Restoring Cytokine Balance in HIV-Positive Individuals with Low CD4 T Cell Counts

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Abstract

HIV infects and destroys CD4+ T cells leading to a compromised immune system. In a double-blinded study, a group of HIV-infected individuals with CD4+ T cell counts below 350 cells/mm3 were given either an empty liposomal supplement or a liposomal glutathione (L-GSH) supplement to take over a 3-month period. Baseline measurements in HIV-positive subjects show a significant decrease in levels of interleukin (IL)-12, IL-2, and interferon (IFN)-γ, along with a substantial increase in the levels of IL-6, IL-10, transforming growth factor (TGF)-β, and free radicals, compared to healthy individuals. Supplementation of HIV-positive subjects with L-GSH for 3 months resulted in a notable increase in the levels of IL-12, IL-2, and IFN-γ, with a concomitant decrease in the levels of IL-6, IL-10, and free radicals, and stabilization in the levels of TGF-β, IL-1, and IL-17, compared to their placebo counterparts. Levels of free radicals in CD4+ T cells stabilized, while GSH levels increased in the treatment group. Those in the placebo group showed no significant difference throughout the study. In summary, supplementation with L-GSH in HIV-infected individuals with CD4+ T cell counts below 350 cells/mm3 can help restore redox homeostasis and cytokine balance, therefore aiding the immune system to control opportunistic infections.

Keywords: HIV, AIDS, redox homeostasis, cytokines, glutathione

Introduction

Currently, there are 36.9 million people living with HIV.1 The majority of the people living with HIV are in low- to middle-income countries, particularly in sub-Saharan Africa.1 HIV, a retrovirus, preferentially infects and destroys CD4+ T lymphocytes resulting in the loss of cell-mediated immunity. The CD4 T cell counts in the peripheral blood of healthy individuals range between 500 and 1,500 cells/mm3 of blood. However, the CD4 T cell counts in individuals with acquired immunodeficiency syndrome (AIDS) are lower than 200 cells/mm3 of blood.3 Loss of CD4 T cells leads to the impairment in the immune functions resulting in immunodeficiency and a high probability that the host will succumb to opportunistic infections.4

Effector CD4+ T cells are classified into various subtypes based on the type of cytokines that they produce such as T helper type-1 (Th1), Th2, and Th17.2–9 Levels of pro- and anti-inflammatory cytokines in the peripheral blood can serve as an indicator of the nature of immune responses.3 Infection with intracellular pathogens often tailor the immune response toward a Th1 pathway, resulting in the production of interleukin (IL)-2 (T cell growth factor) and interferon (IFN)-γ (enhances the effector responses against intracellular pathogens).4 IL-12, secreted by dendritic cells, is required to activate the Th1 pathway.3 On the contrary, IL-4 and IL-10 promote the differentiation of naïve CD4 T cells to Th2 subtypes, which then secrete IL-4, IL-5, and IL-10. These cytokines mediate antibody production.5 Th17 cells play a central role in promoting tissue inflammation and activation of neutrophils to combat infections.5

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IL-6 is important for differentiation of T<sub>H</sub>17 subsets, which then secrete IL-17, IL-6, and tumor necrosis factor (TNF)-α. These cytokines can cause oxidative stress and recruit granulocytes leading to tissue inflammation. Conversely, regulatory T cells (Treg) secrete IL-10 and transforming growth factor (TGF)-β, which can suppress effector mechanisms in macrophages and T cells.

Glutathione (GSH), a tripeptide antioxidant, is important for the maintenance of the cellular redox state and cellular homeostasis. Low levels of GSH have been shown to play an important role in the apoptosis of CD4+ T cells.

Studies have shown that diminished levels of GSH in individuals with HIV infection are, in part, due to the decrease in the production of GSH de novo synthesis enzymes. TGF-β has been shown to downregulate the expression of the enzyme, glutamate–cysteine ligase (rate-limiting step enzyme involved in the synthesis of GSH), leading to decreased production of GSH. Diminished levels of GSH in turn can increase the production of free radicals. This phenomenon occurs because of the increased levels of proinflammatory cytokines. Enhanced levels of reactive oxygen species (ROS) and oxidative stress thus increase with decreased levels of GSH.

Studies have also demonstrated that deficient levels of GSH in individuals with HIV infection can impair the production of cytokines thereby altering immune responses against Mycobacterium tuberculosis (Mtb) infection. Conversely, increased levels of TGF-β have been shown in studies to decrease levels of GSH. Our laboratory has previously reported that supplementing HIV-positive subjects with GSH reduced the levels of TGF-β and IL-10 (anti-inflammatory cytokines), as well as the proinflammatory cytokines, IL-6.

Currently, 18.2 million people were receiving antiretroviral treatment (ART) worldwide. In addition to coinfections, serious non-AIDS events cause substantial disease and death despite human HIV suppression with ART. Elevated levels of inflammatory markers that persist in individuals with HIV infection despite virological control via ART can thus lead to increased mortality risk. This persistent immune activation in patients with controlled viremia is associated with inappropriate immune cell trafficking and activation, tissue damage, and dysplasia. We therefore determined in our double-blinded clinical trial whether liposomal GSH (L-GSH) can restore cytokine balance and redox homeostasis in a subset of 30 HIV-positive individuals who had CD4 T cells below 350/mm<sup>3</sup> in blood and experienced no rise in their CD4 T cell counts in the last 9–12 months despite being on ART. Antiretroviral therapy initiation during acute HIV infection would attenuate changes in these biomarker levels.

Our results indicate that supplementing HIV-positive individuals who have low CD4 T cell counts (and have a greater risk for opportunistic infections) with L-GSH for a 3-month period of time resulted in modulation in the levels of both pro- and anti-inflammatory cytokines, restoration of T<sub>H</sub>1 cytokines and redox homeostasis, and this may protect these high-risk groups from susceptibility to opportunistic infections.

**Materials and Methods**

**Subject recruitment**

A double-blinded clinical trial was conducted in HIV-positive subjects with CD4+ T cell counts below 350 cells/mm<sup>3</sup>. Participants were recruited after final Institutional Review Board approval by Western University of Health Sciences and the Riverside University Health Systems Medical Center. The sample size included 30 participants between the ages of 30 and 65 years, without any preference for race, ethnicity, or gender. Inclusion criteria required for participants to have a documented diagnosis of HIV-1 infection by any equivalent test to a licensed enzyme-linked immunosorbent assay (ELISA) test kit and confirmation by western blot. HIV-1 culture, HIV-1 antigen, plasma HIV-1 RNA, or second antibody tests by a method other than ELISA were considered to be an acceptable confirmatory test. Participants were required to have a CD4 count lower than 350 and a poor CD4 rise over the last two CD4 counts within 9–12 months. Poor CD4 rise was defined as an increase of absolute CD4 count of <50 between the first and last CD4 of the last 9–12 months before the study. Participants were required to have their latest HIV RNA counts to be <200 copies within the last 6 months as well as not having a change in the participant’s antiretroviral regimen within the last 6 months. Exclusion criteria included any participant who was currently taking or had taken L-GSH within the last 6 months, was allergic to L-GSH and/or soy, who has had chemotherapy within the last year, and who was currently pregnant, lactating, or had been pregnant within the last 6 months; pregnancy was considered a reason for study termination. Participants with severe illness, in the opinion of the HIV specialist, including compromised liver function, mental illness, or substance abuse that could prevent adherence to visits and treatment were also excluded from the study. Change in antiretroviral regimen during the study was considered a reason for study termination (Table 1).

Thirty HIV subjects (15 individuals with CD4 count of below 200 cells/mm<sup>3</sup> and 15 individuals with CD4 counts of 200–350 cells/mm<sup>3</sup>) were recruited from two Riverside County Public Health Department HIV clinics (Table 1). Within each group of CD4+ T cell counts, HIV-positive patients were further divided into a placebo and treatment group. Those who received a placebo took an empty liposome supplement. Those who received the L-GSH took a liposome filled with GSH supplementation. Both supplements were supplied by ReadiSorb with no variation in labeling. Both placebo and L-GSH groups were instructed to take one and half teaspoons of their respective supplement, once in the morning and once in the evening after ramping up the dose over 7 days. The amount of reduced GSH for those who received the L-GSH supplementation was about 1,260 mg. The patients were instructed to take the supplement for 3 months, where they returned for a final blood draw of 40 ml of blood (Table 1).

A total of 17 healthy participants (non-HIV) were also recruited as additional controls for measurement of baseline levels of cytokines, free radicals, and GSH. Healthy volunteers neither consumed placebo nor L-GSH. Healthy subjects were recruited from Western University of Health Sciences. After signing a consent form, 40 ml of blood was drawn from each participant on each of two visits, one before treatment and one at 3 months post-treatment (Table 1).

**Liposomal glutathione**

L-GSH (ReadiSorb GSH) and placebo were provided by Dr. Guilford (Your Energy Systems, Palo Alto, CA). L-GSH...
is made up of reduced GSH, purified water, lecithin, glycerin, and potassium sorbate. The liposomes in ReadiSorb GSH are derived from lecithin, which is an extract of non-GMO soy oil and suspended in a liquid solution. Glycerin is used to support the stability of liposomes and allows for an extended shelf life. Potassium sorbate is used as a preservative to prevent yeast and mold growth. Liposomes avoid the digestive system by penetrating the mucosal membranes of the mouth and gastrointestinal tract. Since there is no acid secretion in the mucosal cells of the oral cavity, the L-GSH does not get destroyed. Liposomes are able to penetrate the mucosal tissues because they are made up of phospholipids just like the cell membrane. This allows for rapid release into the blood stream.

**Isolation of plasma, red blood cells, and monocytes**

Plasma, red blood cells (RBCs), and peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of HIV-positive subjects and healthy individuals by density centrifugation. This procedure involves centrifugation of blood layered on Ficoll-Paque at a 1:1 ratio at 600 g for 30 min. Plasma (the top layer) was collected and stored at −80°C, while PBMCs (the second layer) were further washed three times with 1× phosphate-buffered saline and then resuspended in RPMI containing l-glutamine and 5% human AB serum. This solution was plated on a 96-well tissue culture plate, precoated with 0.005% poly-l-lysine, and incubated overnight at 37°C with 5% CO2 to allow monocyte adherence.

**Isolation of CD4+ T cells**

Following overnight incubation of PBMCs, the non-adherent cells were collected and centrifuged at 1,800 rpm. CD4+ T cells were isolated from the PBMCs following the standardized protocol using reagents from the StemCell Company (EasySep Human CD4 Positive Selection Kit).

**Quantifying GSH levels in the plasma and CD4+ T cells of healthy and HIV patients**

Measurement of total and oxidized glutathione was performed using the GSH colorimetric assay from Arbor Assay. Reduced glutathione (rGSH) was calculated by subtracting the oxidized glutathione from the total GSH. All measurements were corrected for total protein levels. Baseline levels of total GSH and rGSH in the CD4+ T cells isolated from healthy subjects and HIV-positive individuals with CD4 T cell counts below 350 cells/mm³. Levels of MDA were also measured in the isolated plasma, RBCs, CD4 T cells from the HIV-positive individuals with CD4 T cell counts below 350 cells/mm³ at 3 months postsupplementation with either empty liposomes or L-GSH.

**Assays of cytokines in plasma**

Levels of IL-6, IL-10, TGF-β, IL-12, IL-2, IFN-γ, TNF-α, IL-1, and IL-17 in plasma were measured by sandwich ELISA. The assay kits were procured from eBioscience. Baseline levels of cytokines were measured in the plasma isolated from healthy subjects and HIV-positive individuals with CD4 T cell counts below 350 cells/mm³. Levels of cytokines were also measured in the plasma samples from the HIV-positive individuals with CD4 T cell counts below 350 cells/mm³ at 3 months postsupplementation with either empty liposomes or L-GSH.

**Statistical analyses**

Statistical data analyses were performed using GraphPad Prism version 7. Baseline levels of GSH, rGSH, MDA, IL-12, IL-2, and IFN-γ were compared between healthy individuals and HIV group, baseline and final visit for HIV placebo group, and baseline and final visit for HIV treatment group. Placebo and treatment groups were further analyzed by separating into two subgroups based on the CD4 T cell counts (CD4 T cell counts below 200 cells/mm³ and CD4 T cell counts 200–350 cells/mm³).

**Results**

**Assay of GSH levels in plasma and CD4+ T cells**

The levels of both total GSH and rGSH were significantly lower in the plasma and CD4+ T cells isolated from HIV-positive individuals with CD4+ T cell counts below 350 cells/mm³ compared to healthy subjects (Fig. 1A–D). There were no changes in the levels of both total GSH and rGSH in the CD4+ T cells isolated from the HIV placebo group at 3 months postsupplementation with empty liposomes, compared to the respective baseline measurements (Fig. 1E). In contrast to the placebo group, L-GSH supplementation resulted in a significant increase in the levels of both total GSH and rGSH in the CD4+ T cells isolated from HIV-positive individuals at 3 months postsupplementation (p < .05) (Fig. 1F, G). Further analysis of HIV L-GSH treatment subgroups based on CD4+ T cell counts indicated that there was a twofold increase in the levels of both total GSH and rGSH in HIV-positive subjects with CD4+ T cells below 200 cells/mm³, and a significant threefold increase in the levels of both total GSH and rGSH in HIV-positive subjects with CD4+ T cells between 200 and 350 cells/mm³ at 3 months postsupplementation with L-GSH (Fig. 1I, J). There were no changes in the levels of total GSH in the HIV placebo subgroups with CD4+ T cells below 200 cells/mm³ and with CD4+ T cells between 200 and 350 cells/mm³ at 3 months postsupplementation with empty liposomes (Fig. 1H).
Assay of MDA levels in plasma, RBCs, and CD4+ T cells

We also observed a significant increase in the baseline levels of MDA in the plasma, RBCs, and CD4+ T cells isolated from individuals with HIV infection with CD4+ T cell counts below 350 cells/mm³ compared to the healthy volunteers (Fig. 2A–C). L-GSH treatment resulted in a significant reduction in the levels of MDA in the RBCs isolated from HIV-positive subjects with CD4 T cells below 200 cells/mm³ and in CD4 T cells between 200 and 350 cells/mm³ at 3 months post-supplementation (Fig. 2F, G). L-GSH treatment also resulted in a significant reduction in the levels of MDA in the plasma samples isolated from HIV-positive subjects with CD4 T cells below 350 cells/mm³ at 3 months post-supplementation (Fig. 2E). L-GSH treatment stabilized the levels of MDA in CD4 T cells of HIV-positive subjects at 3 months post-supplementation (Fig. 2D).

Levels of IL-6 before and after treatment with L-GSH

We observed a significant increase in the levels of IL-6 in the plasma samples isolated from HIV-positive individuals with CD4 counts below 350 cell/mm² at the baseline time point compared to healthy volunteers (Fig. 3A). IL-6 levels in the placebo group showed no significant changes after 3 months of receiving empty liposomes (Fig. 3B). The amount of IL-6 remained about the same throughout this period of time. However, the levels of IL-6 in HIV-positive individuals decreased significantly at 3 months post-supplementation with L-GSH (Fig. 3C).

Levels of IL-10 before and after treatment with L-GSH

Baseline levels of IL-10 levels were significantly elevated in the plasma of HIV-positive individuals with CD4 counts below 350 cells/mm³ when compared to the healthy group (Fig. 3D). There was a statistically significant decrease in the levels of IL-10 in the HIV group at 3 months post-supplementation with L-GSH (Fig. 3F).

Levels of TGF-β before and after treatment with L-GSH

Baseline levels of TGF-β levels were significantly increased in the HIV group with a CD4 count below 350 cells/mm³ when compared to healthy individuals (Fig. 3G). The levels of this cytokine increased in the HIV placebo group at 3 months post-supplementation with empty liposomes (Fig. 3H). Interestingly, L-GSH supplementation stabilized the levels of TGF-β in the HIV group (Fig. 3I).

FIG. 1. Measurement of total GSH and rGSH. (A) Baseline levels of total GSH in plasma samples of Healthy Baseline, and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm³. Total GSH in plasma samples from HIV patients was significantly lower compared to the healthy individuals. GSH assay was performed using a colorimetric assay kit from Arbor Assays. Data represent mean±SE from 16 healthy individuals and 30 individuals with HIV. (B) Baseline levels of rGSH in plasma samples of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm³. rGSH in plasma samples from HIV-positive subjects was significantly lower compared to the healthy individuals. Data represent mean±SE from 16 healthy individuals and 30 individuals with HIV. (C) Baseline levels of total GSH in CD4+ T cells of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm³. CD4+ T cells were isolated using the EasySep Human CD4 Positive Selection Kit from nonadherent PBMCs isolated from whole blood of healthy participants and HIV patients. Total GSH in CD4 T cells from HIV patients was significantly lower compared to the healthy individuals. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV with placebo supplementation. (D) Baseline levels of rGSH in CD4+ T cells of Healthy Baseline and HIV-positive subjects with CD4 T cells below 350 cells/mm³. Levels of rGSH in CD4 T cells from HIV patients were significantly lower compared to the healthy individuals. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV with placebo supplementation. (E) Total GSH in CD4+ T cells from the HIV placebo group with CD4+ T cell counts below 350 cells/mm³. There were no significant changes in the levels of total GSH in the CD4 T cells at 3 months post-supplementation with empty liposomes. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV placebo. (F) Total GSH in CD4+ T cells from the HIV-L-GSH treatment group with CD4 T cells below 350 cells/mm³. There was a significant increase in the levels of total GSH in the CD4 T cells from the HIV-positive subjects at 3 months post-L-GSH treatment. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (G) Total GSH in CD4+ T cells from the HIV-L-GSH treatment group with CD4+ T cell counts below 350 cells/mm³. There was a significant increase in the levels of rGSH in the CD4 T cells from the HIV-positive subjects at 3 months post-L-GSH treatment. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (H) Total GSH in CD4+ T cells from the HIV-L-GSH treatment group with CD4+ T cell counts below 200 cells/mm³ and between 200 and 350 cells/mm³. There were no changes in the levels of total GSH in the CD4 T cells from the HIV subgroups such as those with CD4 T cell counts below 200 cells/mm³ and participants with CD4 T cell counts between 200 and 350 cells/mm³ at 3 months post-treatment with empty liposomes. Data represent mean±SE from 16 healthy individuals and seven individuals with HIV below 200 cells/mm³ and eight individuals with HIV 200–350 cells/mm³ with placebo supplementation. (I) Total GSH in CD4+ T cells from the HIV-L-GSH treatment group with CD4+ T cell counts below 200 cells/mm³ and between 200 and 350 cells/mm³. There was a significant increase in the levels of total GSH in the CD4 T cells from the HIV-positive subjects with CD4 T cell counts between 200 and 350 cells/mm³ at 3 months post-L-GSH treatment. Data represent mean±SE from 16 healthy individuals and seven individuals with HIV below 200 cells/mm³ and eight individuals with HIV 200–350 cells/mm³ with L-GSH supplementation. (J) rGSH levels in CD4+ T cells from the HIV-L-GSH treatment group with CD4+ T cell counts below 200 cells/mm³ and between 200 and 350 cells/mm³. There was a significant increase in the levels of rGSH in the CD4 T cells from the HIV-positive subjects with CD4 T cell counts between 200 and 350 cells/mm³ at 3 months post-L-GSH treatment. Data represent mean±SE from 16 healthy individuals and seven individuals with HIV CD4 T cell counts below 200 cells/mm³ and eight individuals with HIV 200–350 cells/mm³ with L-GSH supplementation, *p≤0.05; **p≤0.01. GSH, glutathione; rGSH, reduced glutathione; SE, standard error; PBMC, peripheral blood mononuclear cell; L-GSH, liposomal glutathione.
Levels of IL-12 before and after treatment with L-GSH

Baseline levels of IL-12 were significantly lower in the HIV group (CD4 below 350 cells/mm³) compared to the healthy counterparts (Fig. 4A). There were no significant changes in the levels of IL-12 in the HIV placebo group at 3 months post-treatment with empty liposomes (Fig. 4B). In the HIV L-GSH group (CD4 below 350 cells/mm³), there was a significant increase in the levels of IL-12 at 3 months post-treatment compared to the baseline levels (Fig. 4C). Further
evaluation of HIV L-GSH subgroups based on CD4 T cell counts indicated that there was a twofold increase in the levels of IL-12 in individuals with CD4 T cell counts below 200 cells/mm³, and a significant increase in the levels of IL-12 in the HIV L-GSH group with CD4 T cells between 200 and 350 cells/mm³ at 3 months postsupplementation with L-GSH (*p < .05) (Fig. 4D).

**Levels of IL-2 before and after treatment with L-GSH**

Baseline levels of IL-2 were significantly compromised in HIV-positive subjects compared to the healthy group (Fig. 4F). Supplementation with empty liposomes resulted in a further decrease in the levels of IL-2 in the HIV placebo group at 3 months post-treatment compared to the baseline time point (Fig. 4G). L-GSH treatment resulted in a modest increase in the levels of IL-2 in the HIV-positive subjects (Fig. 4H).

**Levels of IFN-γ before and after treatment with L-GSH**

There was a twofold decrease in the baseline levels of IFN-γ in HIV-positive subjects (CD4 below 350 cells/mm³) compared to the healthy subjects (Fig. 4I). L-GSH supplementation in HIV-positive subjects resulted in a significant increase in the levels of IFN-γ at 3 months post-treatment (*p < .05) (Fig. 4K) compared to the placebo group (Fig. 4J). Further analysis of HIV L-GSH subgroups based on the CD4+ T cell counts demonstrated a significant increase in the levels of IFN-γ in both individuals with CD4 T cells below 200 cells/mm³ (*p < .05) and in individuals with CD4 T cell counts between 200 and 350 cells/mm³ (*p < .05) at 3 months post-treatment (Fig. 4L).

**Levels of IL-17 before and after treatment with L-GSH**

Baseline levels of IL-17 were significantly decreased in the HIV group with a CD4 count below 350 cells/mm³ when compared to the healthy individuals (Fig. 5A). There was a further decrease in the levels of this cytokine in the HIV-placebo group at 3 months postsupplementation with empty liposomes (Fig. 5B). Interestingly, L-GSH supplementation stabilized the levels of IL-17 in the HIV group (Fig. 5C).

**Levels of IL-1β before and after treatment with L-GSH**

Baseline levels of IL-1β were significantly lower in the HIV group with a CD4 count below 350 cells/mm³ when compared to the healthy individuals (Fig. 5D). There was a further decrease in the levels of IL-1 in the HIV placebo group at 3 months postsupplementation with empty liposomes (Fig. 5E). Interestingly, L-GSH supplementation stabilized the levels of IL-1 in the HIV group (Fig. 5F).

**Discussion**

Due to low CD4+ T cell counts, HIV and AIDS individuals are at 26–31 times greater risk of developing opportunistic infections such as tuberculosis, compared to non-HIV-infected individuals.¹

GSH, an antioxidant exists in two forms: rGSH and oxidized glutathione disulfide (GSSG). The antioxidant properties of GSH are due to rGSH. During oxidative stress, rGSH was significantly lower in the HIV-positive subjects at 3 months post-treatment compared to HIV patients when compared to the healthy individuals (Fig. 5D). There was a further decrease in the levels of this cytokine in the HIV-placebo group at 3 months postsupplementation with empty liposomes (Fig. 5E). Interestingly, L-GSH supplementation stabilized the levels of IL-17 in the HIV group (Fig. 5C).

**FIG. 2.** Measurement of MDA. (A) Baseline levels of MDA in plasma samples of Healthy Baseline and HIV-positive subjects with CD4 T cell counts below 350 cells/mm³. MDA levels in the plasma samples from HIV patients were significantly elevated compared to healthy individuals. MDA assay was performed using the TBARS assay kit from Cayman Chemical. Data represent mean ± SE from 16 healthy individuals and 30 individuals with HIV. (B) Baseline levels of MDA in RBCs of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm³. MDA levels in the RBCs from HIV patients were significantly enhanced compared to healthy patients. Data represent mean ± SE from 16 healthy individuals and 30 individuals with HIV. (C) Baseline levels of MDA in RBCs of HIV-positive subjects with CD4+ T cell counts below 350 cells/mm³. MDA levels in the RBCs from HIV patients were significantly higher compared to healthy individuals. Data represent mean ± SE from 16 healthy individuals and 30 individuals with HIV. (D) MDA levels in the plasma samples from the HIV placebo group with CD4+ T cell counts below 350 cells/mm³. There were no significant changes in the levels of MDA in the samples collected at 3 months postsupplementation with empty liposomes. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV placebo. (E) MDA levels in the plasma samples from the HIV-L-GSH treatment group with CD4+ T cell counts below 350 cells/mm³. There was a significant decrease in the levels of MDA in the plasma samples from the HIV-positive subjects at 3 months post-L-GSH treatment. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (F) MDA levels in the RBCs from the HIV-L-GSH treatment group with CD4+ T cell counts below 350 cells/mm³. There was a significant decrease in the levels of MDA in the RBCs from the HIV-positive subjects at 3 months post-L-GSH treatment. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (G) MDA levels in RBCs from the HIV-L-GSH treatment group with CD4+ T cell counts below 200 cells/mm² and between 200 and 350 cells/mm². L-GSH treatment resulted in a significant reduction in the levels of MDA in the RBCs isolated from HIV-positive subjects with CD4 T cells below 200 cells/mm² and in CD4 T cells between 200 and 350 cells/mm² at 3 months postsupplementation. Data represent mean ± SE from 16 healthy individuals and seven individuals with HIV below 200 cells/mm² and eight individuals with HIV 200–350 cells/mm² with L-GSH supplementation. (H) MDA levels in the CD4+ T cells from the HIV placebo group with CD4+ T cell counts below 350 cells/mm³. There was a significant increase in the levels of MDA in the CD4+ T cells isolated from the HIV-positive subjects at 3 months posttreatment with empty liposomes. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV placebo. (I) MDA levels in the CD4+ T cells from the HIV-L-GSH treatment group with CD4 T cell counts below 350 cells/mm³. There was stabilization in the levels of MDA in the CD4+ T cells isolated from the HIV-positive subjects at 3 months post-L-GSH treatment. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV-L-GSH, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.005; ****p ≤ 0.0005. MDA, malondialdehyde; RBC, red blood cell.
is oxidized to neutralize the ROS, leading to the formation of GSSG. GSSG contains no antioxidant properties.\textsuperscript{24,25}

We demonstrated that the total and reduced forms of GSH were significantly compromised in macrophages, NK cells, and T cells isolated from the peripheral blood of HIV-1-infected individuals.\textsuperscript{11,15–17,26} Decreased levels of GSH in individuals with HIV-1 infection were accompanied by diminished levels of enzymes, such as $\gamma$-glutamyl-cysteinylligase and glutathione synthetase in the RBCs.\textsuperscript{10} We also established that compromised levels of GSH in immune cells derived from the peripheral blood of individuals with HIV-1 infection led to increased survival of \textit{Mtb} inside macrophages.\textsuperscript{11,15–17,26} Augmenting the levels of GSH in macrophages derived from individuals with HIV infection resulted in improved control of \textit{Mtb} infection.\textsuperscript{10,15–17,26} Furthermore, cytokines that are responsible for controlling intracellular infections such as TNF-\textit{z}, IL-1\beta, IL-2, IFN-\gamma, and IL-12 were found to be compromised, while IL-10, an immunosuppressive cytokine, was elevated in the plasma samples of HIV-1-infected individuals.\textsuperscript{17} We have successfully demonstrated that supplementing individuals with HIV-1 infection (CD4 T cell counts between 200 and 900/mm\textsuperscript{3}) for 13 weeks orally with L-GSH (supplied by Your Energy Systems) significantly increased the levels of cytokines, such as IL-2, IL-12, and IFN-\gamma, which are important for mediating effective immune responses against intracellular infections.\textsuperscript{17}

Using the knowledge of HIV infection mechanism as well as our previous findings on the effects of GSH, we conducted a double-blinded study to determine the effects of L-GSH supplementation in restoring a balance in the cytokine levels and redox homeostasis in HIV and AIDS patients with CD4+ T cell counts below 350 cells/mm\textsuperscript{3}.

We observed that the levels of total GSH and rGSH were compromised in plasma and CD4 T cells isolated from HIV-positive individuals with CD4 T cells below 350 cells/mm\textsuperscript{3} compared to healthy individuals (Fig. 1A–E). Supplementing
HIV-positive individuals with L-GSH resulted in an increase in the levels of both total GSH and rGSH in individuals with CD4 T cells below 200 cells/mm³ and in subjects with CD4 T cells between 200 and 350 cells/mm³ (Fig. 1F–J). Furthermore, HIV-positive individuals with higher CD4+ T cell counts (200–350 cells/mm³) responded better to L-GSH treatment than those with lower CD4+ T cell counts (below 200 cells/mm³) by demonstrating a significant increase in the levels of both total GSH and rGSH (Fig. 1I, J).

Diminished production of GSH in HIV-positive subjects correlated with increased levels of MDA in the plasma, RBCs, and CD4 T cells (Fig. 2A–C). L-GSH supplementation for 3 months significantly reduced the levels of MDA in the plasma and RBCs of HIV-positive subjects (Fig. 2E–G), and stabilized the levels of MDA in the CD4+ T cells of individuals with HIV infection (Fig. 2I).

Our results also indicate that there was a significant increase in the baseline levels of IL-6 in HIV-positive individuals with CD4 levels below 350 cells/mm³ when compared to healthy subjects (Fig. 3A). HIV-positive individuals who received placebo for 3 months showed no significant change in their levels of IL-6 (Fig. 3B). However, L-GSH treatment for 3 months resulted in a significant decrease in the levels of IL-6 in HIV-infected individuals (Fig. 3C). It has been shown that exacerbated production of IL-6 can lead to systemic oxidative stress. Our findings thus strongly suggest that the exacerbated oxidative stress is partly due to the increase in IL-6 because when HIV-positive individuals took L-GSH for 3 months the levels of both MDA and IL-6 decreased significantly (Figs. 2A–I and 3A–C).

We also observed that the baseline levels of immunosuppressive cytokines such as IL-10 and TGF-β were significantly elevated in HIV-positive participants with CD4 levels below 350 cells/mm³ when compared to healthy volunteers (Fig. 3D, G). IL-10 has been shown to prevent antigen-specific T cell proliferation by inhibiting the antigen-presenting capacity of monocytes and dendritic cells through downregulation in the expression of major histocompatibility (MHC) complex II molecules on antigen-presenting cells, thereby reducing or preventing optimal antigen-specific proliferative T cell response. IL-10 has also been shown to inhibit chemokine production and can interfere with the formation of granuloma. Supplementation with L-GSH resulted in a significant downregulation in the levels of IL-10 in participants with HIV infection (Fig. 3F).

A significant increase in the levels of TGF-β was observed in participants with HIV infection who consumed empty liposomes for 3 months (Fig. 3G). In contrast to the placebo group (Fig. 3H), HIV-positive subjects who received L-GSH supplementation for 3 months were able to maintain the levels of TGF-β (Fig. 3I). These findings indicate that supplementation of L-GSH in HIV-positive individuals with CD4 T cell levels below 350 cells/mm³ can prevent further increase in the levels of TGF-β than if these individuals were not to receive the L-GSH supplementation (Fig. 3H, I). Studies have shown that TGF-β has the ability to downregulate the de novo synthesis of GSH resulting in decreased levels of GSH. Increased levels of TGF-β in HIV-positive individuals therefore explain the cause for decreased levels of GSH. With the availability of useable GSH, via L-GSH,

**FIG. 3.** Assay of IL-6, IL-10, and TGF-β. (A) Baseline levels of IL-6 in plasma samples of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm³. IL-6 levels were significantly elevated in the plasma samples from HIV patients compared to the healthy individuals. IL-6 was assayed in the plasma samples by sandwich ELISA using assay kits procured from eBioscience. Data represent mean±SE from 16 healthy individuals and 30 individuals with HIV. (B) IL-6 levels in the plasma from the HIV placebo group with CD4+ T cell counts below 350 cells/mm³. There were no changes in the levels of IL-6 in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with empty liposomes. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV placebo. (C) IL-6 levels in the plasma from the HIV-L-GSH group with CD4+ T cell counts below 350 cells/mm³. There was a significant decrease in the levels of IL-6 in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with L-GSH. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (D) Baseline levels of IL-10 in plasma samples of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm³. IL-10 levels were significantly increased in the plasma samples from HIV patients compared to the healthy individuals. IL-10 was assayed in the plasma samples by sandwich ELISA using assay kits procured from eBioscience. Data represent mean±SE from 16 healthy individuals and 30 individuals with HIV. (E) IL-10 levels in the plasma from the HIV placebo group with CD4+ T cell counts below 350 cells/mm³. There were no changes in the levels of IL-10 in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with empty liposomes. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV placebo. (F) IL-10 levels in the plasma from the HIV-L-GSH group with CD4+ T cell counts below 350 cells/mm³. There was a significant decrease in the levels of IL-10 in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with L-GSH. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (G) Baseline levels of TGF-β in plasma samples of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm³. TGF-β levels were significantly increased in the plasma samples from HIV patients compared to the healthy individuals. TGF-β was assayed in the plasma samples by sandwich ELISA using assay kits procured from eBioscience. Data represent mean±SE from 16 healthy individuals and 30 individuals with HIV. (H) TGF-β levels in the plasma from the HIV placebo group with CD4+ T cell counts below 350 cells/mm³. There was a significant increase in the levels of TGF-β in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with empty liposomes. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV placebo. (I) TGF-β levels in the plasma from the HIV-L-GSH group with CD4+ T cell counts below 350 cells/mm³. There was a stabilization in the levels of TGF-β in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with L-GSH. Data represent mean±SE from 16 healthy individuals and 15 individuals with HIV-L-GSH, *p≤0.05; **p≤0.01; ***p≤0.005; ****p≤0.0005. IL, interleukin; TGF, transforming growth factor; ELISA, enzyme-linked immunosorbent assay.
which can bypass the de novo synthesis pathway that is being inhibited by TGF-β, there is restoration in the levels of GSH, redox homeostasis, and maintenance of TGF-β levels.

IL-12, a polarizing cytokine, signals the differentiation of naive CD4+ T cells into the Th1 subset. IFN-γ produced by the Th1 subset of CD4 T cells activates the effector functions of macrophages to kill intracellular pathogens. Baseline levels of IL-12 were significantly diminished in HIV-positive individuals with CD4 below 350 cells/mm³ compared to healthy individuals (Fig. 4A). Supplementation with L-GSH significantly increased the levels of IL-12 in HIV-positive individuals to the same level as healthy individuals (Fig. 4C). Further analysis of HIV L-GSH subgroups indicated a significant increase in the levels of IL-12 (p < .05) in subgroups with CD4 T cell counts between 200 and 350 cells/mm³ and a threefold increase in subgroups with CD4 T cell counts below 200 cells/mm³ (Fig. 4E). Consistent with the previous results, the most notable changes were observed when L-GSH is supplemented in HIV-positive subjects with a higher level of CD4+ T cell counts (i.e., between 200 and 350 cells/mm³).

IL-2, a T cell growth and differentiation factor, is necessary for sustenance of T cells. IL-2 also regulates cellular metabolism and glycolysis, all of which are necessary for long-term T cell survival. Baseline levels of IL-2 were significantly lower in HIV-positive subjects with CD4 below 350 cells/mm³ than healthy individuals (Fig. 4F). Compromised levels of IL-2 can therefore affect the viability of T cells in HIV-positive subjects. L-GSH supplementation stabilized the levels of IL-2 in individuals with HIV infection (Fig. 4H).

IFN-γ activates the effector functions of macrophages, neutrophils, and CD8 T cells, and can enhance antigen presentation through increased expressions of MHC class I and II molecules. In conjunction with TNF-α, IFN-γ can induce nitric oxide production by macrophages leading to improved killing of intracellular pathogens. Consistent with our
previous results of IL-12 and IL-2, we expected that the levels of IFN-\(\gamma\) will also be diminished in HIV-positive subjects compared to healthy individuals. In line with our hypothesis, the baseline levels of IFN-\(\gamma\) were decreased by fourfold in HIV-positive subjects compared to the healthy group (Fig. 4I). L-GSH supplementation for 3 months, resulted in a significant increase in the levels of IFN-\(\gamma\) in both the HIV subgroup with CD4 T cell counts below 200 cells/mm\(^3\) \((p < .05)\) and in the HIV subgroup with CD4 T cell counts between 200 and 350 cells/mm\(^3\) \((p < .05)\) (Fig. 4L). As for the HIV-positive individuals with higher CD4+ T cell counts (between 200 and 350 cells/mm\(^3\)), after 3 months of L-GSH supplementation there was a significant 10-fold increase in the levels of IFN-\(\gamma\) compared to baseline \((p < .05)\). Once again the same trend can be seen that the most notable effect is observed in those HIV patients with a higher CD4+ T cell count.

Levels of proinflammatory cytokines such as IL-17 and IL-1\(\beta\) levels were significantly decreased in the HIV group with a CD4 count below 350 cells/mm\(^3\) at the baseline time point when compared to the healthy individuals (Fig. 5A, D). Interestingly, L-GSH supplementation stabilized the levels of IL-1 and IL-17 in the HIV group (Fig. 5C, F).

Our study findings are consistent with the results from other research groups reporting that HIV-positive individuals on ART have persistent chronic inflammation and decreased immune functions, which in turn can increase the risks for developing cardiovascular and chronic kidney diseases.\(^{29-32}\) Therefore, GSH restoration may not only decrease the risks for developing opportunistic infections in HIV patients receiving ART but can also aid in improving immune functions and reducing the complications arising due to chronic inflammation.

In summary, the total and reduced forms of GSH were significantly compromised in the plasma and CD4 T cells from individuals with HIV infection compared to the healthy subjects. When given the placebo, there were no significant

**FIG. 4.** Assay of IL-12, IL-2, and IFN-\(\gamma\). (A) Baseline levels of IL-12 in plasma samples of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm\(^3\). IL-12 levels were significantly decreased in the plasma samples from HIV patients compared to the healthy individuals. IL-12 was assayed in the plasma samples by sandwich ELISA using assay kits procured from eBioscience. Data represent mean ± SE from 16 healthy individuals and 30 individuals with HIV. (B) IL-12 levels in the plasma samples from the HIV placebo group with CD4+ T cell counts below 350 cells/mm\(^3\). There were no significant changes in the levels of IL-12 in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with empty liposomes. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV placebo. (C) IL-12 levels in the plasma samples from the HIV-L-GSH group with CD4+ T cell counts below 350 cells/mm\(^3\). There was a significant increase in the levels of IL-12 in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with L-GSH. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (D) IL-12 levels in the plasma samples from the HIV placebo group with CD4+ T cell counts below 200 cells/mm\(^3\) and between 200 and 350 cells/mm\(^3\). There were no changes in the levels of IL-12 in the plasma samples from the HIV subgroups such as those with CD4 T cell counts below 200 cells/mm\(^3\) and participants with CD4 T cell counts between 200 and 350 cells/mm\(^3\) at 3 months post-treatment with empty liposomes. Data represent mean ± SE from 16 healthy individuals and seven individuals with HIV below 200 cells/mm\(^3\) and eight individuals with HIV 200–350 cells/mm\(^3\) with placebo supplementation. (E) IL-12 levels in the plasma samples from the HIV-L-GSH treatment group with CD4+ T cell counts below 200 cells/mm\(^3\) and between 200 and 350 cells/mm\(^3\). There was a significant increase in the levels of IL-12 in the plasma samples from the HIV-positive subjects with CD4 T cell counts between 200 and 350 cells/mm\(^3\) at 3 months post-L-GSH treatment. Data represent mean ± SE from 16 healthy individuals and seven individuals with HIV below 200 cells/mm\(^3\) and eight individuals with HIV 200–350 cells/mm\(^3\) with L-GSH supplementation. (F) Baseline levels of IL-2 in plasma samples of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm\(^3\). IL-2 levels were significantly compromised in the plasma samples from HIV patients compared to the healthy individuals. IL-2 was assayed in the plasma samples by sandwich ELISA using assay kits procured from eBioscience. Data represent mean ± SE from 16 healthy individuals and 30 individuals with HIV. (G) IL-2 levels in the plasma samples from the HIV placebo group with CD4+ T cell counts below 350 cells/mm\(^3\). There was a further significant decrease in the levels of IL-2 in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with empty liposomes. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV placebo. (H) IL-2 levels in the plasma samples from the HIV-L-GSH group with CD4+ T cell counts below 350 cells/mm\(^3\). The levels of IL-2 stabilized in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with L-GSH. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (I) Baseline levels of IFN-\(\gamma\) in plasma samples of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm\(^3\). IFN-\(\gamma\) levels were diminished in the plasma samples from HIV patients compared to the healthy individuals. IFN-\(\gamma\) was assayed in the plasma samples by sandwich ELISA using assay kits procured from eBioscience. Data represent mean ± SE from 16 healthy individuals and 30 individuals with HIV. (J) IFN-\(\gamma\) levels in the plasma samples from the HIV placebo group with CD4+ T cell counts below 350 cells/mm\(^3\). There were no significant changes in the levels of IFN-\(\gamma\) in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with empty liposomes. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV placebo. (K) IFN-\(\gamma\) levels in the plasma samples from the HIV-L-GSH group with CD4+ T cell counts below 350 cells/mm\(^3\). There was a significant increase in the levels of IFN-\(\gamma\) in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with L-GSH. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (L) IFN-\(\gamma\) levels in the plasma samples from the HIV-L-GSH treatment group with CD4+ T cell counts below 200 cells/mm\(^3\) and between 200 and 350 cells/mm\(^3\). L-GSH treatment resulted in a significant increase in the levels of IFN-\(\gamma\) in the plasma samples isolated from HIV-positive subjects with CD4 T cells below 200 cells/mm\(^3\) and in CD4 T cells between 200 and 350 cells/mm\(^3\) at 3 months post-supplementation. Data represent mean ± SE from 16 healthy individuals and seven individuals with HIV below 200 cells/mm\(^3\) and eight individuals with HIV 200–350 cells/mm\(^3\) with L-GSH supplementation, *\(p \leq 0.05\); **\(p \leq 0.01\). IFN, interferon.
changes in the levels of total and reduced forms of GSH in the CD4 T cells. L-GSH treatment resulted in a significant increase in the levels of total and reduced forms of GSH in CD4 T cells derived from individuals with HIV infection. Levels of IL-6 were significantly increased in HIV-positive subjects. This was reinforced by the significant increase of MDA in HIV-infected individuals. With administration of L-GSH for a period of 3 months, the levels of IL-6 and MDA were decreased to the levels corresponding to that of healthy subjects. TGF-β levels were found to be elevated in HIV-positive individuals. With L-GSH supplementation, the increase of TGF-β was dampened. Importantly, L-GSH supplementation restored the levels of GSH in HIV-positive subjects. Participants with HIV infection also exhibited
increased levels of IL-10 and decreased levels of IL-12, IL-2, and IFN-γ making them increasingly susceptible to opportunistic infections due to impaired immune responses. With L-GSH supplementation for 3 months, there was a decrease in the levels of IL-10 and an increase in the levels of IL-12, IL-2, and IFN-γ, especially in HIV-positive individuals with greater CD4+ T cell counts (between 200 and 350 cells/mm³). The levels of IL-1 stabilized in the plasma samples isolated from the HIV-positive subjects at 3 months post-treatment with L-GSH. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (D) Baseline levels of IL-1 in plasma samples of Healthy Baseline and HIV-positive subjects with CD4+ T cell counts below 350 cells/mm³. IL-1 levels were significantly compromised in the plasma samples from HIV patients compared to the healthy individuals. IL-1 was assayed in the plasma samples by sandwich ELISA using assay kits procured from eBioscience. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV-L-GSH. (E) IL-1 levels in the plasma samples from the HIV placebo group with CD4+ T cell counts below 350 cells/mm³. There was a further significant decrease in the levels of IL-1 in the plasma samples isolated from the HIV positive subjects at 3 months post-treatment with empty liposomes. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV placebo. (F) IL-1 levels in the plasma samples from the HIV-L-GSH group with CD4+ T cell counts below 350 cells/mm³. The levels of IL-1 stabilized in the plasma samples isolated from the HIV positive subjects at 3 months post-treatment with L-GSH. Data represent mean ± SE from 16 healthy individuals and 15 individuals with HIV-L-GSH, *p ≤ 0.05; **p ≤ 0.01.

Table 1. Subject Characteristics: Shows Characteristics of the Study Population

|                          | Placebo (%) | L-GSH (%) | p-value |
|--------------------------|-------------|-----------|---------|
| Age                      | 51.4 years  | 50.1 years| .278    |
| HIV RNA                  | 21.8        | 19.0      | .338    |
| CDA viral load           | 219.6       | 250.9     | .369    |
| Descent                  | n           | n         | Total (%) |
| African American         | 3           | 1         | 14.3    |
| White                    | 4           | 4         | 28.6    |
| Hispanic                 | 4           | 9         | 46.4    |
| Asian                    | 2           | 0         | 7.1     |
| Unknown                  | 1           | 0         | 3.6     |
| Gender                   | n           | n         | Total (%) |
| Female                   | 3 (21.4)    | 2 (14.3)  | 17.9    |
| Male                     | 10 (71.4)   | 12 (85.7) | 78.6    |
| Transition M → F*        | 1 (7.1)     | 0         | 3.5     |

*Transition from male to female.
L-GSH, liposomal glutathione.
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Author Disclosure Statement

No competing financial interests exist.

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