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Pathophysiology of Adipose Tissue Macrophages indicating a potential reservoir site for HIV infection that might be troublesome to eradicate HIV-1

*N Ahmed[1]

Abstract

Human Immunodeficiency Virus Type 1 (HIV-1) is a condition that affects over 60 million people worldwide. Viral infection can be controlled using highly active antiretroviral therapy (HAART) to suppress HIV-1 replication and hinder disease progression, but viremia quickly rebounds following HAART interruption. It is highly likely that the persistence of virus occurs predominantly in latently infected CD4+ T cells, a new strategy to eradicate HIV-1. Previously it has been shown that the viral reservoir is seeded rapidly following the initial infection of rhesus macaques with SIV, even before the establishment of a systemic infection, so destroying this reservoir may be vital to the effective clearance of HIV. We reasoned that long-lived tissue macrophages contribute significantly to the SIV/HIV reservoir, such as those that may be found in adipose tissue.

Keywords: Adipose tissue, Macrophages, HIV-1

Introduction

It is currently understood that CD4+ T lymphocytes and the monocyte/macrophage lineage serve as a viral reservoir.[1,4,5] It has been published that monocyte/macrophages are important in the pathogenesis of AIDS and have suggested that the dynamic changes of this lineage could provide a new marker for AIDS progression.[4-6] Recently some scientists utilized lung tissue showing that increased monocyte turnover and apoptosis of CD163+ interstitial macrophages can be used as an indicator of AIDS progression.[7,8] In the lung tissue, interstitial macrophages (IM) originate from the blood monocytes and have a short life span. Alveolar macrophages (AM) have a slower turnover rate and appear to be longer-lived cells in the lung during steady -state homeostasis.[8] We were able to track monocyte turnover using BrdU, a molecule that is taken into cells undergoing S-phase, so only actively dividing cells were labeled.[9] In order to track long-lived cells, we used dextran, a polysaccharide that persists in cells for long periods to identify long-lived cells.[10] Our lab also detected SIV DNA in alveolar macrophages implying long-lived AM could serve as a virus reservoir.[8] More research needs to be done to identify the phenotype and function of long-lived macrophages as well as various reservoir sites in HIV-1 infected individuals under HAART therapy.

HIV-1 alters adipose tissue biology by interfering in many pathways, including mechanisms using PPARγ, a transcription factor for adipose differentiation. HIV-1 infection-mediated interference of PPARγ-dependent pathways in adipose tissue, affecting macrophages, likely alters the local environment and leads to lipodystrophy in patients after ART therapy.[11] In patients with HIV-associated lipodystrophy, researchers have found increased pro-inflammatory cytokines and increased macrophage numbers in the subcutaneous adipose tissue, indicating that adipose tissue macrophages play a role in HIV-1 and AIDS pathogenesis.[12-15] Using the NHP model, our group is characterizing and phenotyping adipose tissue macrophages that might have an important role in AIDS pathogenesis. The long-term goal is to identify all tissue reservoir sites present during HAART and prevent the development of these reservoirs that arise when treatment is present so that patients may discontinue HAART, which is currently impossible.

Materials and methods

In our lab, we maintained different groups of both

1. Dr. Nursarat Ahmed, Associate Professor & Head, Department of Microbiology, Gazi Medical College, Khulna, Bangladesh.
Email: monipakhi@yahoo.com

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SIV-infected and uninfected rhesus macaques. When necropsies were performed, we used to collect many tissues including blood, lung, liver, lymph nodes, spleen, muscle, bone marrow, gut, and adipose tissue. The three types of adipose tissue used to isolate mononuclear cells were brown adipose tissue, mesenteric adipose tissue, and surface adipose tissue.

Confocal Staining

A portion of the adipose tissue was sent to the histopathology lab after fixation with Z-fix to make paraffin-embedded tissue slides. Many of these were kept for future study. Several tissue slides were prepared by the histopathology lab that were 0.7 µm in thickness. We removed the paraffin from several slides and stained them with different antigen surface markers so that the cell populations within the fat tissue, including macrophages, could be identified. We used Hematoxylin and Eosin (H&E) staining, as well as antibodies for CD206 and CD163 to label macrophages and Yo-pro to label cell nuclei. After the slides were stained, they were photographed using a Nikon Fluorescence microscope.

Single cell isolation from Adipose tissue

Three types of adipose tissue were separated and washed with PBS to remove blood, hair, and other debris from the tissues following necropsy. The tissues were then cut into small pieces and digested using type-II collagenase to break up the collagen that provided structure to the tissue. The fat tissue was incubated at 37°C in the shaker for 45 minutes and mixed every 10 minutes to ensure that the collagen was properly broken down. After incubation, the samples were centrifuged for 5 minutes to separate the yellow fat tissue from the mononuclear cells. The yellow fat was aspirated using a transfer column and the remaining sample was re-suspended in 10 ml of R-10 media, filtered, collected to a new 50 mL tube, and kept on ice. Any residual tissue that had not been digested had collagenase added to it for 30 minutes to break it down and the procedure was repeated. Once the sample had been re-suspended in R-10 media and filtered, the two mononuclear cell preparations were combined. The mononuclear cell sample was washed and re-suspended in 10 mL of R-10 media and the cells were counted using a light microscope.

Staining Method

An aliquot of isolated cells containing 2×106 cells was collected and used for flow cytometry. The cells were washed with PBS-2%FCS, mixed with a surface antibody cocktail, vortexed, and incubated at room temperature for 20 minutes in the dark. After incubation,1 mL of BD lysis solution was added and incubated at room temperature for 10 minutes in the dark. Cells were washed with PBS-2%FCS to remove all unbound antibodies and centrifuged for 5 minutes at 1,700 rpm. The supernatant was aspirated and the pellet was re-suspended in 0.25 ml of 1% paraformaldehyde. The stained and fixed cells were transferred through a filter tube and spun for 1 minute. Labeling of the samples was completed and they were brought to the Flow refrigerator for processing. The surface antibody cocktail included antibodies to detect lymphocytes and monocytes/macrophages including CD45, CD163, CD14, CD4, HLA-DR, CD206, CD11b, CD3-20, and CD8.

Results

Hematoxylin and eosin (H&E) are one of the principal stains in histology. To understand the basic histology of Mesenteric fat we stained our slide with H&E, shown below in Figure 01. H&E stain causes cell nuclei to take on a deep blue or purple color while structures that are composed of proteins take on a red or pink color. The adipocytes appeared as largely white masses surrounded by pink membranes of loose connective tissue fibers. In between adipocytes, there were smaller cells of unknown identity where macrophages and other immune cells may localize. Adipocytes are large cells, filled with a single large vacuole containing fat, appearing as a white circle, with one nuclear in each, as shown in Figure 01.

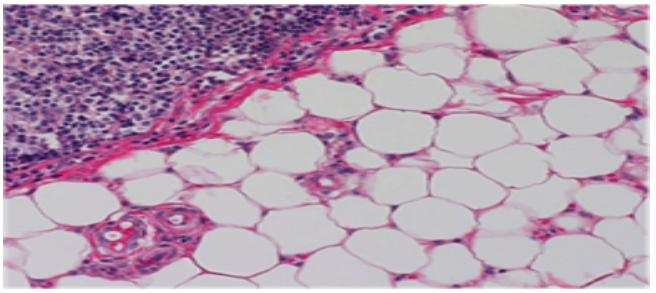

Figure 01: HE staining of mesenteric fat tissue

Mesenteric fat was stained with a rabbit antibody for CD206 (1:50 dilution) and a secondary antibody that was goat anti-rabbit Alexa-488 (1:1000 dilution). The tissue was visualized using a light microscope and color pictures were taken using a Nikon camera. The pictures are shown below in Figure 02, the bright green dotted area is positive for CD206 indicating CD206+ macrophages between the adipocytes. Both images

are from the same sample, but under different light conditions.

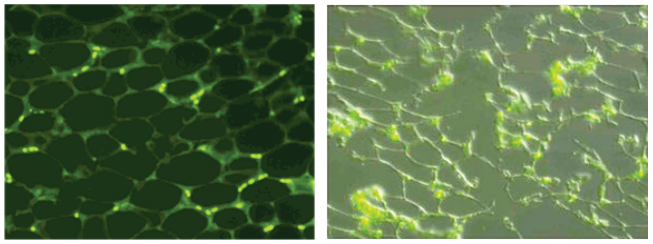

Figure 02: CD206 staining of mesenteric fat

Mesenteric fat was stained with a rabbit antibody for CD206 (1:50 dilution) and a secondary antibody that was goat anti-rabbit Alexa fluor 568 (1:1000 dilution). Mesenteric fat was also stained with Yo-Pro to label cell nuclei. The tissue was visualized using a light microscope and color pictures were taken using a Nikon camera. The pictures are shown below in Figure 03. The CD206 cell surface antigen would label the outside of the cell, shown using red fluorescence, while the Yo-Pro would label the cell nuclei inside the cell, shown using green fluorescence. To the left, each marker is shown alone. To the right, an overlay of both fluorescent markers is shown to demonstrate regions of co-localization. As indicated by the white arrows, there are several locations where there is co-localization of the markers, so these regions are positive for CD206+ macrophages.

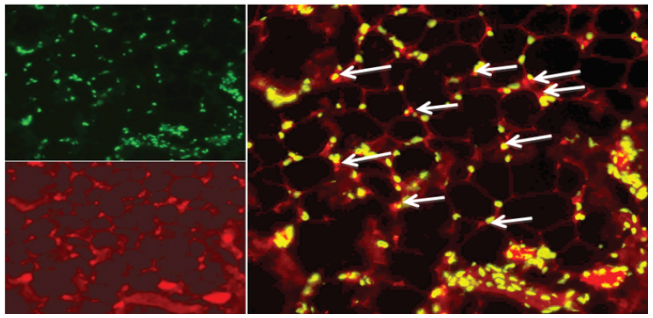

Figure 03: CD206 and Yo-Pro staining of mesenteric fat

Mesenteric fat was stained with a rabbit antibody for CD163 (1:20 dilution) and a secondary antibody that was goat anti-rabbit AL-568 (1:1000 dilution). Mesenteric fat was also stained with Yo-Pro to label cell nuclei. The tissue was visualized using a light microscope and color pictures were taken using a Nikon camera. The pictures are shown below in Figure 04. The CD163 would label the outside of the cell, shown using red fluorescence, while the Yo-Pro would label the cell nuclei inside the cell, shown using green fluorescence. To the left, each marker is shown alone. To the right, an overlay of both fluorescent markers is shown to show regions of co-localization. As indicated by the white arrows, several locations are positive for CD163 indicating the presence of CD163+ macrophages in mesenteric fat in between the adipocytes.

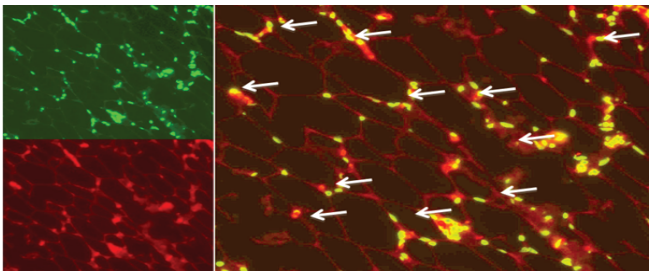

Figure 04: CD163 and Yo-Pro staining of mesenteric fat

The mesenteric fat that was collected was attached to mesenteric lymph nodes on the same cross-sections, so the lymph nodes were also analyzed for macrophage and nuclei co-localization. Below in Figure 05, there are images collected of macrophages found in the lymph node, with cell nuclei shown using green fluorescence and CD163 shown using red fluorescence. The CD163-staining (in red) and Yo-Pro/nuclei staining (in green) are shown to the right of Figure 05 and the overlay is shown to the left. As in Figures 03 and 04, there are many CD163+ macrophages present in the lymph node, as evidenced by co-localization of the nuclei and macrophage markers for CD163, indicated by white arrows.

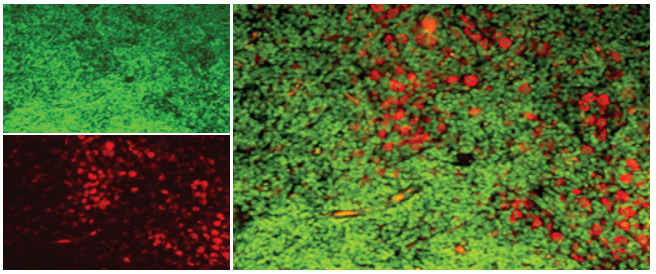

Figure 05: CD163 and Yo-Pro staining of lymph node in mesenteric fat

The sample of mesenteric fat was prepared for analysis by flow cytometry as well as using immunological staining. The mononuclear cells were extracted from mesenteric fat and stained with antibodies specific for HLA-DR, CD3-20, CD11b, CD206, and CD163. These markers were used to identify the different types of mononuclear cells in the sample so that the presence

of macrophages, monocytes, and T-cells could be identified. In Panel A, singlet cells were gated regarding FSC-A and FSC-H indicating all the cellular aggregates in the sample. In Panel B, all the lymphocyte populations were separated from monocytes/macrophages based on CD3/CD20 and HLA-DR. CD3-20 is a marker for lymphocytes, T cells, and B cells, so all the cells that were CD3-20 negative but HLA-DR+ were selected and further analyzed. Previously it was reported that all the tissue macrophages that were either long-lived or short-lived cells are HLA-DR+ and CD11b+. So, In Panel C, All the tissue macrophage populations were separated from HLA-DR negative fractions based on HLA-DR+ and CD11b+. All the cells that were HLA-DR+ and CD11b+ were selected and further analyzed in panel D. Each kind of macrophage population, either long-lived or short-lived, was separated from each other based on two surface antigens, CD206 and CD163. Quadrant 1 shows only CD206+ cells, quadrant 2 shows both CD163+ and CD206+ cells, and quadrant 3 shows only CD163+ cells. Mesenteric fat contains different kinds of macrophage populations where double positive CD206+ CD163+ cell frequencies are very high in mesenteric fat in comparison to other cell types.

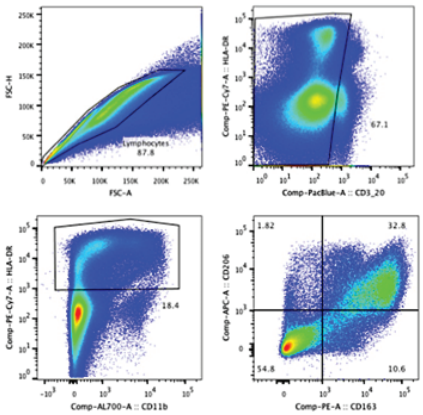

Figure 06: Flow cytometry – mesenteric fat

Flow cytometry was also performed on a sample of brown fat and subcutaneous fat. The same procedure was followed as was used to analyze Figure 06 to obtain a panel where macrophages were separated from each other based on two surface antigens, CD206 and CD163. The brown fat is shown to the left of Figure 07, and the subcutaneous fat is shown to the right of Figure 07. As shown in Figure 07, double-positive CD163+CD206+ cells frequencies are low in brown fat but in subcutaneous fat, the frequency is similar to mesenteric fat.

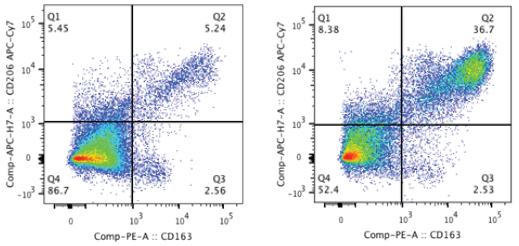

Figure 07: Flow cytometry of brown fat (left) and subcutaneous fat (right)

Conclusion

The overall structure of mesenteric adipose tissue was observed in a cross-section on a paraffinized slide stained with H&E so that cell nuclei could be identified. Mesenteric adipose tissue and adjacent mesenteric lymph node in the same histology slide attached with mesenteric fat was stained with Yo-pro and antibodies for macrophage markers CD206 and CD163, as shown in Figures 03, 04, and 05. There was co-localization of Yo-pro, CD206 and CD163, indicating the presence of CD206+ and CD163+ macrophages in mesenteric adipose tissue and adjacent mesenteric Lymph nodes. The flow cytometry data in Figures 06 and 07 show different macrophage populations in fat tissue those are CD206+CD163+ (double-positive) macrophages, and few cell populations are CD163+ (single-positive) macrophages.

We worked on adipose tissue and tried to understand the phenotype and characterization of long-lived and short-lived adipose tissue macrophages, which might have an important role in AIDS pathogenesis. We also compared the frequencies of long-lived and short-lived macrophages in different groups (either infected or uninfected monkey models) that might change during infection. We traced all the body's long-lived and short-lived macrophages by using dextran. The short-lived cells will phagocyte dextran and die soon but long-lived cells still have dextran for a longer period. By tracing dextran in long-lived macrophages, we tried to determine if adipose tissue can serve as a reservoir site for HIV/SIV in primates undergoing HAART and whether we can deplete long-lived reservoir cells from adipose tissue and other sites of the body then it might be possible for an infected patient to discontinue HAART. BrdU was also used in this study as a marker for actively dividing cells in the tissues with short-lived macrophages and for blood monocytes. Long-lived cells will not uptake BrdU, so they will only take in dextran. Our long-term goal is to identify all the tissue reservoir sites that harbor large amounts of viruses under anti

retroviral therapy and to eliminate all the long-lived macrophages from the reservoir sites to successfully control HIV infection in patients.

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