Title: Distinct CD3⁺CD14⁺ T Cell-Monocytes are dynamic complexes that harbor HIV and are increased with glucose intolerance

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CD3+CD14+ complexes higher in HIV+ with diabetes

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Summary

Persistent systemic inflammation in persons with HIV (PWH) is accompanied by an increased risk of metabolic disease. Yet, changes in the innate and adaptive immune system in PWH who develop metabolic disease remain poorly defined. Using unbiased approaches, we show that PWH with prediabetes/diabetes have a significantly higher proportion of circulating CD14+ monocytes complexed to T cells. The complexed CD3+ T cells and CD14+ monocytes demonstrate functional immune synapses, increased expression of proinflammatory cytokines, and greater glucose utilization. Furthermore, these complexes harbor more latent HIV DNA compared to CD14+ monocytes or CD4+ T cells. Our results demonstrate that circulating CD3+CD14+ T cell-monocyte pairs represent functional dynamic cellular interactions that likely contribute to inflammation and, in light of their increased proportion, may have a role in metabolic disease pathogenesis. These findings provide an incentive for future studies to investigate T cell-monocyte immune complexes as mechanistic in HIV cure and diseases of aging.
Graphical Abstract

CD3+ CD14+ T cell Monocyte complexes

0 minutes

Transient complexes

Inflammation

240 minutes

Stable complexes Increased with Diabetes

IL-10 CD4+ T Reg

0 minutes

240 minutes

Highlights

- Persons with HIV and diabetes have increased circulating CD3+ CD14+ T cell-monocyte complexes.
- CD3+ CD14+ T cell-monocytes are a heterogenous group of functional and dynamic complexes.
- We can detect HIV in T cell-monocyte complexes.
- The proportion of CD3+ CD14+ T cell-monocyte complexes is positively associated with blood glucose levels and negatively with plasma IL-10 and CD4+ T regulatory cells.
Introduction

Persons with HIV (PWH) on antiretroviral therapy often have heightened, chronic inflammation that may contribute to age-related diseases such as diabetes and atherosclerosis.\textsuperscript{1-8} This increased risk of age-related diseases offers an opportunity to investigate how inflammation contributes to the development of cardiometabolic disorders.\textsuperscript{9} We used unbiased approaches to comprehensively characterize alterations in immune cell populations that distinguish PWH with prediabetes or diabetes from those with HIV alone.

Common flow cytometry analysis workflows may overlook interacting cell populations. Typically, presumed single cells suspensions in droplets of buffered fluid are illuminated by the laser beam to capture cell marker expression. During analysis, steps are taken to eliminate cell aggregates which have commonly been presumed to arise ex-vivo due to cell clumping via adhesion molecules and other factors introduced during sample processing, particles on dying cells, or lack of separation of cells due to high-stream flow rates.\textsuperscript{10,11,12} Recent studies, however, have demonstrated the existence of immunologically relevant cell-cell complexes in the blood of individuals with immune perturbation due to infection, vaccination, and cancer.\textsuperscript{13,14,15} Burel et al. found that the presence of CD3\textsuperscript{+} T cell - CD14\textsuperscript{+} monocyte complexes in freshly isolated blood was strongly correlated with their presence in cryopreserved peripheral blood mononuclear cells (PBMCs), suggesting that their occurrence in cryopreserved PBMCs is not an artifact of processing or storage.\textsuperscript{13} The reorganization of adhesion molecules at the immunological synapse between the monocyte and the T cells indicated an activating interaction between these cells.\textsuperscript{13} Similar T-cell complexes have been observed with antigen-presenting B cells.\textsuperscript{16,17}

In the context of HIV, the ineffective killing of HIV-infected macrophages by cytotoxic T lymphocytes may lead to prolonged immunologic synapses resulting in greater inflammation due to the secretion of cytokines and chemokines.\textsuperscript{18} The hypothesis is supported by a similar
phenomenon in diseases such as familial hemophagocytic lymphohistiocytosis, where
lymphocytes engage but fail to kill myeloid cells, which leads to prolonged expression of
proinflammatory cytokines and an elevated systemic inflammatory response. ¹⁹

PWH have chronic systemic inflammation which persists despite effective suppression of
plasma viremia on treatment and is thought to contribute to the development of diabetes and
metabolic disease more broadly. Furthermore, once PWH develop diabetes, the altered immune
responses in the context of continued hyperglycemia may create a reinforcing cycle that leads
to further inflammation and greater metabolic dysregulation. To investigate these relationships
further, we used unbiased analyses to investigate alterations in immune factors in the presence
and absence of glucose intolerance in PWH.
Results

Descriptive characteristics of the cohort

The study cohort was comprised of 38 participants with HIV on antiretroviral therapy with long-term suppression of plasma viremia: 14 without diabetes (hemoglobin A1c [HbA1c] <5.7% and fasting blood glucose [FBG] <100 mg/dl), and 24 with prediabetes (HbA1c 5.7-6.4% and/or FBG 100-125 mg/dl) or diabetes (HbA1c ≥6.5%, FBG≥126 mg/dl and/or on anti-diabetic medication) (Table S1). Individuals with prediabetes and diabetes were combined into a single metabolic disease group. The clinical characteristics of the participants were largely similar, except for parameters linked to glucose intolerance such as body mass index (p<0.05), waist and hip circumference (p<0.05, p=0.01 respectively), and fasting blood sugar (p<0.001). Individuals without glucose intolerance were younger (median age 39 [IQR 35, 53] versus 49 [IQR 40, 56]), but this difference did not reach statistical significance (p=0.1). There were no notable differences observed in the HIV-related laboratory values or antiretroviral therapy regimens.

Circulating cells of the innate and adaptive immune system differ by metabolic health in PWH

Mass cytometry was used to examine immune cells in cryopreserved peripheral blood mononuclear cells (PBMCs) of all participants (Figure S1A). We identified six primary clusters including CD4+ T cells, senescent CD4+ T cells (termed ‘CGC’ cells due to co-expression of CX3CR1, GPR56, and CD57 surface markers), CD8+ T cells, monocytes, B cells, and NK cells (Figure 1A). A comparison of clusters (abundance/size differences) between individuals with diabetes, prediabetes, and those without revealed several clusters in participants with glucose intolerance that were apparently fewer in PWH without diabetes depicted with the red dotted circles, all p<0.05 (Figure 1B, Figure S1B). Other cell types in the blue clusters that were more abundant in PWH without diabetes included classical monocytes, CD8+ naïve T cells, γδ T cells,
memory B cells, CD4\(^+\) T regulatory cells, and CD14\(^+\) CD16\(^{++/-}\) Monocytes (Table S2). The heatmap represents the relative expression (median) of the surface immune markers on cells and the median fold difference in cluster sizes. Magenta clusters were more abundant in prediabetic/diabetic PWH, whereas blue clusters were more abundant in non-diabetic PWH (Figure 1C). CGC\(^+\) CD4\(^+\) T cells, a population we have previously reported as associated with both metabolic and cardiovascular disease conditions in treated HIV, were also increased with diabetes.\(^{2,20,21}\)
A

Monocytes

B cells

CD4+ T cells

CGC+ CD4+ T cells

NK cells

CD8+ T cells

Legend
1. CD57+ CD161+ NK cells
2. Classical monocytes
3. CD4 Naive T cells
4. CGC+ CD8+ T cells
5. CD4 TEM/TH1
6. Mature B cells
7. CGC+ CD4+ T cells
8. CD57+ CD161+ NK cells
9. CD8+ Naive
10. CD161+ CD4+ T cells
11. CD8+ NKT cells
12. CD8 TEMRA
13. CD8 TEM
14. CD8 TEMRA
15. γδ T cells
16. CD4 TEM
17. CD57- CD8+ TEM
18. CRTH2+ CD4+ TCM
19. CRTH2+ CD8+ T cells
20. Non-classical Monocytes
21. CD161+ CGC+ CD8+ T cells
22. Memory B cells
23. CX3CR1+ GPR56+ CD57-CD4+ T cells
24. CRTH2+ Intermediate monocytes
25. CD4 T regulatory cells
26. CTLA4+ FOXP3+ B cells
27. CTLA4+ FOXP3+ CD8+ TEM
28. CRTH2+ FOXP3+ NK cells
29. CD3+ B cells
30. CD161+ CD8+ NKT cells
31. CD3+ CD4- CD8-
32. CD3+ CD14+
33. CRTH2+ CD38+ NC Monocytes
34. Plasmablasts
35. CD3+ CD14+ Monocytes
36. PD1+CD8 TCRγδ
37. CD14+ CD16+ Monocytes

B

Without Diabetes

Prediabetes

Diabetes

Overlay

C

UMAP1

UMAP2

median

fold
difference

no difference

median

scaled
expression

0

0.4

0.8

1

0

0.2

0.6

0.4

0.2

0

0

group

pre-DM/DM

no DM
Figure 1. Phenotypic characterization of PBMCs in non-diabetic, prediabetic/diabetic PWH highlights differences in PBMCs by metabolic disease

(A) UMAP and corresponding heatmap of 1.5 million CD45+ cells from the PBMCs of 38 participants depicting clusters of monocytes, CD4 T cells, CD8 T cells, B cells, monocytes, and NK cells.

(B) UMAPs stratified by metabolic disease highlighting differences using an overlay approach. Clusters higher in prediabetic/diabetic are in magenta and those higher in non-diabetic are in blue.

(C) Heat map shows all the markers that were used to define clusters in the UMAPs. The median fold difference bar (purple clusters are higher in prediabetics/diabetics and blue are higher in non-diabetic PWH). Clusters in the heat map are grouped according to the bigger clusters (CD4+ T cells, CD8+ T cells, Monocytes, B cells, NK cells, DN (double negative CD3+ T cells), and immune complexes).

Figure 2. We employed the T-REX workflow as an unbiased, granular approach to visualize distinct cell populations that varied between non-diabetic and prediabetic/diabetic PWH and marker enrichment modeling (MEM) to identify the markers that define each of the different clusters.

22,23 The UMAP (Figure 2A) displays the distribution of cells from both non-diabetic (blue) and prediabetic/diabetic (red) PWH and the clusters that were greater than 95% different between the two groups (Figure 2B). We found CD14+ clusters outside of the primary monocyte population, adjacent to the CD4+ T cells, CD8+ T cells, CD19+ B cells, and NK cells (Figure 2C).

Unexpected markers (FOXP3, CTLA4, and CRTH2) also defined the clusters with immune complexes, suggesting additional potential biomarkers for further investigation (Figure 2D).

While FOXP3 and CTLA4 are constitutively expressed on CD4+ T regulatory cells, they can be expressed on activated cells. We also compared the cluster sizes identified using T-REX between non-diabetic vs. prediabetic/diabetic using non-parametric analysis. All immune complex cluster sizes except cluster 3 (CD19+ CD14+ complex) remained significantly higher (Figure 2E). UMAPs highlighting the expression of CD14 and FOXP3 on all CD45+ cells within the clusters with monocyte complexes, and not in other cells present in PBMCs are shown (Figure 2F). In summary, with T-REX, the same clusters were different when we generated the UMAPs stratified by diabetes status in Figure 1b. The complexes were present within each
cluster of T cells/ B cells/ NK cells and Monocytes. We chose to focus on CD3$^+$ CD14$^+$ T-cell monocyte complexes going forward, in the context of HIV reservoir and diabetes.

When we analyzed CD3$^+$CD4$^+$ T cells, we found several subclusters within CD4$^+$ T cells that were also CD14$^+$ (Figure S2A). The CD4$^+$ T cell - CD14$^+$ monocyte clusters were mostly present in people with prediabetes/diabetes (Figure S2B-C). The T-REX workflow generated a similar result, with the three larger clusters that were higher with glucose intolerance also co-expressed CTLA4, FOXP3, and CD14 (Figure S2D). Similarly, several CD3$^+$CD8$^+$ T cell subclusters were CD14$^+$ (Figure S3A). Stratification by the metabolic group demonstrated that the CD8$^+$CD14$^+$ subclusters were mainly present in the prediabetic/diabetic participants (Figure S3B). This was confirmed using T-REX analysis, with two of the main clusters labeled preDM/DM1 and 2, which included cells with CD14, CTLA4, and FOXP3 co-expression (Figure S3C-D).
Figure 2. Classical monocytes complex with T cells, NK cells, and B cells are increased in the peripheral blood of PWH with glucose intolerance.

(A) UMAP of CD45^+ cells from non-diabetic (blue), prediabetic/diabetic PWH (red).

(B) Clusters identified by the T-REX algorithm that are increased and decreased with prediabetes/diabetes (0 trends towards non-diabetic and 100 towards prediabetic/diabetic).

(C) Clusters higher with prediabetes/diabetes (preDM/DM) or without diabetes (NonDM) are shown along with the heat map that highlights markers that define the cells within the clusters.

(D) The enrichment scores of markers (increased and decreased) for selected clusters are shown over the UMAP, adjacent to the clusters. Among the markers that characterize the clusters, magenta (increased markers), and blue (decreased).

(E) Violin plots showing proportions of select subclusters that are significantly different between non-diabetes (non-DM and prediabetes/diabetes (preDM/DM).

(F) UMAPs show CD14 and FOXP3 expression on the clusters.

Statistical analysis by Mann-Whitney test (E). See Figures S2 and S3.
CD3+ T cell - CD14+ monocyte complexes increase in longitudinal samples

To confirm that the complexes identified by mass cytometry were monocytes complexed to other immune cells and not artifacts, we stained PBMCs with a flow cytometry panel to identify cells with CD3+ and CD14+ expression (Figure 3A). The Z channel shows CD3 expression on lymphocytes and monocytes (Figure 3A). Based on the two-dimensional plots, CD3+CD14+ T cell complexes are among the larger monocyte cells (larger and more granular). We then sorted CD14+ monocytes and CD3+CD14+ T cell-monocyte complexes and used light microscopy to image the cells. Compared to CD14+ cells alone, the CD3+ CD14+ complexes had a higher proportion of cells with presumed immunological synapses due to the proximity of cells and flattening at the connecting points (Figure 3B).

We next analyzed longitudinal PBMC samples from 37 of the 38 participants who had a second visit 2-3 years after the first, to assess whether the proportion of CD3+ CD14+ complexes remained stable or varied over time. CD3+ CD14+ T cell-monocyte complexes ranged from 0.07% to 11.5% of CD3+ T cells by flow cytometry at the first visit and 0.55% to 9.5% at the second visit. We observed a significant increase (p=0.03) in the proportion of CD3+ CD14+ T cell – monocyte complexes, as a proportion of CD3+ live cells, from the first to second visit (Figure 3C). When stratified by diabetes status, there was a greater increase in CD3+ CD14+ T cell-monocyte complexes among the non-diabetic compared to the prediabetic/diabetic PWH (Figure 3D). This observation suggests that a higher proportion of circulating T cell–monocyte complexes in prediabetes/diabetes represents more of a terminal state and that once PWH have prediabetes or diabetes, then metabolic disease and related complications are frequent, while some of the PWH without diabetes may still be progressing in this direction as they age.
Figure 3. CD14+ classical monocytes form complexes primarily with CD8+ and central memory T cells.
(A) Two-dimensional flow cytometry plot of PBMCs showing CD14, CD3, and Live/Dead stains on PBMCs. CD3+ cells were gated on live cells (including lymphocytes and monocytes), followed by a two-dimensional plot of CD8+ and CD14+ cells.
(B) Bright-field images of CD14+ and CD3+ CD14+ cells.
(C) Violin plot showing % CD3+ CD14+ T-cell-monocyte complexes in longitudinal time points 2-3 years apart.
(D) Box plots plot showing % CD3+ CD14+ T-cell-monocyte complexes in longitudinal time points stratified by diabetes status.
(E) Dot plot showing proportions of CD4+ and CD8+ T cells among CD3+ CD14+ complexes.
(F) Dot plot showing proportions of memory CD3+ T cells ( naïve, CD45RO+CCR7+, TEM, CD45RO+CCR7+, TCM, CD45RO+CCR7+, TEMRA, CD45RO+CCR7+).
Statistical analyses were performed using Mann Whitney U test (C) and Wilcoxon matched pair signed ranks test (D).

T cell-CD14+ monocyte complexes are positively associated with fasting blood glucose and hemoglobin A1C.

Based on the observed differences in CD3+ CD14+ complexes by diabetes status, we posited that the complexes may drive glucose intolerance. We used partial Spearman rank correlation analysis adjusted for age, sex, and BMI to assess whether the circulating immune complexes were associated with fasting blood glucose and hemoglobin A1C. We found that CD8+ T cell–CD14+ monocyte complexes were associated with fasting blood glucose (p<0.05) and hemoglobin A1C (p<0.01) (Figure 4A). The NK cell – CD14+ monocyte cluster was also positively associated with fasting blood glucose (p<0.05) but not hemoglobin A1C. CD4+ T cell – CD14+ monocyte complexes were only positively associated with hemoglobin A1C (p<0.01) and not fasting blood glucose (Figure 4A).

Greater systemic inflammation, in aggregate, is associated with an increased risk of cardiovascular and metabolic disease. There were no significant differences in plasma cytokines among the 38 participants by group comparison, including IL-1β, IFN-γ, TNF-α, IL-6, IL-17, and IL-10 (Data not shown). We performed Spearman rank correlation analyses adjusted for factors related to HIV (CD4:CD8 ratio, duration of antiretroviral therapy) and diabetes.
We found that the immune complexes with monocytes [Clusters 18, 19, 26, 27, 28, and 29] were strongly correlated with each other. Notably, all clusters were negatively correlated with circulating CD4\(^+\) T regulatory cells (CL 25) (Figure 4B). CD8\(^+\) T cells (p<0.01), B cells (p<0.05), and NK cells (p<0.05) complexed with monocytes were also negatively correlated with plasma IL-10 (p<0.05). The CD4\(^+\) CD14\(^+\) T cell-monocyte cluster was negatively correlated with IL-10 but this was not statistically significant (p=0.067). Cluster 32 was comprised of CD3\(^+\) T cells (both CD4 and CD8) complexed with CD14\(^+\) monocytes that were less correlated with the other complexes. This was a much smaller cluster than all other complexes and positively correlated with plasma sCD163 (p=0.075) and plasma IL-17A (p=0.03). We considered whether the relationship between the immune complexes and IL-10/CD4 T regulatory cells might depend on the level of blood glucose. To test this hypothesis, we included an interaction term (hemoglobin A1C*IL-10 and hemoglobin A1C*CD4 T regulatory cells) in a linear regression model with the immune complexes as the dependent variables (Figure S4A). The coefficients for the interaction terms were negative and statistically significant (Figure S4B), suggesting that the effect of IL-10 and CD4\(^+\) T regulatory cells on the immune complexes decreases as hemoglobin A1C increases or vice versa. In other words, the relationship between the immune complexes and hemoglobin A1C is weaker for individuals with higher levels of IL-10 or CD4 T regulatory cells.
Figure 4. T cell-Monocyte immune complexes positively associated with blood glucose are negatively associated with IL-10.

(A) Heatmap shows partial Spearman correlation of immune complex clusters and CD4 T regulatory cells and hemoglobin A1c, fasting blood glucose adjusted for age, sex, and BMI. UMAP with clusters is included for reference.

(B) Heatmap shows partial Spearman correlation of immune complex clusters and CD4 T regulatory cells and inflammatory cytokines (IL-10, IL-6, sCD14, sCD163, IL-15, IL-17, and IL-1β) adjusted for CD4:CD8, antiretroviral therapy duration, and hemoglobin A1C.

See Figure S4.

CD4+ T cells complexed with CD14+ monocytes include TH1, TH17, and TH2 phenotypes

To better characterize the CD4+ T cells complexed with CD14+ monocytes, we first analyzed the memory subsets using CCR7 and CD45RO (Figure 5A). Similar to our analysis with total CD3+ T cells, CD14+ monocytes were largely complexed with CD4+ TCM and TEM cells in both non-diabetic and prediabetic/diabetic PWH (Figure 5B). Several markers of activation were used to define activated CD4+ T cells (CD137/OX40 and HLADR/CD38). Representative two-dimensional flow charts show the expression of these markers on CD4+ T cells from non-diabetic and prediabetic/diabetic PWH (Figure 5C). In general, there was a significantly higher proportion of activated, CD137+ OX40+ CD4+ T cells and HLADR+ CD38+ CD4+ T cells in prediabetic/diabetic PWH compared to non-diabetic (Figure 5D, left panels). Focusing on immune complexes, we observed that CD4+ CD14+ T cells had a higher proportion of CD137 and OX40 expressing cells (Figure 5D, top right panel). Irrespective of metabolic status, all cells within immune complexes were HLADR+ CD38+. Circulating activated T cells and immune complexes were correlated with fasting blood glucose (Figure 5F). Using chemokine receptor markers, we defined CD4+ T helper subsets within the T cell-monocyte complexes. CD4+ T cells from prediabetic/diabetic PWH compared to non-diabetic had a significantly higher proportion of TH2, TH17, and TH1 CD4+ T cells (Figure 5G). A similar trend was observed with CD4+ CD14+ T cell-monocyte complexes which had higher proportions of Th1 and Th17 cells. In summary, the T-cell monocyte immune complexes consist of activated immune cells, enriched for inflammatory memory subsets.
Figure 5. CD4+ T cells complexed with CD14+ monocytes in prediabetic/diabetic PWH are activated with a larger proportion of TH17 and TH1 cells.

(A) Two-dimensional flow cytometry plots show memory CD4+ T cells (Naïve, CD45RO- CCR7+; TCM, CD45RO+ CCR7+; TEM CD45RO+ CCR7-; TEMRA CD45RO- CCR7-).

(B) Memory CD4+ T cell subsets in non-diabetic and prediabetic/diabetic PWH have a similar distribution of memory T cell subsets.

(C) Representative plots showing CD137/OX40 and HLA-DR/CD38 co-expression on CD4+ T cell – CD14+ monocyte complexes stratified by diabetes.

(D) Dot plots show % CD137+ OX40+ CD4+ T cells and % CD137+ OX40+ CD14+ complexes.

(E) Dot plots show % HLA-DR+ CD38+ CD4+ T cells and % HLA-DR+ CD38+ CD14+ complexes.

(F) Activated T cells -monocyte immune complexes are correlated with fasting blood glucose.

(G) PWH with pre-diabetes/diabetes have a higher proportion of TH2 (CRTH2/CCR4), TH17 (CCR6/CD161), and TH1 (CXCR3) cells stratified by diabetes.

(H) CD4+ T helper cells within CD4+ CD14+ T cell-monocyte complexes have higher proportions of TH2, TH17, and TH1 cells.

Statistical analyses were performed using the Mann-Whitney U test (D-E), Spearman correlation (F), and the Kruskal-Wallis test (G-H).

T cell-monocyte pairs form stable complexes and have particles consistent with HIV

In order to understand the durability of the T cell-monocyte complexes given their abundance, we sorted CD3+ T cell-CD14+ monocyte immune complexes and imaged them over 4.5 hours.

Time-lapse imaging of the CD3+ CD14+ T cell-monocyte immune complexes shows dynamic and heterogeneous relationships between CD14+ monocytes and CD3+ T cells (Figure 6A-B, movies CD and E). We observed several stable complexes over a period of 5 hours (Figure 6C, D) and a proportion of monocytes (antigen-presenting cells, APCs) that transiently interacted with T cells (Figure 6E).

Macrophages are HIV reservoirs that may effectively form synapses and infect CD4+ T cells. We hypothesized that HIV transfer may be enhanced by T cell-monocyte complexes in circulation. We sorted CD3+ CD14+ T cell-monocyte complexes and employed transmission electron microscopy (TEM) to capture the T cell and monocyte pairs interacting with high resolution. We observed T cells in immunological synapses with monocytes (Figure 6F-i,ii) with 100nm particles in T cells-monocyte pairs (Figure 6F-i,ii) and T cells among sorted CD3+ CD14+ T cell-monocyte complexes consistent with HIV (Figure 6G).
Figure 6. CD3+ CD14+ T cell - monocytes form stable complexes

(A) Phase-contrast microscopy of sorted CD3+ CD14+ T cell-monocyte complexes at time 0.
(B) Pie chart showing the percentage of (CD14 antigen presenting cells (APCs) that are stably associated with T cells, transiently associated with T cells or not associated with T cells over 4.5 hrs. 

(C & D) Show insets of stable complexes, right hand panel shows the time overlay and the color code. A yellow asterisk (*) in c marks a T cell that proliferates. Scale bars – purple pseudocolor defines T cell and green marks APC.

(E) Time series demonstrating transient interactions between APC and three T cells (marked 1,2,3). Blue arrowheads and numbers mark point of interactions between APC and T cells.

(F) TEM of CD3⁺ CD14⁺ T cell-monocyte complexes. Inset highlights ultrastructural cell-cell interactions (i) and (ii) and the presence of 100nm diameter particles (black arrow).

(G) TEM of CD3⁺ T cell among sorted CD3⁺ CD14⁺ T cell-monocyte complexes 3 days post-culture. Enlarged image (i) highlighting 100nm diameter particles (black arrow).

Scale bars are 50µm A, 20µm C-E, 4µM F, 500nm F(i), F(ii), G (i), and 1µm G. 

See movies CD and E.

Using ddPCR, we quantified HIV DNA in sorted CD4⁺ T cells, CD14⁺ monocytes, and CD3⁺ CD14⁺ T cell-monocyte complexes from 6 PWH on antiretroviral therapy. Representative images show LTR (blue) and RNase P copies (green) in CD3⁺ CD14⁺ complexes (Figure 7A), CD4⁺ T cells (Figure 7B), and CD14⁺ cells (Figure 7C). We found that CD3⁺ CD14⁺ T cell-monocyte complexes had significantly more HIV DNA copies per million cells compared to paired single CD4⁺ T cells and CD14⁺ monocytes (Figure 7D-E). Monocytes are reported to be infected with latent human CMV, therefore used ddPCR to quantify CMV. We did not detect CMV DNA using three different sets of primers against US28, gB, and IE-1 (data not shown) in immune complexes from all 6 participants (ages 24 to 59). Taken together, we were able to detect HIV using ddPCR, which was highest in CD3⁺ CD14⁺ T cell-monocyte complexes.

If the immunological synapses between CD3⁺ T cells and CD14⁺ monocytes are an antigen-driven process, we posited that clonally expanded T cells could be present within the CD3⁺ CD14⁺ complexes. Among 4 representative participants with higher HIV DNA copies in their CD3⁺ T cell-CD14⁺ monocyte complexes, we index-sorted single complexes per well and sequenced the αβ TCR chains. The majority of the TCRs were single clones with a few clonal TCRs (>2 T cells with identical CDR3), (Figure 7F). We used TCRmatch (http://tools.iedb.org/tcrmatch/) to predict the antigen specificity of the clonal TCRs among those
identified. The majority of the clonal (CDR3 >2) TCRs that we found in public databases were predicted to bind viral antigens (CMV, EBV, SARS-CoV2), with one predicted to bind HIV [CASKEGGAGGYTF] (Table S3). In addition, two CDR3 were predicted to bind an epitope from the coagulation factor VIII. In summary, these data suggest that circulating T cell-monocyte complexes may be partly driven by the presentation of herpes viral antigens. In PWH, monocytes may be complexed with HIV-infected T cells, which places the monocyte in proximity to infected cells to allow the formation of functional synapses.

**T cell-monocyte complexes have RNA transcriptomes enriched for genes important for activation and adhesion**

We performed a single-cell transcriptomic analysis of PBMCs from a participant with a large proportion of T cell-monocyte complexes as determined by CyTOF and flow cytometry. Using CITE seq and RNA transcriptomic profiles, we classified immune cell complexes/doublets that would have been excluded from further analysis based on traditional analysis approaches. UMAP shows the different cell types that were classified based on their transcriptomic profile (Figure 7G). We found several clusters that had immune complexes including a T cell-non-classical monocyte cluster, T cell-classical monocyte cluster, B cell-monocyte cluster, T cell-B cell cluster, and megakaryocyte-T cell cluster. UMAPs and violin plots show CITE-seq expression of several surface markers, CD14, CD16, CD3, CD4, CD69, and CD8 (Figure 7H-I).

Classical monocytes paired with T cells had an almost equal representation of T cells with clonal and non-clonal TCRs. Non-classical monocytes on the other hand were mostly paired with clonal TCRs (Figure 7J).
Figure 7. CD3\(^*\) CD14\(^+\) T cell-monocyte complexes have more copies of HIV compared to CD4\(^+\) T cells and CD14\(^+\) monocytes alone and are transcriptionally more activated through direct antigen presentation.

(A) Representative ddPCR plot showing HIV (blue) and RNase P (green) copies in sorted CD3\(^+\) CD14\(^+\) T cell-monocyte complexes.

(B) Representative ddPCR plot showing HIV (blue) and RNase P (green) copies in sorted CD3\(^+\) CD4\(^+\) T cells.

(C) Representative ddPCR plot showing HIV (blue) and RNase P (green) copies in sorted CD14\(^+\) monocytes.

(D) Violin plot shows HIV viral copies per million cells from the 6 participants in each of the sorted cell types – CD14\(^+\), CD3\(^+\) CD4\(^+\), and CD3\(^+\) CD14\(^+\).

(E) The line plot shows HIV viral copies in paired samples.

(F) Circos plot shows paired TCR\(\alpha\beta\) CDR3 sequences expressed on T cells complexed with monocytes from one representative participant.

(G) UMAP of PBMCs processed for 10X sequencing from a participant with a high proportion of immune complexes, highlighting T cell-monocyte doublets, B cell-T cell doublets, and B cell-monocyte doublets.

(H) UMAPs highlight surface marker expression on PBMCs using CITE seq barcoded antibodies.

(I) Violin plots show the difference in the surface marker expression levels between all the cells.

(J) TCR sequences were obtained, and the stacked bar chart shows the total number of cells with TCRs and is color-coded based on the clonality of the cells, with 2 or greater considered clonal.

(K) Bar plots depict protein translation by measuring puromycin uptake (puromycin), and changes in uptake with the addition of inhibitors (2DG, oligomycin, and 2DG plus oligomycin(DGO)) for each subset of cells [CD3\(^-\) CD14\(^+\), CD14\(^+\) monocytes, CD16\(^+\) monocytes, and CD4/CD8 T cell subsets (naive, TCM, TEM and TEMRA) for 15 individuals (Blue: NonDM, Green: PreDM and Magenta: DM).

(L) Bar plots show percent glucose dependence (top) and percent mitochondrial dependence (bottom).

(M) The proportion of CD3\(^+\) CD14\(^+\) T cell-monocyte complexes decreases with the inhibition of oxidative phosphorylation by oligomycin. This is no change in glucose inhibition by 2DG.

Statistical analysis using Kruskal Wallis (D), Wilcoxon test (E), Mann Whitney Test (L, M) See Figure S5, S6 and S7 and Tables S5-S7

Differential gene expression of CD3\(^+\) CD14\(^+\) doublet cluster versus classical monocytes (singlets) showed increased expression of genes that enrich for T cell activation [CCL5, IL7R, CD3E, CD2, CD8B, LCK, CD7, and HLA-DPA1] and the adaptive immune response [CD3E, PRF1, GZMM, LIME1, SKAP1, and CD247] (Figure S5A, Table S4). Genes involved in lipid localization [THBS1, RETN, ACSL1, TSPO, CD36, IRS2], granulocyte activation, and neutrophil-mediated immunity [S100A12, S100A8, S100A9, CDA, MNDA, RETN, CD36, GLUT3, LYZ] were significantly higher in monocytes than in the T cell-monocyte complexes. On the other hand, differential gene expression of CD3\(^+\) CD14\(^+\) T cell-monocyte complexes versus
T cells enriched for genes involved in antigen processing and presentation [HLA-DRA, CD74, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1, HLA-DPB1, and HLA-DRB5] (Figure S7B, Table S5). Non-classical monocytes complexed with T cells were also transcriptionally different compared to singlet non-classical monocytes. Similar to the classical monocytes, the T cell-non-classical monocyte complexes enriched for genes involved in the adaptive immune response [CD3E, IL7R, CD8B, PRF1, GZMM, CD247, SKAP1, LIME1, CD7, and TNFAIP3 – bold genes were also upregulated in T cell-classical monocytes versus monocytes]. Several overlapping pathways were overrepresented in CD16+ single-cell non-classical monocytes over the T cell-non-classical monocyte complexes including neutrophil-mediated immunity [SERPINA1, FCER1G, FTH1, LGALS3, CD68, ATP6V0C, PSAP, CFD, STXB2, SLC11A1, S100A11, CMTM6], granulocyte activation and platelet degradation [TIMP1, SERPINA1, FCER1G, PSAP, CFD, ALDOA, STXB2, LYN, and PECAM1] (Figure S5C, Table S6). The differential gene expression between CD3+ CD16+ T cell-monocyte complexes versus T cells had an over-representation of genes that enriched for several pathways including IL-10 production [FCER1G, LILRA5, LILRB1, VSIR, PYCARD, and LGALS9] and negative regulation of T cell activation [LST1, LYN, HMOX1, LGALS3, CD74, MNDA, LILRB1, VSIR, PTPN6, CD86, CEBPB, and LGALS9]. Pathways highly represented in T cells compared to these CD3+ CD16+ T cell-monocyte doublets included protein localization to the membrane (Figure S5d Table S6).

Our analysis of immune complexes from 10x sequencing data is confounded by complexes that may be formed due to technical artifacts, where more than one cell ends in a single droplet. Therefore, we used existing 10X sequencing data from sequenced PBMCs from individuals receiving cardiac surgery (n=10), to create artificial clusters by combining single T cells and monocytes. The differential gene expression between T cells-classical monocytes complexes compared to artificial doublets (single memory T cells and classical monocytes grouped for comparison) showed higher levels of GNLY, HLA-DRA, HLA-DPA1, HLA-DPB1, HLA-DRB1,
MT-ND4, and MT-ND3 with overrepresentation of several pathways including MHCII antigen presentation and TCR signaling (Figure S6A-C, Table S6). Taken together, these data suggest that in vivo, monocytes form durable complexes with a subset of clonal memory T cells that persist ex-vivo. The T cell-classical monocyte complexes have transcriptomes that suggest an activated inflammatory response which differs from the artificial doublets.

Changes in metabolism can be very informative of the inflammatory state and functional profile of immune cells. While immune cells can rely on both glucose and mitochondria for energy production, activated cells mostly rely on glycolysis. We measured glucose and mitochondrial dependencies of PBMCs ex vivo using SCENITH. Based on the puromycin uptake, CD3+ CD14+ T cell-monocyte complexes and CD14+ monocytes at baseline appear to be more metabolically active than T cells (Figure 7K). While both CD14+ monocytes and CD3+ CD14+ immunologic complexes were mainly glucose-dependent, the latter had a higher mitochondrial dependence (Figure 7L). We next posited that if the immune synapse formation/maintenance is a high energy function based on the presence of mitochondria close to the synapse (Figure S7A-B), then incubation of PBMCs with metabolic pathway inhibitors (2DG, oligomycin) would affect the proportions of immune complexes detectable by flow cytometry. While the proportion of CD3+ CD14+ complexes remained the same after inhibition of glycolysis with 2DG, there was a significant decrease in the percent of detectable CD3+ CD14+ immune complexes with inhibition of oxidative phosphorylation (Figure 7M). Taken together, these initial studies suggest that the cells that make up the CD3+ CD14+ immune complexes may use both glycolysis and oxidative phosphorylation, though inhibition of mitochondrial ATP synthesis reduced the proportion of complexes.
Discussion

This study reveals that circulating immunologic monocytes complexed with T cells are increased in the presence of glucose intolerance in PWH, suggesting that these immune cell interactions may contribute to the elevated burden of diabetes and metabolic disease associated with long-term HIV infection. Importantly, the immune complexes have RNA transcriptomes that demonstrate an inflammatory profile. Additionally, there is an inverse association between the immune complexes and anti-inflammatory responses like IL-10 and CD4+ T regulatory T cells. Many of these complexes remain intact over a 4-hour time-lapse ex-vivo, which suggests that they form prolonged synapses that may be an additional driver of inflammatory response.

Although immune complexes in blood have traditionally been ignored, recent findings implicate them in immune perturbation. Among the possible explanations for the accumulation of circulating immune complexes, are the presence of HIV within these complexes and the fact that they are increased with glucose intolerance, maybe a reflection of a bidirectional and self-reinforcing loop that exists between complex-driven inflammation (driven by HIV) and elevated blood sugar.

Monocytes are important leukocytes that constitutively leave the circulation to survey tissue. CD14+ classical monocytes represent close to 90% of total monocytes and are more likely to become macrophages after transmigration into tissues. On the other hand, CD14loCD16+ non-classical monocytes tend to differentiate into dendritic cells and may re-enter the blood circulation. In PWH on antiretroviral therapy, replicating and integrated HIV can be detected in circulating monocytes. Although CD14loCD16+ non-classical monocytes may be more prone to HIV infection, due to the study design using CD3 and CD14 to detect the immune complexes, we show here that HIV viral particles within CD14+ cell-classical monocyte complexes. Our imaging studies allow us to speculate that HIV could be transferred from either cell type. Within tissues, macrophages infected with HIV can effectively transmit HIV to CD4+ T
cells, suggesting these cellular interactions may be an important contributor to T cell loss and the establishment of the cellular reservoir.\textsuperscript{25,35,36}

HIV cure will require the elimination of viral reservoirs, which has been a challenge due to the inability of the immune system to eliminate latently infected cells.\textsuperscript{18,37} The field recognizes that there is a lot of heterogeneity in the viral reservoir which includes both cell types as well as their viral activation states including the latent virus that is not transcriptional active and the transcriptionally active virus which can be in a spectrum plus or minus viral protein expression.\textsuperscript{37} The immune-complex imaging suggests that these complexes are heterogeneous, many of which remain intact over a 5-hour window. Thus, these complexes may adhere to inflamed endothelium and be recruited together and help establish and replenish viral reservoirs within tissues. Future studies will investigate the recruitment of these cells to better understand this. Interestingly, our T cell receptor sequencing data consisted of clonal cells however there were no dominant clones to suggest that these complexes are driven by a single antigen. However, based on previously published public TCRs which are limited by what has been studied, a large proportion of the TCRs expressed were against EBC, CMV, HIV, and SARS-CoV2. While this may be biased by predominant TCRs in public databases, it implies that other chronic infections such as EBV and CMV may also be important. While we were able to detect HIV by droplet digital PCR, we did not detect CMV DNA within these complexes. However, additional studies that use multiple modalities for detecting viruses (HIV, CMV, EBV, Influenza antigens, DNA, RNA transcripts) are warranted.

Chronic systemic inflammatory responses in PWH may partly explain the high incidence and prevalence of diabetes.\textsuperscript{38,39} Similarly, there is growing evidence inflammation is important in the pathogenesis of type 2 diabetes.\textsuperscript{40} IL-10 is an anti-inflammatory cytokine that is expressed by several cell types including macrophages, Th2 cells, regulatory T cells, dendritic cells, and B
cells. Several studies including the Leiden 85-plus study have shown that immune cells from individuals with metabolic syndrome or type 2 diabetes expressed lower levels of IL-10 upon stimulation. While untreated HIV infection may be associated with increased expression of IL-10, which in turn is thought to suppress T-cell responses. Over time and with antiretroviral therapy treatment, IL-10 expression decreases and may be associated with metabolic disease in PWH. Furthermore, a study looking at the effects of exercise suggests that PWH are unable to increase IL-10 expression when compared to HIV-negative individuals. In this study, the relationship between the T cell-monocyte complexes and IL-10 and the interaction between hemoglobin A1C and IL-10 reinforces the notion that complexes arise in the setting of inflammation. Taken together, a state of systemic inflammation in PWH, compounded with diabetes and a lack of adequate inhibitory responses, could enhance the presence of the T cell-monocyte immune complexes.

A recent study on immunometabolism of CD4+ T cells in the context of HIV showed that the infectivity of the CD4+ T cells was more dependent on the metabolic activity of the T cells and less on the activation status. They separated CD4+ T cells based on their metabolic activity and then infected with HIV and observed a higher magnitude of HIV infection with those that were more metabolically active (both oxidative phosphorylation and glycolysis). Interestingly, they also showed fewer cells with latent HIV infection, when they partially inhibited glycolysis using 2DG, suggesting that the steps required for HIV to establish latency are glucose dependent. In our study, CD3+ CD14+ immune complexes were metabolically active with dependence on glucose than oxidative phosphorylation. Based on the recent study, this would make the T cells and possibly monocytes better able to support an HIV viral reservoir. Importantly, the formation of these immune complexes is an energy-demanding process, and the inhibition of oxidative phosphorylation may have been sufficient to affect some of these immunological synapses as previously published.
In summary, we have defined specific features of metabolically active, dynamic T cell-monocyte immune complexes increased with glucose intolerance in the setting of chronic HIV infection. In PWH, these cells also represent a fraction of the HIV reservoir that is likely to be recruited into the tissue to establish a tissue reservoir. The complex interplay between inflammatory and metabolic disorders makes these cells particularly interesting. Future studies investigating these cells in vivo will provide insight into their role in the development of metabolic disease and complications that may arise from this.

Limitations of the study

This is a cross-sectional study that is not able to show causality, therefore while the inflammatory immune complexes are increased in persons with HIV with increased glucose tolerance and appear to carry HIV, we are currently unable to make conclusions as to whether these immune complexes are important in the pathogenesis of the metabolic disease. Another limitation is that our conclusions cannot be extended to HIV-negative individuals at this time. Future studies in mouse models and human cohorts with and without metabolic disease, including PWH are needed to understand the extent to which the immune complexes may be important in the pathogenesis of a metabolic disease or may be associated with complications related to metabolic disease.
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Energy Metabolism Profiling with SCENITH
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Oligomycin A | N/A | Sigma Aldrich | #75351-5MG
2-deoxy-D-glucose 2mM | N/A | Sigma Aldrich | #D8375

Software and Algorithms

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Resource availability

Requests for further details on protocols and data included in this study are available upon request from the lead contact, celestine.wanjalla@vumc.org

Study Participants

All PWH were previously recruited for the HIV, Adipose Tissue Immunology and Metabolism cohort (HATIM) at the Vanderbilt Comprehensive Care clinic from 2017 to 2020 (first visit) and 2021 to 2022 (second visit). The cohort was developed to study contributors to metabolic disease in treated HIV infection and included individuals without diabetes (hemoglobin A1c [HbA1c] <5.7% and fasting blood glucose [FBG] <100 mg/dl), with prediabetes (HbA1c 5.7-6.4% and/or FBG 100-125 mg/dl), and diabetes (HbA1c ≥6.5%, FBG≥126 mg/dl and/or on
medications to treat diabetes). All PWH were on antiretroviral therapy with sustained viral suppression for a minimum of 12 months before the study, with a CD4+ T cell count > 350 cells/ml (Supplemental Table 1). The cohort excluded individuals with inflammatory illnesses, substance abuse (amphetamines and cocaine), greater than 11 alcoholic drinks per week, and active hepatitis B and C. The study is registered at ClinicalTrials.gov (NCT04451980) and additional details are previously published. This study was approved by the Vanderbilt University of Medicine Institutional Review Boards. Participants in this study provided written informed consent and questions answered where applicable. Records and samples are stored with de-identified labels. The investigators carried out studies by the guidelines of the United States Department of Health and Human Services.

Mass cytometry

Cryopreserved PBMCs were thawed in RPMI media containing Nuclease S7 (20ul). The PBMCs were pelleted, washed in PBS without calcium or magnesium, and resuspended in 1ml RPMI media followed by incubation with 10uM Cisplatin (3 minutes before quench with RPMI/10% fetal bovine serum) to stain for LIVE/Dead cells. Cells were then washed twice with PBS/1% BSA. For all 37 antibodies included in the panel, we made a master mix with the surface markers (Supplemental Table 2). The cells were incubated with the master mix of antibodies for 30 minutes at room temperature, followed by two washes with PBS, and a fix with 1.6% paraformaldehyde. The cells were washed two additional times with PBS and then resuspended in 1ml of cold methanol and stored at -20°C. One day prior to running the samples on the Helios, the cells were washed twice with PBS and resuspended in PBS/1% BSA. Cells were then stained with intracellular markers (CTLA4 and FOXP3) for 20 minutes at room temperature. The cells were then stained with 2ul (250nM) of DNA intercalator (Ir) in PBS with 1.6% PFA. Just prior to running the cells were washed in PBS followed by Millipore water. For
analysis, we resuspended 500,000 cells/ml of Millipore H₂O. \(1/10\) volume of equilibration beads were added to the cells, which were then filtered and run on the Helios.

FCS files from the Helios cytometer were bead-normalized using the premessa R package's normalizer GUI method. FCS files were then submitted to a gating scheme using Flowjo to clean the data of debris (DNA-), Fluidigm beads (175++165++), events that showed obvious doublet character by Gaussian parameters (EventLength high Residual high), and dead cells (cisplatin\(^*\)). Using Flowjo, various cell types were targeted for analysis including CD45+ lymphocytes, CD4\(^+\) T cells, CD8\(^+\) T cells, Monocytes, B cells, and NK cells (refer to gating scheme figure if appropriate). Gated subsets were exported from Flowjo for further analysis.

The R programming language (version 4.2.1) was used for the subsequent analysis steps. CD45+ lymphocyte exports were downsampled to 40000 events per sample, CD4\(^+\) T cell exports were downsampled to 40000 events per sample, CD8\(^+\) T cell exports were downsampled to 40000 events per sample, Monocyte exports were downsampled to 30000 events per sample, B cell exports were downsampled to 30000 events per sample, and NK cell exports were downsampled to 30000 events per sample. All FCS file handling in R was done using the flowCore package. Each downsampled subset was passed through the following workflow: Subset parameters were transformed using the function \(\text{asinh}(x/5)\). A nearest neighbor search was done to produce a weighted adjacency matrix with a number of nearest neighbors set to the dimension of subset + 1. The Leiden community detection algorithm was used to cluster the adjacency matrix. Uniform Manifold Approximation and Projection (UMAP) was done for subset visualization using the uwot R package.

Tracking Expanding populations (T-REX) and Marker Enrichment Modeling (MEM) of enriched features
The T-REX algorithm was performed as published. In brief, we classified cells of interest measured using mass cytometry including CD45$^+$ cells, CD3$^+$ CD4$^+$ T cells, CD3$^+$ CD8$^+$ T cells, CD3$^+$ CD19$^+$ HLA-DR$^+$ monocytes, and CD3$^+$ CD56$^+$ CD16$^+$ NK cells. UMAP analyses were performed for concatenated non-diabetic participants (group 1) and prediabetic/diabetic participants (group 2). This was followed by K nearest neighbor (KNN) analyses to search for the nearest neighbors for each cell. The difference in the percent change per cell between group 1 and group 2 is calculated based on the abundance of these cells in each group in the KNN region. ≤5% and ≥95% changes in cell percentages were considered significant, which we clustered using DBSCAN. The phenotype of the clusters that were significantly different between the groups was determined using the MEM package.

**Flow cytometry**

PBMCs were stained with fluorescently tagged antibodies as previously published. In brief, thawed and washed PBMCs were stained with Aqua (Live/Dead marker) for 10 minutes at room temperature, followed by the addition of fluorescently tagged antibodies against T cells [CD3-BV786, CD4-PerpCP cy5.5, CD8-A700], monocytes [CD14-APC], T-cell memory subsets [CD45RO and CCR7]. CD19 antibody was included in the Aqua channel (therefore we did not evaluate CD19 cells for flow cytometry). For the SCENITH assay, we used antibodies [anti-puromycin, Zombie, CD3, CD4, CD8, CD14, CD16, CD19, CD27, CD28, CD38, CCR7, CD45RO, CX3CR1, CXCR3, PD1, CXCR5, IgD, and KLRG1].

**Droplet digital PCR**

We sorted CD3$^+$ CD4$^+$ T cells, CD14$^+$ monocytes, and CD3$^+$ CD14$^+$ T cell-monocyte complexes into separate Eppendorf tubes with PBS. Cells were pelleted and resuspended in lysis buffer [Tritonx100 (0.1%), Tris HCL (10mM), and Proteinase K (400ug/ml)] at 55°C for 10 hours. Additional proteinase K was added during the heat inactivation stage at 95°C for 5 minutes. For
HIV DNA quantitation, we used LTR primers (LTR 5’AGC ACT CAA GGC AAG CTT TA-3’, and reverse primer -LTR 5’ TGT ACT GGG TCT CTC TGG TTA G-3’, and probe 5’-FAM-GCA GTG GGT TCC CTA GTT AGC CAG AGA G-3IABkFQ3’). 56 HIV transcripts were quantified as copies/million cells. 19ul of the ddPCR SuperMix (LTR primers & RPP30 housekeeping gene primers and probes), and 6ul of cell lysates were mixed and aliquoted per well (96-well twin tec plate) and droplets generated with an AutoDG. Droplets were read using a plate reader, and the positive droplet threshold was manually set using the negative droplet control (media only).

Single-cell T-cell receptor (TCR) sequencing
We sequenced TCRs from single cells as previously published. 57 In brief, we index sorted single CD3+ CD14+ T cell-monocyte complexes into 96 well plates with 3µL of lysis buffer. We used primers with uniquely tagged barcodes with a unique molecular identifier (UMI) for reverse transcription. This allowed for multiplexing. cDNA was pooled and amplified using the KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), as per the manufacturer’s instructions. We quantified individual TCR gene expression using the UMIs 58-60. Nested PCRs were done targeting the TCR. 2µL of pooled cDNA was enriched for TCRab genes using the TSOend primer and the constant region primers, TCRA or TCRB. These products were purified and pooled. We created indexed libraries for sequencing using Truseq adapters and quantified using the Jetseq qPCR Library Quantification Kit (Meridian Biosciences Inc., OH, USA), as per the manufacturer’s instructions. We sequenced samples on an Illumina MiSeq using a 2×300bp paired-end chemistry kit (Illumina Inc., CA, USA). Sequencing reads were quality-filtered and demultiplexed to assign reads to individual wells and sequences mapped to the TCRA and TCRB loci and assigned TCR clonotypes using the MIXCR software. 61 Circos plots were generated using the visual genomics analysis studio (VGAS). 62
**Time-lapse imaging**

CD3⁺ CD14⁺ T cell-monocyte complexes were sorted as above and resuspended in RPMI with 10% fetal bovine serum (FBS). The cells were then plated on poly-L-Lysine pre-coated coverslips at a density of 15,000 - 40,000 complexes per 100 µl media. The cells on the coverslip were placed in a 24-well plate and time-lapse imaging was captured using an EVOS M5000 imaging system. Image J Version 1.53t 24 August 2022 was used for image analysis.

**Transmission electron microscopy**

CD3⁺ CD14⁺ T cell-monocyte complexes, CD3⁺ CD4⁺ T cells, and CD14⁺ monocytes were sorted as above and plated on poly-L-Lysine pre-coated coverslips. For day 3 samples, we added RPMI media supplemented with human IL-2 [10ng/mL]. For doublet imaging, the cells were plated on a poly-L-lysine coated coverslip for 1-2 hours. Then when the cells are bound to the coverslip, the media was aspirated and then the cells were fixed with 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer. After secondary fixation, samples were washed for five mins with 0.1 M sodium cacodylate buffer (7.3 pH). From there, we performed two washes of five mins with diH₂O to ensure the plates were cleaned. While keeping all solutions and plates at room temperature, the samples were incubated with 2.5% uranyl acetate, diluted with H₂O, at 4 °C overnight. Following this, samples were dehydrated using an ethanol gradient series. After dehydration, the ethanol was replaced with Eponate 12™ mixed in 100% ethanol in a 1:1 solution, then incubated at room temperature for 30 mins. This was repeated three times for 1 hour using 100% Eponate 12™.

The plates were finally placed in new media and cured in an oven at 70 °C overnight. The plates were cracked upon hardening, and the cells were separated by submerging the plate in liquid nitrogen. An 80 nm thickness jeweler’s saw was used to cut the block to fit in a Leica UC6 ultramicrotome sample holder. The section was placed on formvar-coated copper grids.
counterstained in 2% uranyl acetate for 2 mins. Then the grids were counterstained by Reynold’s lead citrate for two mins. Images were acquired by TEM on either a JEOL JEM-1230, operating at 120 kV, or a JEOL 1400, operating at 80 kV. 64

**Single-cell RNA sequencing**

We performed 10X sequencing on PBMCs from one prediabetic participant with a significant proportion of CD3+ CD14+ T cell-monocyte complexes as determined by flow cytometry. PBMCs were thawed and washed with PBS (no calcium or magnesium), and the pellet was resuspended in 100µL of cell staining buffer. The cells were incubated with Fc receptor-blocking solution (5 µL, TruStain). Cells were incubated on ice for 10 minutes. A Total Seq C master mix containing surface antibodies (CD3 clone UCHT1 #300479, CD4 clone SK3 #344651, CD8a clone SK1 #344753, CD14 clone 63D3 #367137, CD16 clone 3G8 #302065, CD69 clone FN50 #310951) was prepared. 3µL of the master mix was added to each sample, which was incubated on ice for 30 minutes. The cells were washed three times and resuspended in 0.04% BSA in PBS. We submitted twenty thousand cells that were loaded onto the Chromium Single Cell 5’ assay (10x Genomics). Cells were encapsulated and feature barcoded, with library preparation, cDNA amplification, and sequencing (50bp paired-end reads, on NovaSeq 6000 S2 platform). We aligned the raw sequencing reads to the human genome (hg38) using STAR v.2.7.2a. We identified cells using cell ranger (version 7.1). Downstream analysis was performed using the Seurat V4. 65 We filtered out cells with ≤ 200 genes, umi ≤ 400 or > 4000, and ≤ 10% mitochondrial genes expressed. We used DoubletFinder with default settings to identify heterotypic doublets. We then performed downstream analyses on the RNA count matrix including feature selection, scaling and centering counts, principal component analysis, Uniform Manifold Approximation, and Projection (UMAP) reduction, shared nearest neighbor graph construction, and finally cluster determination. Annotation was performed using cell-
specific gene markers. Doublets were identified by the expression of multiple cell lineages and confirmed by DoubletFinder classification.

Statistical analysis
Statistical analysis in this study was performed in Graph pad Prism version 9.5.0 and R version 4.2.1.

Data and code availability
Gene expression data from this study have been deposited in the NIH Gene Expression Omnibus (GEO) accession numbers: GSE229707 and GSE230276.

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Declaration of interests
The authors have no competing interests.


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