell populations, including innate cells such as monocytes, dendritic cells, natural killers; and adaptive cells such as $CD4^+$ and $CD8^+$ T lymphocytes, and B lymphocytes (**Figure 4**). Live cells were first selected from the single cell population (**Figure 4A**). From the live cell population, monocytes were selected based on size and granularity and then gated for $CD14^+$ $CD16^-$ to select the classical monocyte population (**Figure 4B**). Then, cells were gated based on CD3 expression. From the $CD3^-$ population we identified dendritic cell identification, gated on $CD19^-$ $CD56^-$, then HLA-DR^+ $CD14^-$, and finally $CD123^+CD11c^+$, (**Figure 4C**). From the $CD3^-$ population, HLA-DR^- cells were selected, then the population was gated for $CD16^+CD56^-$ to select the natural killer cell population (**Figure 4D**). Also, from the $CD3^-$ population, to identify the B lymphocyte population the $CD19^+$ cells were gated (**Figure 4E**). Finally, $CD4^+$ and $CD8^+$ T lymphocyte populations were selected from the $CD3^+$ population (**Figure 4F**).

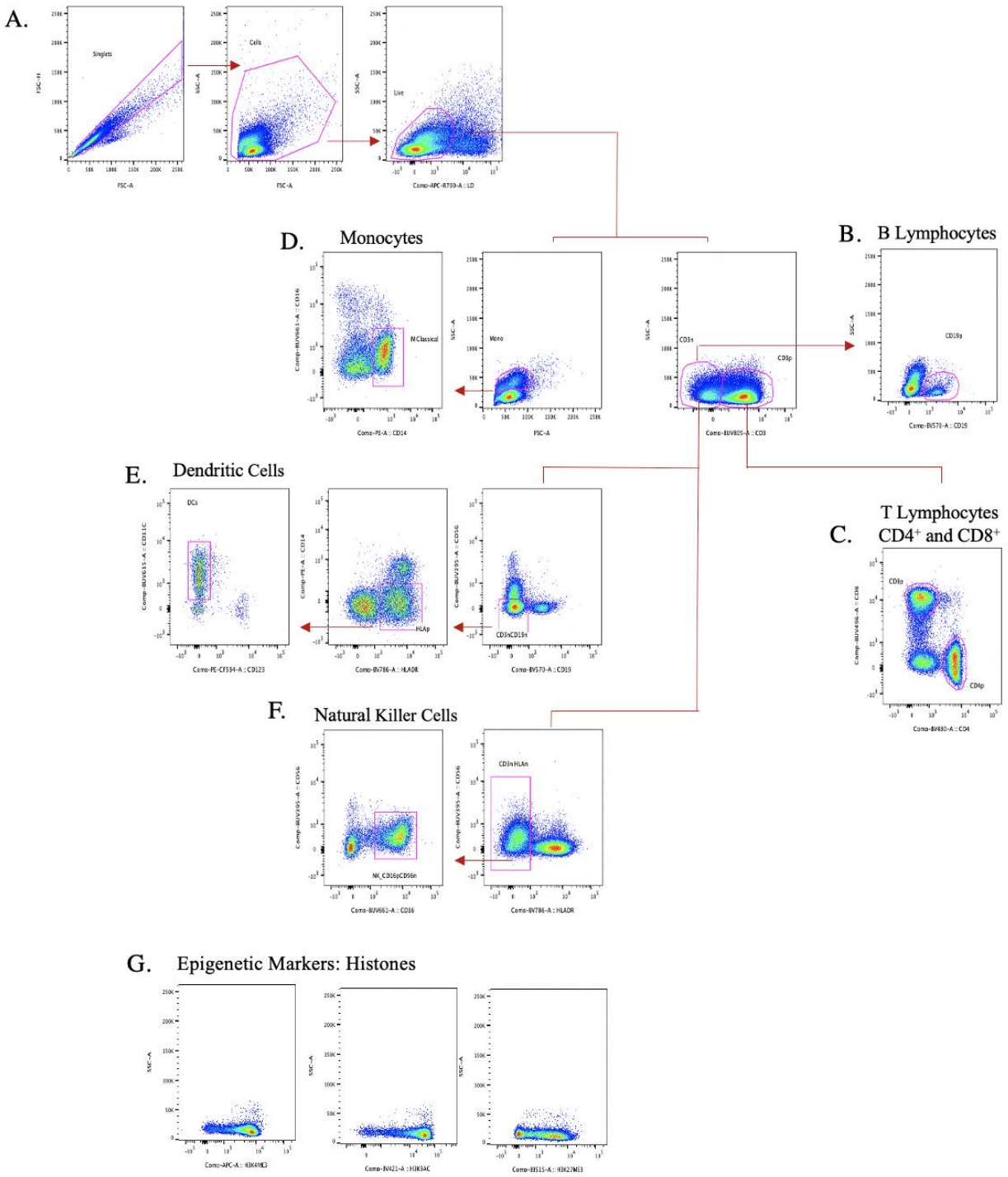

Figure 4. Sequential gating strategy to identify immune cell populations and epigenetic modifications of histones. Representative dot plots of multiparametric flow cytometry panel using two-parameter density plots. The pink gates represent the selection of cell populations, and the red arrows represent the sequential gating. (A) Single cells were first selected and then gated for live cells. (B) From the live cell population, the monocytes were selected and then gated for $CD14^{+}$ and $CD16^{-}$ to select the classical monocyte population. (C) From the live cell population, cells were gated based on CD3 expression. From the $CD3^{-}$ population, the cells were gates for $CD19^{-}$ and $CD56^{-}$, then $HLA\text{-}DR^{+}$ and $CD14^{-}$, and finally $CD123^{+}$ and $CD11c^{+}$ to select the dendritic cell population. (D) From the $CD3^{-}$ population, $HLA\text{-}DR^{-}$ cells were selected, then the population was gated for $CD16^{+}$, $CD56^{-}$ to select the natural killer

cell population. (E) From the $CD3^-$ population, $CD19^+$ cells were gated to select the B lymphocyte population. (F) From the $CD3^+$ population, cells were gated for the $CD4^+$ and $CD8^+$, and the respective populations were selected. (G) Epigenetic modifications, H3K27Me3, H3K4Me3, and H3K9Ac, were analyzed from each cell population. The median frequency was determined using FlowJo.

To evaluate the difference in immune cell frequency between DS (n = 59) and non-DS (n=51) individuals, a PCA was performed (**Figure 5**). The frequencies of DCs, monocytes, NKs, $CD4^+$ and $CD8^+$ T cells, and B lymphocytes out of the total cell populations were determined by using Flow Jo. To determine the significance between immune cell profiles, a two-tailed T test was performed. The results indicate that there is a significant variable (p = < 0.0001, CI = 0.95) contributing to the different clusters of immune cell frequencies between DS and non-DS (**Figure 5**).

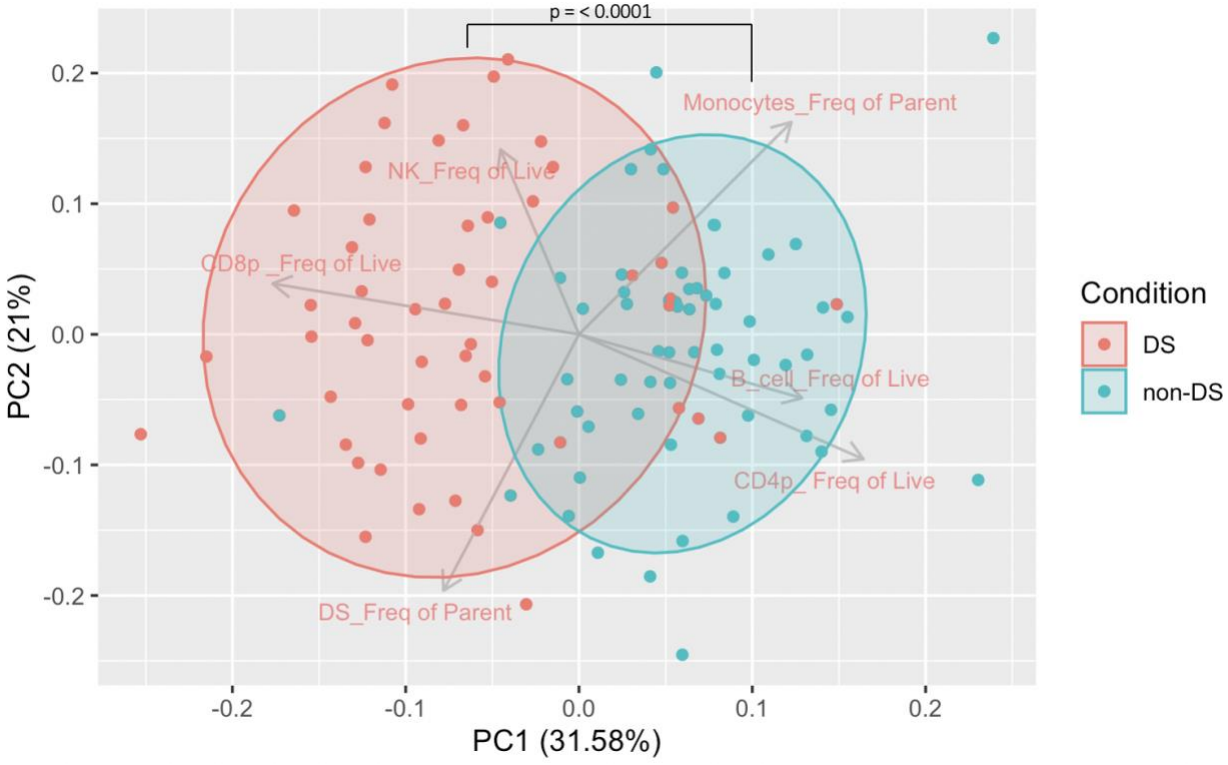

Figure 5. Immune cell frequencies are different between Down syndrome and non-Down syndrome groups. A principal component analysis (PCA) of immune cell frequencies of total live cells stratified by Down syndrome (DS, n = 51) and non-Down syndrome individuals (non-DS, n= 59) was performed. Each dot represents an individual sample of DS (red) and non-DS (blue). Six cell populations were measured, and the distribution is represented by a grey arrow. An unpaired two-tailed (p = <0.0001) with a confidence interval of 95% was performed, represented by the ellipses.

It was found that DS individuals have a different immune cell profile as compared to non-DS individuals (**Figure 6**). The percentage of immune cell populations from the total live cells were compared between individuals with DS (n= 59) and those without DS (n = 51) (**Figure 6**). DS individuals had a significantly higher frequency of natural killer cells (p=0.0104) (**Figure 6A**). The frequency of dendritic cells (DCs) in DS individuals was slightly lower, but the difference was not significant (p=0.0512) (**Figure 6B**). A lower frequency was observed for monocytes (p=0.0026), CD4+ lymphocytes (p=0.0001), and B lymphocytes (p=0.0001) for DS compared to non-DS individuals (**Figure 6C, D, F**). Conversely, DS individuals had a significantly higher frequency of CD8+ lymphocytes compared to non-DS individuals (p=0.0001) (**Figure 6E**).

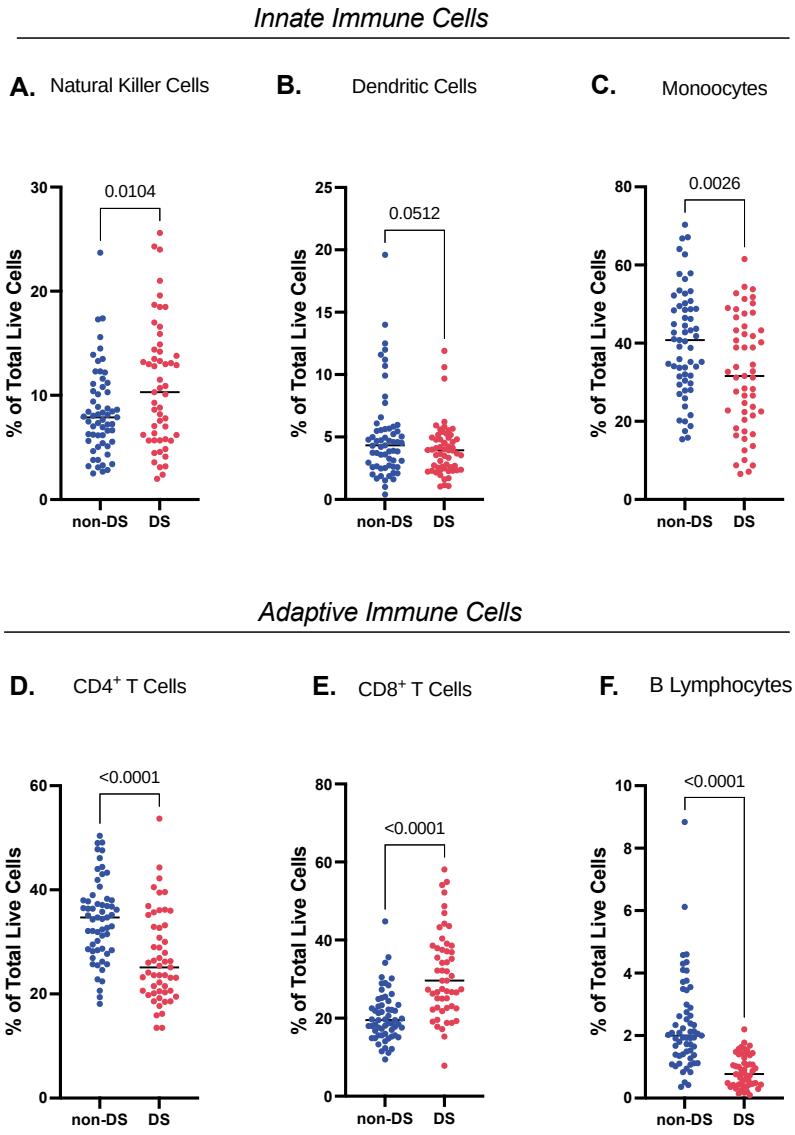

Figure 6. Down syndrome individuals have a higher frequency of natural killer cells and CD8+ lymphocytes and lower monocytes, CD4+ lymphocytes, and B lymphocytes compared to non-Down syndrome. Innate and adaptive immune cell percentages of the total cell live population were measured for each sample (n= 119) and stratified by DS (red) and non-DS (blue). An unpaired parametric two-tailed T test was used to determine significance (a <0.05). (A) A higher frequency of NK cells was found in DS compared to non-DS individuals (p= 0.0104). (B) DS individuals had a slightly lower frequency of DCs, but the difference was not significant (p = 0.0512). (C) A lower frequency of monocytes was found in DS compared to non-DS individuals (p= 0.0026). (D) A lower frequency of $CD4^+$ lymphocytes were found in DS compared to non-DS individuals (p= 0.0001). (E) A higher frequency of $CD8^+$ lymphocytes were found in DS compared to non-DS individuals (p=0.0001). Lower frequency of B lymphocytes was found in DS compared to non-DS individuals (p= 0.0001).

Epigenetic Modifications

Flow cytometry was used to identify the total gross epigenetic modifications of each immune cell type. After isolating the immune cell populations as described in Figure 4, the epigenetic modifications, H3K27Me3, H3K4Me3, and H3K9Ac, were analyzed in each of the following cell populations: monocytes, dendritic cells, natural killers, $CD4^+$ and $CD8^+$ T lymphocytes, and B lymphocytes. The per cell level expression as measured as the median of fluorescence intensity (MFI) was selected for each histone modification using FlowJo (**Figure 4G**).

Significant differences in total epigenetic modifications in immune cells were observed in four immune cell types in the DS group compared to the non-DS group. H3K4Me3 was found to be significantly higher in NK cells (p=0.0088), and monocytes (p=0.0015) compared to healthy controls, although not significant in dendritic cells (p=0.0517) (**Figure 7A, B**). The level of H3K9Ac was found to be significantly higher in DS individuals in NK cells (p=0.0069), monocytes (p = 0.0024), $CD8^+$ T cells (p = 0.0165), and B lymphocytes (p=0.0018) (**Figure 7C-F**). Moreover, the level of H3K27Me3 was found to be significantly higher in DS individuals in NK cells (p=0.0390) and monocytes (p = 0.0015) (**Figure 7G-H)**. These findings suggest that Down Syndrome (DS) individuals have an altered epigenetic profile in various immune cell types compared to healthy controls.

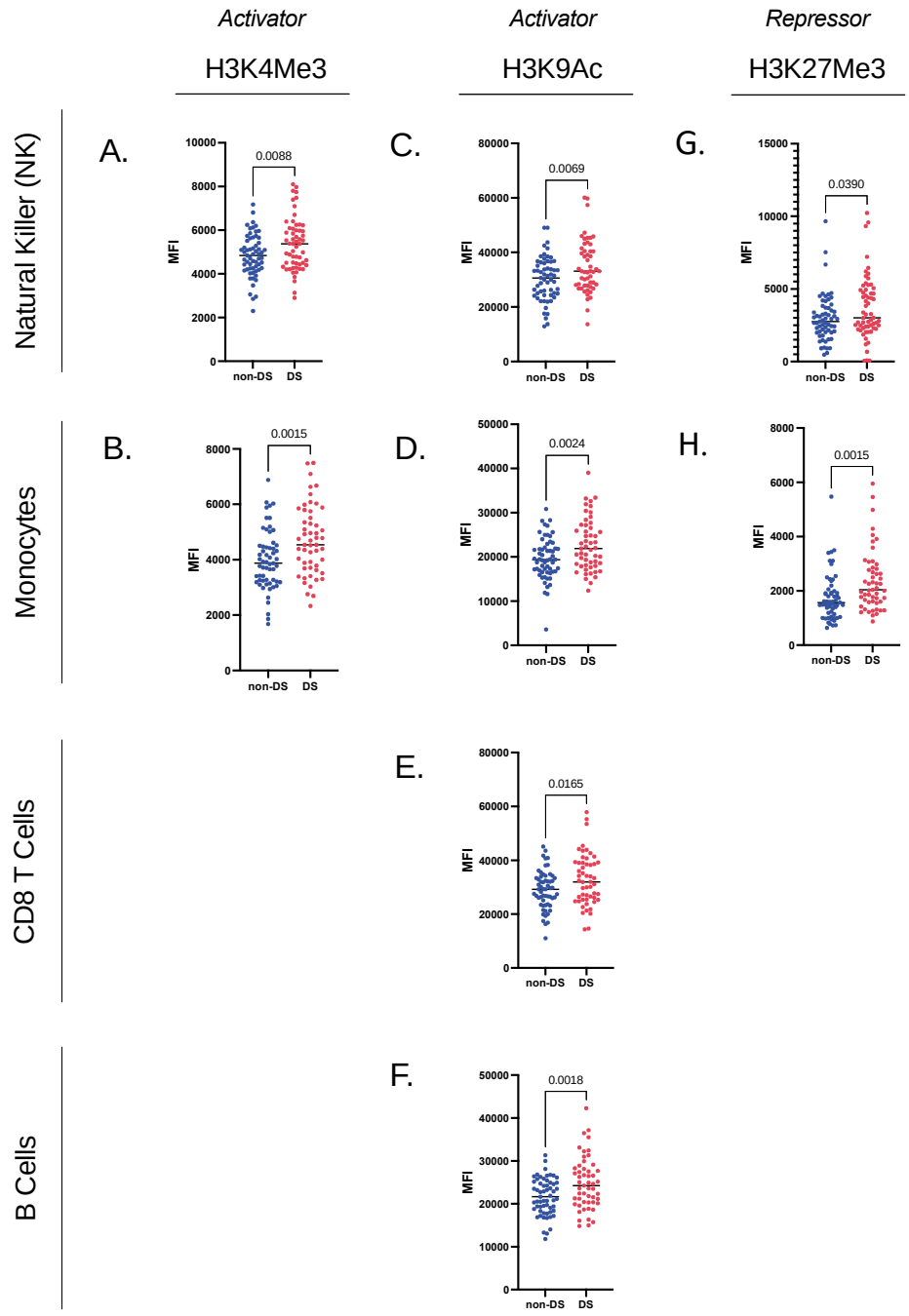

Figure 7. **Down syndrome individuals have a higher per cell level expression (MFI) of H3K4 and H3K27 methylation and H3K9 acetylation in four cell types compared to health controls.** H3K4Me3, H3K9Ac (transcription activators), and H3K27Me3 (transcription repressor) were measured for each sample (n= 119) and stratified by DS (red) and non-DS (blue). An unpaired parametric two-tailed T test was used to determine significance (a <0.05). (A-B) The level of H3K4Me3 was found to be significantly higher DS individuals in NK cells (p=0.0088) and monocytes (p = 0.0151). (C-F) The level of H3K9Ac was found to be significantly higher DS individuals in NK cells (p=0.0069), monocytes (p = 0.0024), CD8 T cells (p = 0.0165), and B lymphocytes (p=0.0018). (G-H) The level of H3K27Me3 was found to be significantly higher DS individuals in NK cells (p=0.0390) and monocytes (p = 0.0015). MFI: median fluorescence intensity.

Cytokine/Chemokine Association with Epigenetic Modifications

To determine if there was a correlation between the cytokine/chemokine levels and histone epigenetic modifications expression, a Spearman's correlation matrix was used (**Figure 8, Supplemental Figure 4**). The correlogram includes histones epigenetic MFIs and cytokines/chemokines that were significantly different ($p < 0.05$) between the DS and non-DS groups (**Supplemental Figure 5**). There was a strong correlation between H3K4Me3, H3K27Me3, and H3K9Ac modifications of each cell type (NK, monocyte, CD8 T cell, and B lymphocyte) to one another. IL-10 was found to have significant positive correlation to NK H3K27Me3, monocyte H3K27Me3, and monocyte H3K9Ac modifications. FLT3L was also significantly positively correlated to H3K4Me3, H3K27Me3, and H3K9Ac for all cell populations other than NK H3K27Me3. MCP-2 and Eotaxin were significantly positively correlated to monocyte H3K27Me3 and H3K4Me3 modifications. On the other hand, TGF-β had a significant negative correlation to monocyte H3K4Me3, H3K27Me3, and H3K9Ac, as well as B cell H3K9Ac.

Cytokines were also found to be correlated to one other. TFG-β1, TGF-β2, and Fractalkine, were significantly negative correlated to Eotaxin, IP-10, MCP-1, IL-10, FLT3L, I-TAC, IL-15, MIP-3α, and SDF-1α. Of note, TGF-β3 was not correlated to any histone modifications or cytokines, except for a negative correlation with fractalkine.

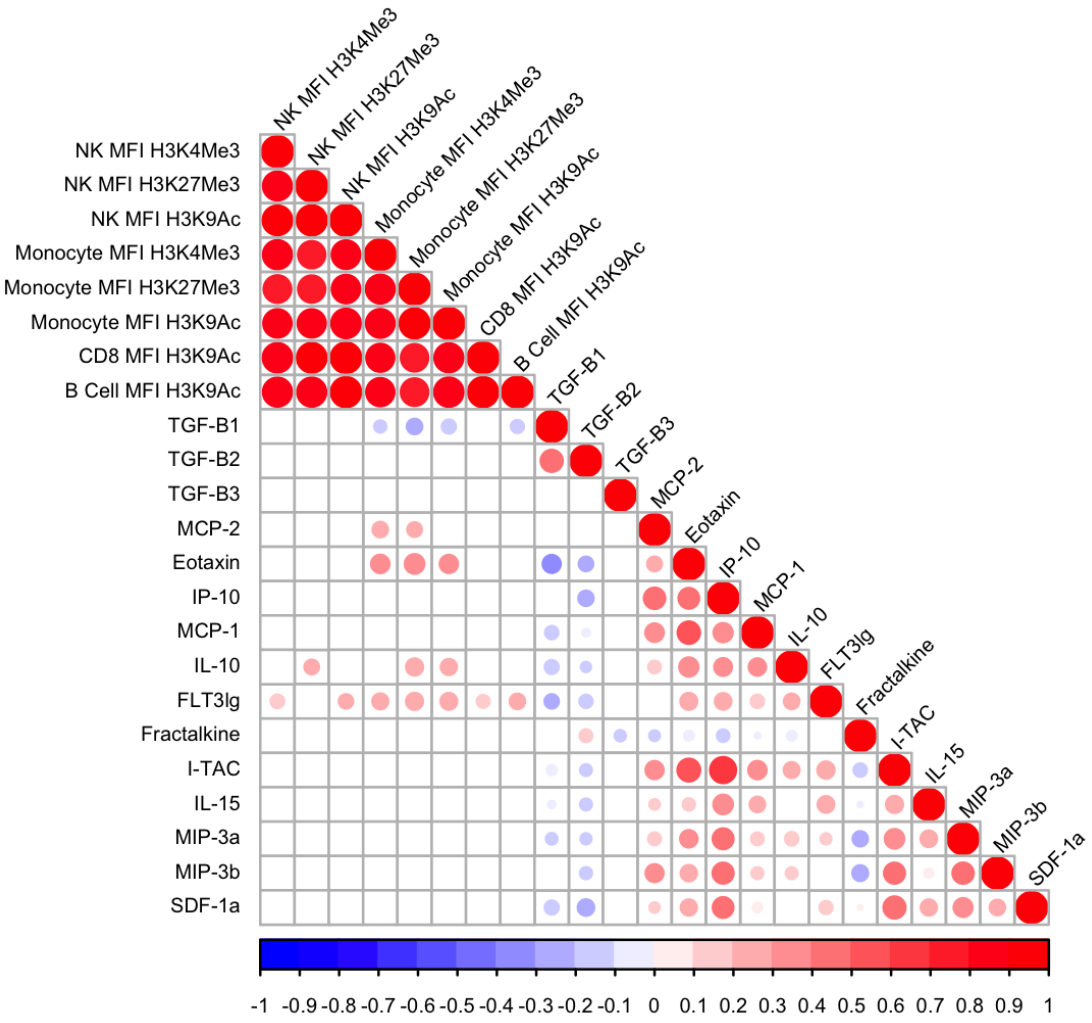

Figure 8. IL-10 and FLTLG have a significant positive correlation to histone modifications.
Spearman's correlation correlogram of significant ($p < 0.05$) histone levels (MFI) and cytokines/chemokines. The strength of the correlation between two variables is represented by the color and size of the circle at the intersection between two variables. Bright red indicates a strong positive correlation which ranges to bright blue indicating a strong negative correlation. Only correlations of significance are shown.

Feature Selection

To identify the minimal number of features that could discriminate DS from non-DS samples we performed a feature selection analysis. From all the analyzed data including cytokines, chemokines, cell frequencies, and epigenetic modifications, 9 features were identified as the small number of features to best discriminate both groups. TGF-β1, B lymphocyte frequency of total live cells, and $CD4^+$ T cell frequency of total live cells were significantly elevated and were the best discriminators of non-DS samples (red annotation on the top of the heatmap, **Figure 9).** On the other hand, $CD8^+$ T cell frequency of total live cells, Eotaxin, I-TAC, IP-10, IL-10, and FLT3L were significantly elevated and the best features to discriminate DS samples (blue annotation on the top pf the heatmap, **Figure 9**).

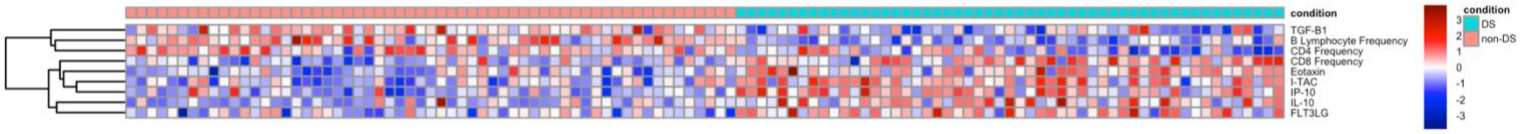

Figure 9. Feature selection shows the minimal number of evaluated features that discriminate DS and non-DS individuals. Heatmap of feature selection analysis for significant cytokines, histone modifications, and immune cell frequencies in Down syndrome (DS, light blue) and non-Down syndrome individuals (non-DS, pink) annotation on the top of the heatmap. TGF-B1, B lymphocyte frequency of total live cells, $CD4^+$ frequency of total live cells, $CD8^+$ frequency of total live cells, Eotaxin, I-TAC, IP-10, IL-10, FTL3L are the features that best discriminate both groups. Red represents significantly increased and blue represents significantly decreased in each group respectively.

Discussion

The current study, using a cohort of 110 individuals with or without DS, from Sao Paulo/Brazil, has brought new knowledge regarding epigenetic modifications in innate and adaptive subsets isolated from DS individuals. These findings were put in the context of the circulating cytokine/chemokine milieu and will be discussed below.

Cytokine Profile

We investigated differences in cytokine profiles between individuals with and without Down syndrome (DS) by conducting a PCA which showed distinct clusters of cytokines between the groups. Further analysis showed the DS group had significantly lower levels of anti-inflammatory cytokines TGF-β1 and TGF-β2 and higher levels of TGF-β3 and IL-10.

TGF-β is known to play a significant role in downregulating the immune response through a negative feedback loop mechanism to decrease the inflammatory response. In this mechanism, TGF-β is initially secreted by various immune cells, including regulatory T cells and macrophages, which then signals suppression of pro-inflammatory cytokines and promotion of anti-inflammatory cytokines [68]. This causes a decrease in the immune response and inflammation, which in turn decreases the production of TGF-β, due to a decrease in the stimulus that initially triggered its secretion. The difference in TGF- β1 and TGF- β2 levels in DS and non-DS individuals can be explained by this feedback loop. It is known that DS individuals have a less robust immune response to a stimulus, which causes a decreased signal of TGF-β1 and TGF- β2 to be released by immune cells, as per our results [20]. In contrast, TGF- β3 levels, which regulates wound healing and cell proliferation, were found to be higher in the DS group [69-71]. Increased production of TGF- β3 is most likely a result of the immune system trying to counteract chronic inflammation.

Chemokines

MCP-1 was found to be upregulated in plasma from DS individuals, which is supported by a previous cytokine analysis of a DS cohort (DS n = 20, non-DS n = 20), however they did not analyze MCP-2 [55]. Increased MCP-1 levels have also been found to be significantly elevated in diseases such as diabetes mellitus type 1 [23], breast cancer [24], and chronic inflammatory neurodegenerative disorders [25]. MIP-3β levels were significantly higher in our DS group and similar results have been found in children less than 3 years old in São Paulo, Brazil [72]. Although we found increased levels of IP-10, there is very limited research on the circulating levels in plasma of DS individuals for this chemokine. Overexpression of IP-10 has been observed and correlated to dysregulation of the kynurenine pathway in DS individuals, which is hypothesized to contribute to increased immune inflammation [73]. Increased IP-10 has already been better correlated with other autoimmune diseases such as thyroid disease, and IP-10, MCP-1, MCP-2, MIP-1α upregulation have all been associated with severity of disease of COVID-10 [31, 32].

Our results also indicated that Eotaxin, I-TAC, and SDF-1α were upregulated in DS individuals compared to non-DS individuals. Limited research on the relationship between Eotaxin and DS exist, but there is a strong correlation between increased Eotaxin levels in rheumatoid arthritis patients [26, 27] and neuroinflammatory diseases such as multiple sclerosis and Alzheimer's disease [28, 29]. Similarly, the role of I-TAC and FLT3 ligand in disease modulation has not been well characterized with respect to DS or other diseases. I-TAC has only been linked to experimental autoimmune encephalomyelitis in a rat model [74]. FLT3 upregulation is known to occur in individuals with GATA2 mutations and acts as an important biomarker for acute myeloid leukemia [38, 39]. Additionally, in a mice model FLT3 ligand levels were found to affect the survival and disease progression due to FLT3 constitutive activation [75]. SDF-1α levels have

been reported to be higher in individuals of Ig4 related diseases, rheumatoid arthritis, osteoarthritis as well as a key factor in cancer tumor growth, but has not been correlated to DS [35, 36].

Immune Cell Populations

Immune cell frequency and epigenetic analysis revealed altered epigenetic profiles in various immune cell types in PBMCs from DS individuals. Significantly higher frequency of natural killer cells and $CD8^+$ lymphocytes were found in DS samples, but a lower frequency of monocytes, $CD4^+$ lymphocytes, and B lymphocytes. Lower frequencies of monocytes, $CD4^+$ lymphocytes, and B lymphocytes have been reported in both DS children and adults and corroborate our findings [47, 49].

Although DS individuals have a less robust immune response compared to healthy individuals, their frequency of $CD8^+$ T cells are consistently higher. Our result of significantly higher frequency of $CD8^+$ T cells in adults ranging from 12 to 56 years of age, is consistent with previous studies on adult DS cohorts [54, 55]. It is hypothesized that upregulation of $CD8^+$ T cells is directly impacted from the increased cytokine signaling. $CD8^+$ T cells have also been reported to be upregulated in viral infections including SARS-CoV-2, and autoimmune diseases such as thyroid disease, multiple sclerosis, and type 1 diabetes [76, 77].

Our results also indicated that DS individuals present a higher frequency of NK cells compared to non-DS individuals. These results are in line with what was previously observed in the literature showing the dysregulation of the innate immune system [46]. Bloemers et al. [43] observed increase in NK population in DS children at the ages 1 to 9 years, and Maccario [43, 44] also observed the same phenomenon in children and adults DS individuals. Also, numbers and activity of NK were evaluated in children and adults with DS and showed significant NK percentage in DS in comparison with healthy individuals [45].

Epigenetic Modifications

Our data has shown that DS individuals also presented significantly higher levels of histone modifications in four immune cell types: NK, monocytes, $CD8^+$ T cells, and B lymphocytes. H3K4Me3 and H3K9Ac, which both activate transcription, were higher in all four cell types. H3K27Me3, which causes transcription repression, was found to be significantly higher in NK and monocytes.

Histone modifications of $CD8^+$ T cells have been reported to affect other autoimmune disorders such as, multiple sclerosis, type 1 diabetes, and aplastic anemia [76]. We hypothesize that the histone modifications change gene expression, which affects immune cell function, including cytokine secretion. In DS specifically, there is limited research on the correlation between histone modification and DS. However, the presence of trisomy 21 has been associated with significant DNA methylation of non-21 chromosomes [78]. Further, DNA methylation in DS individuals has been linked to the epigenetic clock, and has a positive correlation to accelerated ageing [79].

Cytokine/chemokines Influences of Epigenetic Modifications

The correlation analysis between cytokine and histones showed that IL-10 and FLT3L had a significant positive correlation to histone modifications of NKs, monocytes, CD8 cells, and B cells, while TGF- β1 had a significant negative correlation to four histone modifications. TGF-β1, TGF-β, and Fractalkine were negative correlated to approximately all of the other cytokines/chemokines. The feature selection analysis identified the minimum number of analyzed features that discriminate DS from non-DS individuals, including cytokine/chemokine levels and histone modifications. $CD8^+$ T cell frequency, Eotaxin, I-TAC, IP-10, IL-10, and FLT3L were the

minimal features that defined DS individuals being all elevated; while TGF- β1, B lymphocyte frequency and $CD4^+$ T cell frequency were the best definers of non-DS individuals.

TGF-B

TGF-B1 has been shown to regulate gene expression through epigenetic mechanisms, including histone modifications. It has been suggested that TGF-B1 induces the expression of the histone methyltransferases (HMTs), which catalyzes the methylation of histone H3 at lysine 27 (H3K27), resulting in the formation of a repressive chromatin structure that silences gene expression [80]. Our results indicate that TGF-B1 may interreact with histone acetyltransferases (HATs), as a correlation between H3K9Ac and TGF-B1 was also observed [81]. Further, TGF-B1 was negatively correlated with Eotaxin, IP-10, FTL3L, I-TAC, MIP-3β, and SDF-1α which is supported by the negative feedback loop.

IL-10

IL-10 is a potent anti-inflammatory cytokine that has been shown to regulate gene expression in immune cells by modulating histone modifications [66]. IL-10 activates several downstream signaling pathways, including STAT3, which has been linked to the regulation of histone modifications. STAT3 can activate various HATs and recruit them to target genes, resulting in an increase in histone acetylation and gene expression [82]. Conversely, STAT3 can also recruit histone deacetylases (HDACs) to target genes, leading to a decrease in histone acetylation and gene repression [82]. Further, IL-10 has also been shown to activate the transcription factor CREB, which can regulate gene expression by modulating histone modifications by the recruitment of HATs or HDACs to target genes [83]. Therefore, IL-10 may regulate gene expression by altering the balance of histone acetylation and deacetylation at target gene loci through the activation of STAT3 and CREB signaling pathways. This may result in the

regulation of various immune cell functions, including the differentiation and activation of T cells, B cells, and dendritic cells, and the production of pro-inflammatory cytokines.

FLT3L

The cytokine profile analysis showed that the levels of FLT3L were higher in DS individuals compared to non-DS individuals and that there was a significant correlation between FLT3L levels and histone modifications. FLT3L had a significant positive correlation to NK H3K27Me3, Monocyte H3K27Me3, H3K9Ac, and H3K4Me3. FLT3L is a known cytokine that plays a key role in the development of immune cells, particularly dendritic cells [37]. One possible mechanism by which FLT3L could affect histone modifications is through the activation of downstream signaling pathways, such as the Ras-MAPK pathway. The Ras-MAPK pathway has been implicated in the regulation of histone modifications, including H3K9Ac and methylation [84, 85]. It has been proposed that FLT3L signaling through these pathways regulate histone modifications and has been shown to lead abnormal functions of trophoblast cells which cause abortions [85]. Thus, the high levels of FLT3L found in DS individuals, may influence histone modifications, either by directly affecting the activity HATs or HMTs, or by modulating the expression or activity of other factors involved in histone modification, such as histone deacetylases or demethylases.

Limitations and Future Directions

One potential limitation of this study is the use of a specific cohort composed of Down syndrome (n= 51) and non-DS (n= 59) healthy individuals from São Paulo, Brazil. The cohort included individuals between the ages of 12 and 56, and only classified them as under or over 30 years of age. While this cohort allowed for the identification of differences in cytokine levels, immune cell frequencies, and histone modifications, the findings may not be generalizable to

individuals of all ages or those with Down syndrome living in other regions and/or countries. The socioeconomic status of the cohort may have influenced the results. However, despite this limitation, most of our findings corroborate findings from other DS/non-DS cohorts world-wide.

Further research is needed to determine the mechanisms underlying the correlation between cytokine profiles and epigenetic modifications in DS populations, as observed in our study. Future studies should also investigate the functional consequences of the altered epigenetic modifications in immune cells of DS individuals. Understanding the immune system of individuals with DS may lead to the development of targeted therapies and interventions to improve their health outcomes, such as anti-inflammatory treatments. For example, it is known that IFN stimulation leads to the activation of the Jak/STAT pathway, which in turn causes upregulation of inflammatory cytokines [86]. Jak inhibitors may be a therapy of interest to decrease inflammation in DS individuals, however more research is needed [87].

Conclusion

Our study evaluated the phenotypic and epigenetic differences between Down syndrome and non-Down syndrome individuals. Ultimately, circulating cytokine levels were correlated to histone modifications of four immune cell types. These findings suggest that DS has distinct immunological characteristics, which are modulated through cytokine expression and epigenetics. While the study is limited in its generalizability due to the cohort demographics, it provides a foundation for future studies analyzing the role of epigenetics in immune dysregulation in DS and other autoimmune diseases, as well as possible targeted interventions to ameliorate the quality of life of these individuals.

Supplemental Information

Abbreviation	Name	Function
Anti-Inflammatory Cytokines		
IL-10	Interleukin-10	Suppression of T cells and macrophages
TGF-β1	Transforming growth factor-beta 1	Regulation of innate and adaptive cell proliferation, differentiation, and migration
TGF-β2	Transforming growth factor-beta 2	Regulation of innate and adaptive cell proliferation, differentiation, and migration
TGF-β3	Transforming growth factor-beta 3	Wound healing, cell differentiation
Pro-Inflammatory Cytokines		
IL-1β	Interleukin-1 beta	Lymphocyte activation
IL-4	Interleukin-4	B cell proliferation and activation, helper T cell differentiation
IL-5	Interleukin-5	Eosinophils regulation
IL-6	Interleukin-6	Production of acute phase response
IL-7	Interleukin-7	B and T cell proliferation and maturation
IL-8	Interleukin-8	Neutrophil activation
IL-9	Interleukin-9	T cell and mast cell growth
IL-12	Interleukin-12	Helper T cell activation
IL-13	Interleukin-13	B cell and monocyte regulation
IL-15	Interleukin-15	T cell activation
IL-17α	Interleukin-17 alpha	Cytotoxic T cell response
IL-18	Interleukin-18	Helper T cell induction
IL-21	Interleukin-21	Helper T cell and T cell induction
IL-22	Interleukin-22	Wound healing and skin infections
IL-27	Interleukin-27	Naïve T cell differentiation
TNF-α	Tumor necrosis factor- alpha	Macrophage regulation, antiviral response
TNF-β	Tumor necrosis factor- beta	Cytotoxic cell regulation, antiviral response
IFN-α2α	Interferon alpha 2 alpha	Cytotoxic cell regulation, antiviral response
IFN-β	Interferon beta	Macrophage recruitment
IFN-γ	Interferon gamma	Innate cells and T cell regulation

Supplemental Table 1. Cytokine details and functions. Cytokine abbreviation, full name, classification, and function are described [88].

Abbreviation	Full Name	Classification	Function
MCP-1	Monocyte chemoattractant protein-1	Chemokine	Migration of monocytes and macrophages
MCP-2	Monocyte chemoattractant protein-2	Chemokine	Migration of effector cells
MCP-3	Monocyte chemoattractant protein-3	Chemokine	Regulate macrophage and lymphocyte function
MIP-1α	Macrophage inflammatory protein-1 alpha	Pro-inflammatory Chemokine	Recruitment of lymphocytes and dendritic cells, wound healing
MIP-3α	Macrophage inflammatory protein-3 alpha	Pro-inflammatory Chemokine	Recruitment of granulocytes
MIP-3β	Macrophage inflammatory protein-1 beta	Pro-inflammatory Chemokine	Recruitment of lymphocytes, wound healing
IP-10	Interferon-gamma-induced protein 10	Chemokine	Activation of dendritic cells, natural killer cells, and T cells
I-TAC	Interferon-inducible T cell alpha chemoattractant	Chemokine	Recruitment of lymphocytes
FTL3L	FMS-like tyrosine kinase 3 ligand	Chemokine, Growth Factor	Development of dendritic cells
Eotaxin		Chemokine	Recruitment of eosinophils
SDF-1α	Stromal cell-derived factor 1 alpha	Chemokine	Activation of lymphocytes, neutrophils, and hematopoietic stem cells, wound healing
Fractalkine		Chemokine	Recruitment of macrophages and lymphocytes

Supplemental Table 2. Classification of Chemokines. Chemokine abbreviation, full name, classification, and function are described.

Surface Antibodies				
Marker	**Fluorochrome**	**Clone**	**Catalog Number**	**Company**
CD3	BUV805	UCHT1	612895	BD Horizons
CD4	BV480	L200	566159	BD Horizons
CD8	BUV 496	RPA-T8	612942	BD Horizons
CD19	BV570- P	HIB19	624298	BD Horizons Custom
HLA-ADR	BV786	G46-6	564041	BD Horizons
CD14	PE	M5E2	561707	BD Pharmingen
CD16	BUV661	3G8	624285	BD Horizons Custom
CD11c	BUV615	3.9	624297	BD Horizons Custom
CD123	PE-CF594	7G3	562391	BD Horizons
CD56	BUV395	B159	740299	BD OptiBuild
Intracellular Antibodies				
Marker	**Fluorochrome**		**Catalog Number**	**Company**
H3K27Me3	BB515		5499S	Cell Signaling Technology
H3K9Ac	BV421		11857S	Cell Signaling Technology
H3K4Me3	APC Alexa Fluor® 647		12064S	Cell Signaling Technology

Supplemental Table 3. Surface and Intercellular antibodies specifications. Samples were stained with surface antibodies, followed by the intracellular antibodies. The cellular marker, fluorochrome, clone, catalog number, and company are shown for each antibody.

	NK MFI H3K4Me3	NK MFI H3K27Me3	NK MFI H3K9Ac	Monocyte MFI H3K4Me3	Monocyte MFI H3K27Me3	Monocyte MFI H3K9Ac	CD8 MFI H3K9Ac	B Cell MFI H3K9Ac	TGF-B1	TGF-B2	TGF-B3	MCP-2	Eotaxin	IP-10	MCP-1	IL-10	FLT3lg	Fractalkine	I-TAC	IL-15	MIP-3a	MIP-3b	SDF-1a
NK MFI H3K4Me3	1																						
NK MFI H3K27Me3	0.861	1																					
NK MFI H3K9Ac	0.907	0.940	1																				
Monocyte MFI H3K4Me3	0.869	0.770	0.841	1																			
Monocyte MFI H3K27Me3	0.786	0.765	0.833	0.859	1																		
Monocyte MFI H3K9Ac	0.825	0.820	0.893	0.851	0.927	1																	
CD8 MFI H3K9Ac	0.890	0.911	0.959	0.804	0.776	0.864	1																
B Cell MFI H3K9Ac	0.867	0.872	0.938	0.817	0.782	0.885	0.940	1															
TGF-B1	-0.112	-0.112	-0.117	-0.149	-0.243	-0.200	-0.135	-0.174	1														
TGF-B2	-0.038	-0.071	-0.021	-0.133	-0.174	-0.056	-0.025	-0.041	0.498	1													
TGF-B3	0.089	0.126	0.124	0.074	0.115	0.101	0.074	0.082	0.156	0.120	1												
MCP-2	0.193	0.192	0.197	0.243	0.217	0.202	0.157	0.191	0.042	0.045	0.081	1											
Eotaxin	0.287	0.280	0.276	0.343	0.392	0.337	0.230	0.341	-0.330	-0.215	0.114	0.222	1										
IP-10	0.152	0.102	0.132	0.249	0.275	0.190	0.092	0.129	-0.212	-0.238	0.115	0.464	0.437	1									
MCP-1	0.024	0.026	0.005	0.047	0.118	0.045	-0.047	0.050	-0.179	-0.058	0.018	0.348	0.511	0.375	1								
IL-10	0.126	0.217	0.204	0.209	0.297	0.262	0.153	0.202	-0.197	-0.104	0.170	0.161	0.374	0.321	0.303	1							
FLT3lg	0.189	0.162	0.207	0.257	0.295	0.286	0.168	0.242	-0.207	-0.177	0.158	0.084	0.291	0.278	0.183	0.245	1						
Fractalkine	0.049	-0.024	-0.002	-0.012	-0.103	-0.027	0.024	0.042	0.093	0.165	-0.129	-0.115	-0.092	-0.157	-0.029	-0.091	-0.057	1					
I-TAC	0.258	0.191	0.195	0.331	0.320	0.247	0.190	0.209	-0.099	-0.140	0.131	0.334	0.531	0.695	0.342	0.256	0.300	-0.181	1				
IL-15	0.029	0.002	0.049	0.138	0.073	0.043	0.032	0.082	-0.051	-0.144	0.054	0.112	0.154	0.390	0.244	0.028	0.280	-0.025	0.298	1			
MIP-3a	0.013	0.015	0.013	0.110	0.177	0.139	-0.014	0.044	-0.135	-0.124	0.102	0.165	0.308	0.444	0.162	0.183	0.122	-0.236	0.387	0.278	1		
MIP-3b	0.127	0.158	0.121	0.189	0.243	0.185	0.093	0.093	-0.082	-0.147	0.176	0.328	0.271	0.451	0.145	0.143	0.163	-0.254	0.448	0.082	0.458	1	
SDF-1a	0.162	0.079	0.106	0.214	0.202	0.162	0.147	0.159	-0.199	-0.274	-0.010	0.107	0.266	0.436	0.088	-0.033	0.172	0.016	0.499	0.264	0.364	0.245	1

Supplemental Table 4. Correlation values between cytokines and histone modifications. Spearman's correlation test was performed to determine a correlation value between two variables. Positive values indicate a positive correlation, negative values indicate a negative correlation, and zero indicates no correlation.

	NK MFI H3K4Me3	NK MFI H3K27Me3	NK MFI H3K9Ac	Monocyte MFI H3K4Me3	Monocyte MFI H3K27Me3	Monocyte MFI H3K9Ac	CD8 MFI H3K9Ac	B Cell MFI H3K9Ac	TGF-B1	TGF-B2	TGF-B3	MCP-2	Eotaxin	IP-10	MCP-1	IL-10	FLT3lg	Fractalkine	I-TAC	IL-15	MIP-3a	MIP-3b	SDF-1a
NK MFI H3K4Me3	0																						
NK MFI H3K27Me3	3.15E-06	0																					
NK MFI H3K9Ac	2.93E-06	1.88E-06	0																				
Monocyte MFI H3K4Me3	2.93E-06	2.76E-06	3.25E-06	0																			
Monocyte MFI H3K27Me3	3.25E-06	3.22E-06	3.26E-06	2.34E-06	0																		
Monocyte MFI H3K9Ac	3.28E-06	3.26E-06	3.07E-06	2.95E-06	1.97E-06	0																	
CD8 MFI H3K9Ac	2.28E-06	2.42E-06	2.05E-06	3.31E-06	3.18E-06	3.22E-06	0																
B Cell MFI H3K9Ac	2.81E-06	2.45E-06	2.02E-06	3.31E-06	3.31E-06	2.74E-06	1.85E-06	0															
TGF-B1	0.091	0.089	0.056	0.012	0.005	0.005	0.091	0.028	0														
TGF-B2	0.601	0.795	0.676	0.194	0.219	0.345	0.656	0.510	0.00018	0													
TGF-B3	0.589	0.816	0.862	0.643	0.998	0.980	0.876	0.837	0.589	0.984	0												
MCP-2	0.084	0.084	0.152	0.035	0.026	0.063	0.201	0.145	0.075	0.072	0.844	0											
Eotaxin	0.124	0.084	0.105	0.019	0.006	0.018	0.159	0.064	0.000	0.002	0.887	0.00060	0										
IP-10	0.778	0.646	0.620	0.802	0.844	0.977	0.601	0.709	0.056	0.000	0.998	0.00054	0.0076	0									
MCP-1	0.617	0.747	0.696	0.948	0.887	0.998	0.539	0.795	0.012	0.001	0.830	0.001	0.00041	2.84E-06	0								
IL-10	0.206	0.049	0.057	0.053	0.010	0.015	0.163	0.062	0.000	0.018	0.302	0.004	2.98E-06	0.0389	0.0012	0							
FLT3lg	0.043	0.080	0.037	0.003	0.003	0.005	0.042	0.016	0.00017	0.00044	0.781	0.067	0.00011	0.0384	0.011	0.00043	0						
Fractalkine	0.564	0.926	0.827	0.977	0.767	0.855	0.604	0.696	0.206	0.011	0.046	0.005	0.024	1.45E-05	0.000	0.0089	0.125	0					
I-TAC	0.816	0.890	0.841	0.402	0.464	0.716	0.894	0.969	0.011	0.000	0.799	0.000	0.00069	3.03E-06	1.88E-06	0.0279	0.016	0.00043	0				
IL-15	0.809	0.475	0.653	0.743	0.987	0.883	0.640	0.834	0.049	0.000	0.467	0.035	0.025	2.79E-06	4.04E-05	0.1219	0.007	0.0031	3.04E-06	0			
MIP-3a	0.453	0.510	0.464	0.995	0.905	0.908	0.442	0.570	0.033	0.000	0.663	0.011	0.009	3.23E-06	6.35E-06	0.040	0.047	2.14E-06	2.98E-06	3.18E-05	0		
MIP-3b	0.706	0.719	0.851	0.355	0.309	0.439	0.883	0.830	0.055	0.001	0.324	0.001	0.012	2.67E-06	0.0021	0.036	0.168	1.89E-06	1.72E-06	0.00282979	1.74E-06	0	
SDF-1a	0.350	0.873	0.719	0.114	0.225	0.357	0.510	0.475	0.014	0.000	0.345	0.038	0.019	0.000	0.009	0.283	0.005	0.021	1.71E-06	3.18E-06	2.81E-05	0.00035	0

Supplemental Table 5. P-values of correlation between cytokines and histone modifications. Spearman's correlation test was performed to determine the significance ($\alpha = 0.05$) of correlation between two variables. P-values for each are combination are shown.

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