Video Article

A Simple Flow Cytometric Method to Measure Glucose Uptake and Glucose Transporter Expression for Monocyte Subpopulations in Whole Blood

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Abstract

Monocytes are innate immune cells that can be activated by pathogens and inflammation associated with certain chronic inflammatory diseases. Activation of monocytes induces effector functions and a concomitant shift from oxidative to glycolytic metabolism that is accompanied by increased glucose transporter expression. This increased glycolytic metabolism is also observed for trained immunity of monocytes, a form of innate immunological memory. Although *in vitro* protocols examining glucose transporter expression and glucose uptake by monocytes have been described, none have been examined by multi-parametric flow cytometry in whole blood. We describe a multi-parametric flow cytometric protocol for the measurement of fluorescent glucose analog 2-NBDG uptake in whole blood by total monocytes and the classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocyte subpopulations. This method can be used to examine glucose transporter expression and glucose uptake for total monocytes and monocyte subpopulations during homeostasis and inflammatory disease, and can be easily modified to examine glucose uptake for other leukocytes and leukocyte subpopulations within blood.

Video Link

The video component of this article can be found at https://www.jove.com/video/54255/

Introduction

Monocytes are a major component of the human innate immune system that are rapidly mobilized to sites of infection and inflammation[1]. Activation of monocytes is critical for limiting acute damage by pathogens and is also central to the pathogenesis of several chronic diseases, including atherosclerosis[2], cancer[3], and HIV[4,5].

The metabolism of resting and activated monocytes differs dramatically, with resting monocytes utilizing oxidative metabolism and activated monocytes utilizing glycolytic metabolism (*i.e.*, fermentation of glucose to lactate)[6]. Activation of monocytes induces expression of glucose transporters that allows for increased glucose uptake for glycolytic metabolism[7]. Monocyte glucose transporter 1 (Glut1) is one such transporter upregulated during activation and its expression has been shown to lead to production of pro-inflammatory cytokines in vitro and in adipose tissue of obese mice[8]. Infection of a monocytic cell line by Kaposi sarcoma associated herpesvirus leads to cellular upregulation of Glut1[9], and we recently showed that during chronic HIV infection an increased percentage of Glut1-expressing monocytes are present during untreated and combination antiretroviral therapy-treated infection[10]. Taken together, these studies show that glucose uptake and glycolytic metabolism by monocytes are important aspects of many inflammatory diseases. Thus, a simple method to measure monocyte Glut1 expression and glucose uptake during homeostasis and inflammatory disease is likely to be of use to a wide range of researchers.

Human monocytes are heterogeneous, being comprised of three distinct subsets that can be examined by differential expression of the cell surface markers CD14 and CD16[11,12]. Classical monocytes express a high level of CD14 but do not express CD16 ($CD14^{++}CD16^{-}$), intermediate monocytes express a high level of CD14 and an intermediate level of CD16 ($CD14^{++}CD16^{+}$), and non-classical monocytes express a low level of CD14 and a high level of CD16 ($CD14^{+}CD16^{++}$). Monocytes that express CD16 are termed $CD16^{+}$ monocytes, which compared to $CD16^{-}$ monocytes have high expression of inflammatory cytokines and the ability to more effectively present antigens[13,14]. Approximately 10% of monocytes express CD16 during homeostasis with higher percentages observed during inflammation[15]. Monocyte subpopulations are associated with certain disease states and could be useful biological markers of disease and disease progression[16].

Our goal was to identify a method that can measure glucose transporter expression and glucose uptake by human monocytes and monocyte subpopulations in conditions as close to physiological conditions as possible. Previous studies measured monocyte glucose transporter expression and glucose uptake[17,18], though these methods examined isolated monocytes that can have altered protein expression compared to physiological conditions[19], and no previous study has examined human monocyte subpopulations. Using multi-parametric flow cytometry, we describe a method to examine glucose transporter expression and uptake of the fluorescent glucose analog 2-NBDG by total monocytes and monocyte subpopulations (based on CD14 and CD16 expression) within whole unmanipulated blood.

Protocol

NOTE: HIV-infected and HIV-uninfected subjects were recruited from the Infectious Diseases Unit at The Alfred Hospital in Melbourne, VIC, Australia, and from the local community, respectively. Informed consent was obtained from all participants, and the research was approved by The Alfred Hospital Research and Ethics Committee.

1. Glut1 Cell Surface Detection on Monocytes and Monocyte Subpopulations

1. Collect blood in citrate ACD-B anticoagulant tubes and begin the experiments in a biological safety cabinet within 1 hr of collection.
2. Add 100 µl of blood to polypropylene tubes. Add 2 ml of 1x lysing solution (see **Materials Table**) to tubes while on ice, pipetting gently to mix. Incubate for 15 min on ice. Centrifuge at 220 x g for 5 min.
3. Decant and wash twice by adding approximately 2-4 ml of wash solution (0.5% BSA in 1x PBS) and centrifuging at 220 x g for 5 min.
4. Use a pipette to carefully remove as much of the wash solution as possible. Place tubes on ice and re-suspend in 100 µl of wash solution.
5. To identify specific monocyte subpopulations stain cells with the following volume of antibodies per 100 µl cell suspension prepared in step 1.4: 5 µl anti-CD3-PE, 5 µl anti-CD14-APC, 5 µl anti-CD16-PECy7, 5 µl Glut1-FITC or IgG2b-FITC (isotype control tube).
6. Place on ice for 30 min in the dark. Wash 2 times with wash solution. Fix with 200-300 µl of 0.5% formaldehyde made in 1x PBS.
7. Analyze on a flow cytometer capable of detecting 4 colors within 24 hr within the following excitation and emission wavelength: FITC (488, 530), PE (488, 575), PECy7 (488, 780), APC (633, 660)[10].

2. Glucose Uptake by Monocytes

1. Pipette 90 µl of blood collected in step 1.1 in polypropylene tubes. Add 10 µl of a 14.60 µM 2-NBDG working solution to the 90 µl of blood (1.46 mM final concentration) and flick gently to mix. It is critical to limit 2-NBDG exposure to light by covering tubes with aluminum foil.
2. Incubate at 37 °C in the dark for 15-30 min and then immediately place on ice. Add 4 ml of 1x FACS lysing solution to tubes while on ice. Centrifuge at 220 x g at 4 °C for 5 min.
3. Wash once by adding 4 ml of wash solution (0.5% BSA in 1x PBS). Centrifuge at 220 x g at 4 °C for 5 min. Decant and place on ice.
4. Stain cells with antibodies: 5 µl anti-CD3-PE, 5 µl anti-CD14-APC and 5 µl anti-CD16-PECy7. Mix and place on ice for 30 min in the dark.
   NOTE: During this period make sure the flow cytometer is ready for immediate analysis. Acquire cells within the following excitation and emission wavelength: 2-NBDG (488, 530), PE (488, 575), PECy7 (488, 780), APC (633, 660).
5. Add 4 ml of ice cold wash buffer (0.5% BSA in 1x PBS) to tubes. Wash once by centrifugation at 220 x g at 4 °C for 5 min. Decant and add 200-300 µl of ice old PBS and keep on ice in the dark (covered with aluminum foil). Analyze on a flow cytometer within 10 min using excitation and emission wavelength setting as in step 2.4.

3. Data Acquisition and Analysis

NOTE: A knowledge of flow cytometry and data analysis is assumed.

1. Using a flow cytometer capable of at least 4-color analysis, set compensation using unstained and individually stained samples.
   NOTE: Single staining using a FITC-labelled CD4 and CD14 can be used for Glut1 and 2-NBDG compensation.
2. Set up and label appropriate windows before acquiring samples. Draw a gate around the monocyte population, and acquire 100,000 to 300,000 events per sample at medium rate. 50,000 events per compensation sample is sufficient.
   NOTE: Compensation may be conducted prior to sample acquisition or in single cell analysis software, following standard procedures.
3. Export and save data into an appropriate location. Open up single cell analysis software such as FlowJo or other analysis software (**Supplemental Figure 1**) and drag and drop samples as specified (**Supplemental Figure 2**).
4. Double click to open file (**Supplemental Figure 3**). Draw a circle to gate monocytes based on forward and side scatter properties as shown in **Figure 1A** and **Supplemental Figure 4**. Double click the monocyte population. Observe and draw a box around the $CD3^-$ population (**Supplemental Figure 4**).
5. Double click the $CD3^-$ population. To observe the monocyte subpopulations select CD14-APC on the 'x' axis, and CD16-PECy7 on the 'y'-axis, and label accordingly (**Supplemental Figure 5**).
6. Where there are no distinct positive and negative populations, measure the expression of Glut1 or 2-NBDG uptake in the specific monocyte subpopulations. Determine the mean fluorescence intensities (MFI) of Glut1 and 2-NBDG by subtracting the isotype and no 2-NBDG background (**Supplemental Figure 6**).
7. Where defined populations exist, use the IgG2b-FITC to set the gate, and determine the percentage positive cells (**Figure 3**).
   NOTE: Use this procedure to analyze total $CD14^+$ monocytes. Since 2-NBDG uptake is usually marked by a shift in fluorescence intensities the data is best represented by MFI and histograms.

Representative Results

Compensation must be performed for individual fluorochromes to prevent fluorescence spillover. Monocytes are first enriched by gating based on forward and side scatter. The plots presented are representatives of at least six independent experiments conducted on whole blood from six or more participants as previously reported[10]. **Figure 1A** shows the initial gating of monocytes by cell scatter and exclusion of T cells by gating within the $CD3^-$ population. Monocytes are then gated for CD14 expression alone or in combination with CD16 to identify total monocytes or monocyte subpopulations as shown in **Figure 1B** and **Figure 1C**, respectively. For analysis of monocyte subpopulations, the following nomenclature should be applied as previously described[12]: classical monocytes ($CD14^{++}CD16^-$) should express approximately 100-fold greater CD14 MFI than the isotype control and CD16 MFI should be similar to the isotype control; intermediate monocytes ($CD14^{++}CD16^+$) should express approximately 100-fold greater CD14 MFI than the isotype control and approximately 10-fold greater CD16 MFI compared to the isotype control; non-classical monocytes ($CD14^+CD16^{++}$) should have similar MFI for CD14 and the isotype control and approximately 100-fold greater CD16 MFI than the isotype control. Cells without expression of CD14 and CD16 are not considered monocytes and should not be included in gating. Gated monocytes or monocyte subpopulations can then be examined for glucose transporter expression. As indicated in Figure 2, distinct populations of $CD14^+$ $Glut1^+$ monocytes may be observed, most notably in cells obtained from HIV+ individuals, where infection is characterized by a chronic state of inflammation. Similar but rare $CD14^+Glut1^+$ cells may be observed within specific monocyte subsets in HIV uninfected persons (**Figure 3A**), but are more pronounced in HIV+ individuals (**Figure 3B**). Noteworthy, in the absence of distinct populations, it may be appropriate to represent the results as mean or median florescence intensities, which takes into consideration the cumulative increase in Glut1 cell surface expression.

Glucose uptake can be assessed by comparing whole blood incubated with 2-NBDG or vehicle control for gated monocytes or monocyte subpopulations. We previously showed that approximately 50% of monocytes were 2-NBDG positive after a 15 min incubation[10]. This uptake level allows for detection of 2-NBDG without reaching saturation, when differences in monocyte 2-NBDG uptake may no longer exist. Analysis of 2-NBDG uptake by monocytes from HIV uninfected and HIV+ persons revealed a higher uptake by cells from HIV+ persons, which is in agreement with the Glut1 expression data (**Figure 4**-**5**). Overall, these results illustrate that the assays described here can be used to potentially study monocyte metabolic activities in biological contexts that elicit an inflammatory state such as diabetes, cardiovascular diseases, and viral and bacterial infections.

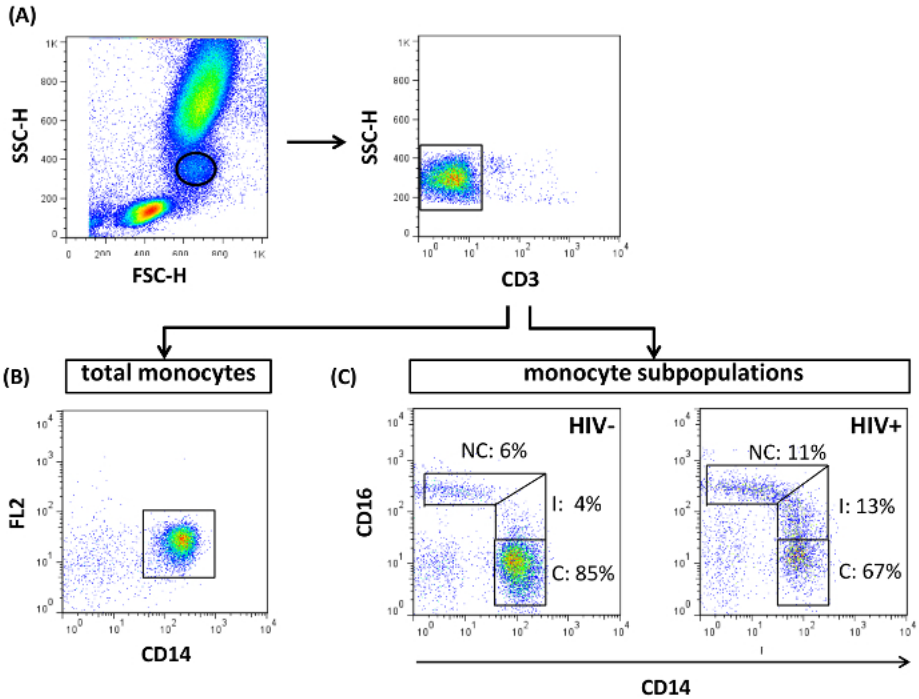


Figure 1: Gating strategy used to analyze total monocytes and monocyte subpopulations from representative HIV- and HIV+ blood samples. Samples of whole blood were analyzed by flow cytometry for monocyte cell surface Glut1 expression within 1 h of collection. (**A**) Cells were gated based on forward and side scatter characteristics and CD3 expression. (**B**) To examine total monocytes, $CD3^-$ cells were then gated for CD14 expression. (**C**) To examine monocyte subpopulations (classical, C; intermediate, I; and non-classical, NC) for HIV-uninfected persons and HIV-infected treatment naïve persons, CD3- cells were then gated based on expression of CD14 and CD16. Please click here to view a larger version of this figure.

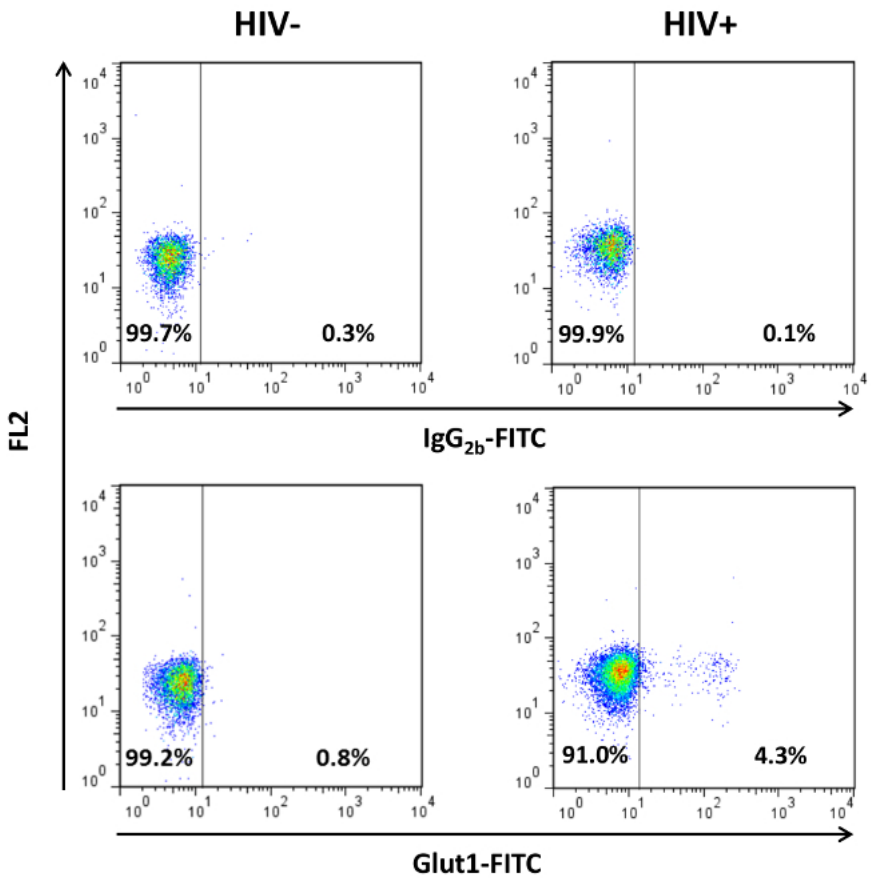


Figure 2: Analysis of cell surface Glut1 expression on total monocytes from representative HIV- and HIV+ blood samples. $CD14^+$ monocytes from HIV-uninfected or HIV-infected treatment naïve persons were stained with FITC-labeled isotype control or Glut1 antibody. Please click here to view a larger version of this figure.

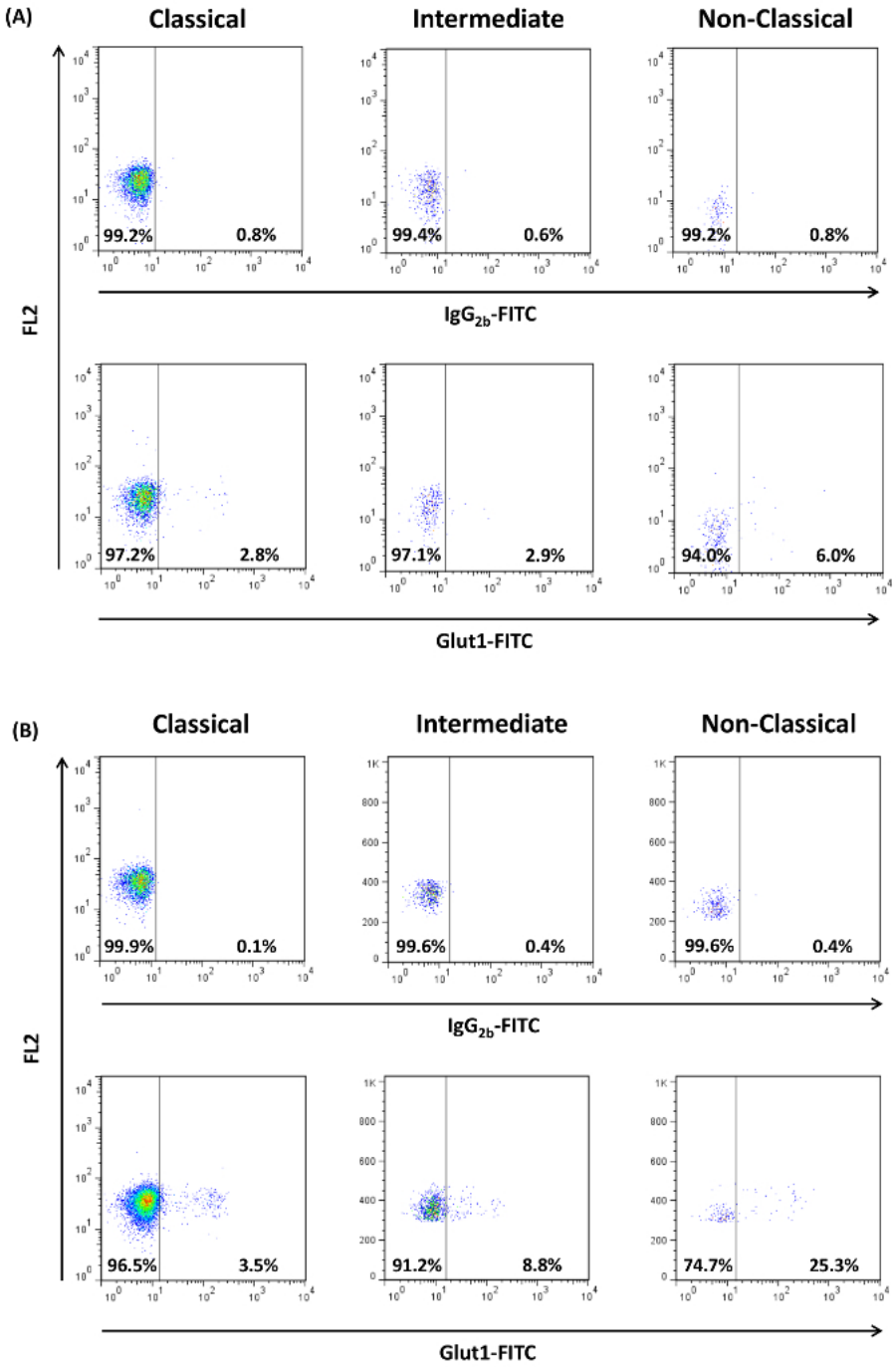


Figure 3: Analysis of cell surface Glut1 expression on monocyte subpopulations from representative HIV- and HIV+ blood samples. Monocyte subpopulations were stained with FITC-labeled isotype control or Glut1 antibody for (**A**) HIV-uninfected or (**B**) HIV-infected treatment naïve blood samples. Please click here to view a larger version of this figure.

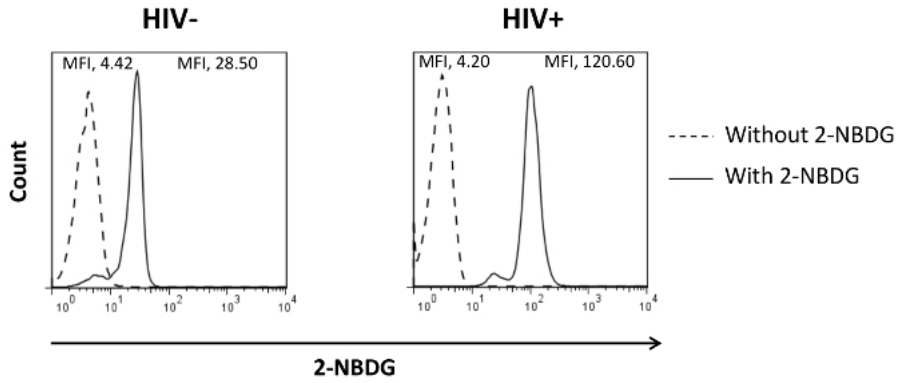


Figure 4: Uptake of 2-NBDG by total CD14^{+} monocytes from representative HIV- and HIV+ blood samples. Blood from HIV-uninfected or HIV-infected treatment naïve persons was incubated with vehicle or 2-NBDG at a final concentration of 1.46 µM for 15 min before washing and incubating with cell surface antibodies to gate monocytes as described in Figure 1. Please click here to view a larger version of this figure.

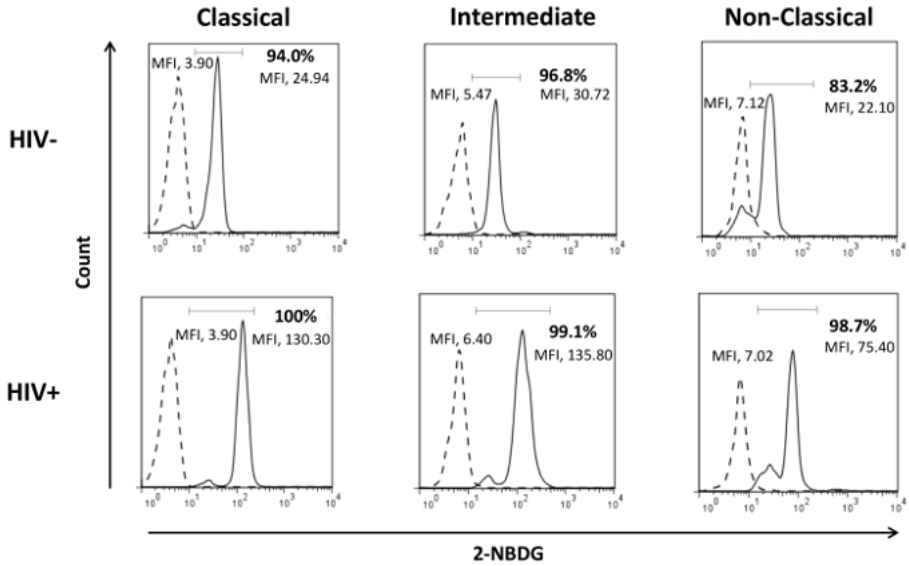
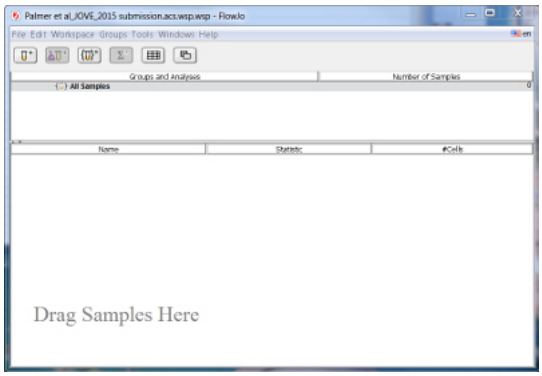
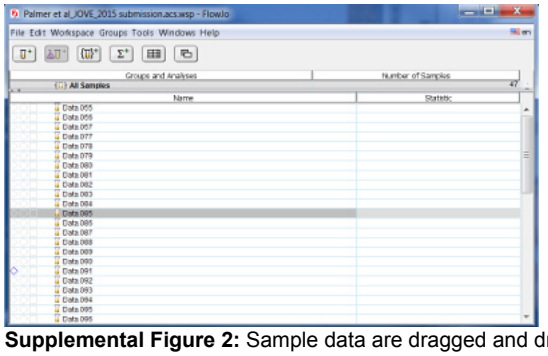


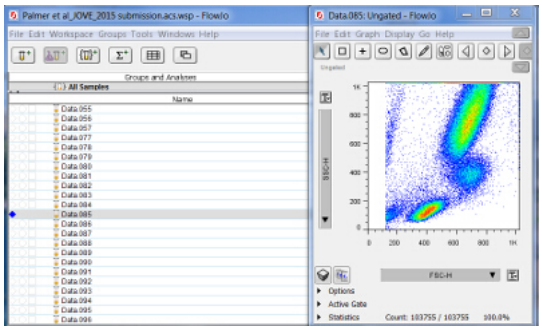
Figure 5: Uptake of 2-NBDG by monocyte subpopulations from representative HIV- and HIV+ whole blood samples. Blood from HIV-uninfected or HIV-infected treatment naïve persons was incubated with vehicle or 2-NBDG at a final concentration of 1.46 mM for 15 min before washing and incubating with cell surface antibodies to gate monocyte subpopulations as described in Figure 1. Please click here to view a larger version of this figure.



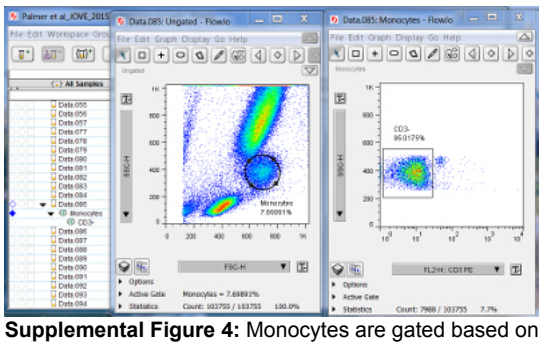
Supplemental Figure 1: Workspace window for flow cytometry cell analysis software. Please click here to view or download this supplementary figure.



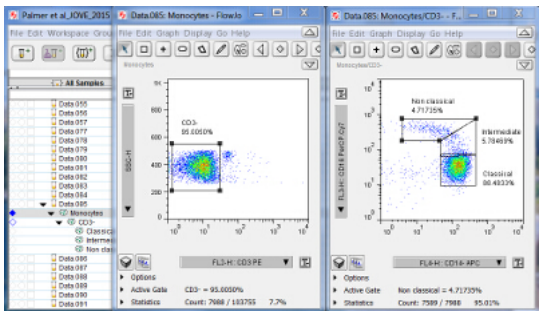
Supplemental Figure 2: Sample data are dragged and dropped into this workspace. Please click here to view or download this supplementary figure.



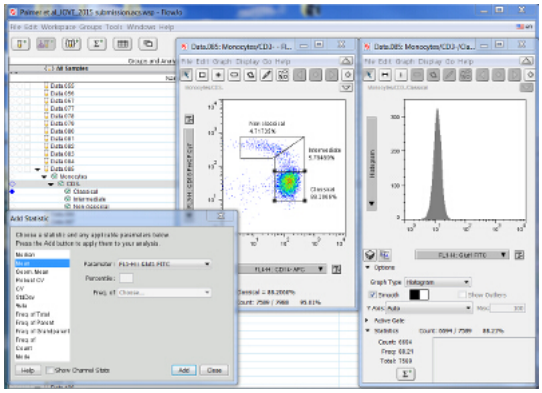
Supplemental Figure 3: Samples in workspace, data 085 is double clicked, and a second window appeared showing the three major cell populations (lymphocytes, monocytes and neutrophils) within the fresh whole blood sample based on forward (FSC) and side scatter (SSC). Please click here to view or download this supplementary figure.



Supplemental Figure 4: Monocytes are gated based on forward (FSC) and side scatter (SSC). The population was double clicked which brought up a new window. CD3 is selected on the 'x' axis and CD3-negative population (gating out lymphocytes) was selected. Please click here to view or download this supplementary figure.



Supplemental Figure 5: The CD3$^-$ monocyte population was double clicked which brought up a new window where monocyte subpopulation could be defined based on CD16 and CD14 expression. Please click here to view or download this supplementary figure.



Supplemental Figure 6: Monocyte subpopulations are selected, and Glut1 cell surface expression (mean fluorescence intensity: MFI) is obtained by selecting 'statistics' on the histogram window, 'mean' from the 'add statistics' window, and selecting Glut1 from the 'parameter' drop down menu. Please click here to view or download this supplementary figure.

Discussion

The protocol described here details a simple method to examine glucose transporter expression and fluorescent glucose analog uptake by monocyte and monocyte subpopulations in whole blood. By assessing 2-NBDG uptake in whole blood, this technique allows for conditions similar to those *in vivo*. A previous study examined 6-NBDG uptake in monocytes separated from whole blood by density centrifugation[17]. However, this study did not examine monocyte subpopulations and separation of monocytes from whole blood can potentially alter expression of certain cell surface molecules[19]. Radioactive glucose tracers have also been used to measure monocyte uptake of glucose[20,21], but monocytes must be previously isolated for this method and usage of radioactivity requires significant safety precautions. Our protocol uses routine biosafety procedures and is minimally manipulative, thus allowing for the flow cytometric measurement of 2-NBDG uptake by monocytes and monocyte subpopulations mimicking in vivo conditions.

2-NBDG enters the glycolytic pathway and has been shown to be metabolized by cells into non-fluorescent molecules[22]. Therefore, it is critical to limit metabolism after 2-NBDG incubation by keeping cells chilled at 4 °C. 6-NBDG is another fluorescent glucose analog that can be used but it is less useful as it does not enter the glycolytic pathway and therefore does not accurately reflect the bioenergetic status of the cells[23].

If fluorochromes with overlapping spectra are utilized, compensation becomes critical to prevent fluorochrome spillover. In this protocol we use monocytes stained individually with CD14 and CD16 to set compensation parameters, but compensation of cell surface markers can also be performed using compensation beads.

Granulocytes can express CD16 but can be excluded by gating out CD15-expressing cells. However, stringent gating of monocytes based on light scatter properties can limit the number of granulocytes included in the analysis. If a flow cytometer is a available with more channels, the granulocyte marker CD66b can also be used to exclude granulocytes from the analysis.

The Glut1 antibody used in this study binds to a cell surface epitope and therefore does not bind to intracellular Glut1. An antibody that binds to an intracellular Glut1 epitope can be used to measure total Glut1 monocyte expression, but cells must be permeabilized before staining[10]. In addition to monocytes, this technique can be used to examine glucose uptake and metabolism in other leukocytes found within blood. We have extensively examined T cell uptake of 2-NBDG using the method described here, and have also examined 2-NBDG uptake by NK cells[24]. For successful detection of Glut1 expression and 2-NBDG uptake it is imperative to limit traces of the red blood cell lysis buffer by washing cells with excess wash buffer according to the protocol, and we found that FITC or APC-labeled Glut1 antibody give better signals than PE or PerCP conjugates. We have not investigated the reasons for this.

Since cells are metabolically active even at low temperatures, it is critical that following the 2-NBDG incubation at 37 °C, that tubes are kept directly on ice and centrifugation conducted at 4 °C. In the absence strong 2-NBDG signal, check that the correct concentration is being used, reduce light exposure in the room and Biosafety cabinet and cover tubes with foil when appropriate. Optimization may be required by setting up a time course 2-NBDG uptake experiment for 5, 15, 20, 30, 60, and 90 min. Typically, optimal time should be 10-60 min, depending on cell types and their activation status.

A major limitation with the 2-NBDG uptake assay is light sensitivity together with the fact that it is being utilized by the cells. Thus it is important to limit the number of samples to ensure that the last sample is analyzed within 30 min of the first one. A biological limitation is that, even though the frequency of Glut1-expressing nonclassical monocytes, and Glut1 expression on nonclassical monocytes were significantly greater than classical monocytes, no differences existed in 2-NBDG uptake between the two subpopulations[10]. This raises the possibility that other Gluts, such as Glut3 and Glut4 expressed on monocytes may be involved in glucose metabolism in different disease settings. It is also possible that the activity of Glut1 may also be regulated post translationally.

A major advantage of our flow cytometric glucose uptake protocol over radioisotope labelling is the ability to combine the technique with immunophenotypic analysis to identify and study specific subpopulations of immune cells in small volume of blood. In addition the APC-conjugated anti-Glut1 may be applied to simultaneously analyze Glut1 cell-surface expression and 2-NBDG uptake. A change in Glut1 expression due to 2-NBDG uptake has not been previously demonstrated, but this possibility cannot be ruled out.

Increased glucose uptake and metabolism by immune cells is a hallmark of activated T cells and monocytes[25-27]. These cells may be activated in response to pathogen infection[28,29], and inflammatory signals in conditions such as autoimmune diseases like lupus[30-32], and obesity and

diabetes[8,33]. Increased glucose metabolism is also required for cancer cell survival, growth and metastasis[34]. Notably, metabolic dysregulation in immune cells has emerged as a hallmark of HIV infection, and is associated with immune activation[24], inflammation[10], and infectivity of $CD4^+$ T cells[35-37]. Therefore, this method will be of interest to a diverse audience including those with an interest in inflammatory mediated diseases, cancer, infectious diseases, immunology and immunometabolism[38].

Disclosures

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTION:

C.S.P conceived the project, designed and conducted experiments, analyzed and interpreted data, and wrote the manuscript. J.J.A interpreted data and wrote the manuscript. T.R.B wrote the manuscript. J.M.M interpreted data, made critical intellectual suggestions, and reviewed the manuscript. S.M.C interpreted data, made critical intellectual suggestions and reviewed the manuscript.

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