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The role of dysfunctional High Density Lipoprotein in immune activation and atherosclerosis associated with Human Immunodeficiency Virus Type 1 infection

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The role of dysfunctional High Density Lipoprotein in immune activation and atherosclerosis associated with Human Immunodeficiency Virus Type 1 infection

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Microbiology, Immunology, and Molecular Genetics

by

Theodoros Kelesidis

2013

ABSTRACT OF THE DISSERTATION

The role of dysfunctional High Density Lipoprotein in immune activation and atherosclerosis associated with Human Immunodeficiency Virus Type 1 infection

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University of California, Los Angeles, 2013

Professor Otto O. Yang, Chair

With improved survival cardiovascular disease (CVD) has become an increasingly important cause of morbidity and mortality among people with treated HIV-1 infection. HIV-1 infection is characterized by a chronic state of immune activation that is an independent predictor of disease progression. Residual immune activation in treated HIV-1 may be contributing to atherosclerosis but there is limited evidence supporting this hypothesis. The mechanisms that connect inflammation, CVD and activation of the immune system in HIV infection remain to be elucidated. Demonstrating mechanisms that are associated with CVD in patients with HIV infection may initiate further studies to explore the efficacy of novel therapeutic interventions that may reduce the CVD and immune activation associated with HIV-1 infection and might improve the prognosis of HIV infected patients.

Lipids can regulate atherogenesis, immunity and HIV-1 immunopathogenesis. Oxidized lipids may be a significant mediator in the interplay between inflammation, immune activation and

atherosclerosis. While normal High Density Lipoprotein (HDL) binds oxidized lipids and protects against CVD, HDL can become oxidized and dysfunctional in the setting of chronic inflammatory diseases. Thus oxidized (dysfunctional) HDL may have multiple roles in the pathogenesis of HIV-1 infection but it is not known whether HIV-1 infected subjects have dysfunctional HDL. We hypothesize that a vicious cycle of HIV-1-induced immune activation, inflammation, production of dysfunctional HDL, and further immune activation explains the increased rate of atherosclerotic disease in HIV-1 infection. The primary goal of this dissertation is to better define the role of dysfunctional HDL in immune activation and atherosclerosis associated with HIV-1 infection.

This was addressed using a variety of approaches. First we showed that immune activation may be a contributing factor to progression of atherosclerosis in HIV-1 infection. Using a cell based assay of HDL function we found that HIV-1 infected subjects have dysfunctional HDL. We then developed novel cell free biochemical assays that measure redox properties of HDL as tools to study HDL function in vivo in the setting of large scale studies in humans. We used these assays to demonstrate that HIV-1 infected subjects have dysfunctional HDL that is independently associated with cardiometabolic risk factors such as obesity and insulin resistance, biomarkers of macrophage and T cell activation, and predictors of disease progression such as CD38 expression on $CD8^+$ T cells and lower nadir $CD4^+$ T cell count. In view of our in these vivo observations that support a role of oxidized HDL in immune activation and CVD associated with HIV-1 infection, we investigated the direct in vitro effects of dysfunctional (oxidized) HDL on T cells, which are important for immune activation, HIV-1 immunopathogenesis and atherogenesis. We found that both *in vivo* modified dysfunctional HDL from HIV-1 infected subjects and *in vitro* oxidized HDL directly upregulated markers of T cell activation *in vitro*. In addition oxidized HDL directly increased T cell proliferation and production of cytokines, specific killing of HIV-1 specific cytotoxic $CD8^+$ T cells (CTLs) and

expression of chemokine receptors (CXCR4, CCR5) on $CD4^+$ T cells important for HIV-1 infectivity, whereas it impaired the ability of HIV-1 specific CTLs to suppress HIV-1 replication. Our preliminary in vivo and in vitro data supported a role of lipids and oxidized HDL in the regulation of the NF-kB pathway in HIV-1 infected subjects but not in uninfected controls.

Together these results suggest that dysfunctional HDL has a major role in immune activation and atherosclerosis associated with HIV-1 infection. Future research could potentially focus on the role of dysfunctional HDL in directly affecting the life cycle of HIV-1 and the antiviral immune responses and the mechanisms that mediate the interplay between oxidized HDL, HIV-1 immunopathogenesis, and atherogenesis. A few proposed ideas are summarized in the Discussion of this Dissertation. This work is innovative and may have an impact on public health since these findings may lead to novel therapeutic interventions (such as HDL mimetics) to reduce CVD, a leading cause of death in treated HIV-1 infected subjects.

The dissertation of Theodoros Kelesidis is approved.

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DEDICATION

This thesis is dedicated to my wife Maria and my family (Iosif, Chrysanthos, and Evangelia) who supported and encouraged the work presented herein with great patience and care.

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LIST OF ABBREVIATIONS

7AAD – 7-aminoactinomycin D

ABCA1 – ATP-binding cassette transporter 1

ACTG – AIDS Clinical Trials Group

ACS – Acute coronary syndrome

aHDL – Anti-inflammatory HDL

AIDS – Acquired immune deficiency syndrome

APC – Allophycocyanine

APCs – Antigen Presenting Cells

APC-H7 – Allophycocyanine/H7

AP-HDL – Acute phase HDL

Apo – Apolipoprotein

ApoA-I – Apolipoprotein A-I

ApoB – Apolipoprotein B

ApoE – Apolipoprotein E

ART – Antiretroviral therapy

β-2 – beta 2

BD – Becton, Dickinson

bDBP – Brachial diastolic pressure

B-LCLs – B lymphocyte cell lines

BMI – Body Mass Index

bSBP – Brachial systolic pressure

CA – Capsid

CAD – Coronary artery disease

CCR5 – C-C Chemokine Receptor 5

CD – Cluster of differentiation

CD1 – Cluster of differentiation 1

CD4 – Cluster of differentiation 4

CD8 – Cluster of differentiation 8

CD14 – Cluster of differentiation 14

CD36 – Cluster of differentiation 36

CD38 – Cluster of differentiation 38

CD69 – Cluster of differentiation 69

CD163 – Cluster of differentiation 163

CE-OOH – Cholesteryl linoleate hydroperoxide

CFSE – Carboxyfluorescein succinimidyl ester

CTL – $CD8^+$ T lymphocyte

C57BL/6J – C57 black 6J

CHD – Coronary heart disease

CI – Confidence Interval

CIMT – Carotid artery intima-media thickness

CMV – Cytomegalovirus

CO_2 – Carbon dioxide

CRP – C-reactive protein

Cr – Chromium

CT – Computed Tomography

CTLs – Cytotoxic CD8+ T cells

CuSO4 – Copper Sulphate

CVD – Cardiovascular disease

CXC – Chemokine Receptor 4

DAPI – 4'-6-Diamidino-2-phenylindole

DC – Dentritic cells

ΔCIMT– Yearly rate of change of carotid artery intima-media thickness

ΔIMT – Yearly rate of change of carotid artery intima-media thickness

ΔLPS – Yearly rate of change of lipopolysaccharide

ΔnHRA – Yearly rate of change in normalized HDL redox activity

ΔsCD14 – Yearly rate of change of soluble cluster of differentiation 14

DCF – 2', 7'-dichlorofluorescein

DCF-DA – Dichlorodihydrofluorescein diacetate

DHR – Dihydrorhodamine

dL – Deciliter

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DOR – Dihydrorhodamine oxidation rate

EDTA – Ethylenediaminetetraacetic Acid

ELISA – Enzyme-linked immunosorbent assay

Env – Envelope

E: T – Effector-to-target cell ratio

EU – Enzymatic Units

FITC – Fluorescein isothiocyanate

FPLC – Fast protein liquid chromatography

FU – Fluorescence units

Gag – Group-specific antigen

GFP – Green fluorescent protein

Gp – Glycoprotein

gp120 – Glycoprotein 120

gp41 – Glycoprotein 41

GSH – Glutathione selenoperoxidase

HBS – (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered saline

HCV – Hepatitis C virus

HDL – High Density Lipoprotein

HDL-C – High Density Lipoprotein Cholesterol

HDLox – Oxidized high density lipoprotein

HEPES – (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HII – HDL-inflammatory index

HIV – Human Immunodeficiency Virus

HLA – Human leukocyte antigen

HLA-DR – Human leukocyte antigen-D related

H_2O_2 – Hydrogen peroxide

HOMA: Homeostasis model assessment for insulin resistance

HPETE – Hydroperoxyeicosatetraenoic acid

HPLC – High Performance Liquid Chromatography

HPODE – Hydroperoxyoctadecadienoic acid

HRA – HDL redox activity

HRP – Horseradish peroxidase

Hs-CRP – High-sensitivity C reactive protein

IC – Inhibitory concentration

IFN-γ – Interferon-gamma

IL – Interleukin

IN – Integrase

IQR – Interquartile range

IRB – Institutional Review Board

KBr – Potassium bromide

LAL – Limulus Amoebocyte Lysate

LCAT – Lecithin-cholesterol acyltransferase

LC/MS/MS – Liquid Chromatography - Tandem Mass Spectrometry

LDL – Low-density lipoproteins

LDLox – Oxidized low density lipoprotein

LDLR – Low-density lipoprotein receptor

LOX-1 – Lectin-like oxidized LDL receptor-1

LOOH – Lipid hydroperoxides

Lp-PLA2 – Lipoprotein-associated phospholipase A2

LPS – Lipopolysaccharide

LT – Lean trained

MA – Matrix

MAPK – Mitogen-activated protein kinase

MCP-1 – Monocyte chemoattractant-1

M-CSF – Macrophage colony-stimulating factor

MetO – Methionine sulfoxide

MFI – Median fluorescence intensities

MHC – Major histocompatibility complex

MI – Myocardial infarction

Min – Minute

MIP – Macrophage Inflammatory Protein

ml – Milliliters

mM – Millimole

mm^3 – Cubic millimeter

µM – Micromole

µg – Microgram

µl – Microliter

µm – Micrometer

M/M – Monocyte/macrophage

MRM – Multiple reaction monitoring

mRNA – Messenger RNA

Mφ – Macrophage

MOI – Multiplicity of infection

MS/MS – Tandem mass spectrometry

m/z – mass-to-charge ratio

NaCl – Sodium Chloride

NC – Nucleocapsid

NCEP – National Cholesterol Education Program

nDOR – Normalized dihydrorhodamine oxidation rate

nHRA – Normalized HDL redox activity

Nef – Negative Factor

NF-kB – Nuclear factor kappa-light-chain-enhancer of activated B cells

ng – Nanogram

NKT – Natural killer T cells

NNRTI – Non-nucleoside reverse transcriptase inhibitors

NRTI – Nucleoside reverse transcriptase inhibitors

nm – Nanometer

OH – Hydroxyl

OPG – Osteoprotegerin

Ox-LDL – Oxidized low-density lipoproteins

OxPAPC – Oxidized L-*α*-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine

OU – Overweight untrained

PAF – Platelet activating factor

PAF-AH – Platelet-activating factor acetylhydrolase

PAFR – Platelet activating factor receptor

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PE – Phycoerythrin

PE-Cy7 – Phycoerythrin-Cy-7

PEG – Polyethylene glycol

pg – Picogram

pHDL – Pro-inflammatory HDL

PI – Proliferation index

PIs – Protease Inhibitors

PLA2 – Phospholipase A2

Pol – Polymerase

PON1 – Paraoxonase 1

RA – Rheumatoid arthritis

RANTES – Regulated on Activation Normal T Cell Expressed and Secreted

ROS – Reactive oxygen species

RT – Reverse transcriptase

RT-PCR – Real Time Polymerase chain reaction

PRRs – Pattern recognition receptors

RANK – Receptor activator of the NF-kB

RANKL – Receptor activator of nuclear factor-kB ligand

RCT – Reverse cholesterol transport

RFI – Relative fluorescence intensity

RNA – Ribonucleic acid

RPMI – Roswell Park Memorial Institute

RT – Resistance training

sCD14 – Soluble cluster of differentiation 14

sCD163 – Soluble cluster of differentiation 163

SD – Standard Deviation

SEM – Standard Error of Mean

SEVR – Subendocardial viability ratio

SIV – Simian immunodeficiency virus

SMART – The Strategies for Management of Antiretroviral Therapy

SPSS – Statistical Package for the Social Sciences

SRs – Scavenger receptors

SR-B1 – Scavenger receptor class B1

TCR – T cell receptor

TG – Triglycerides

TLR – Toll-like receptor

TNF – Tumor Necrosis Factor

TRAIL – TNF-related apoptosis-inducing ligand

TRAF6 – Tumor necrosis factor receptor-associated protein 6

Treg – T regulatory cells

U – Units

UC – Ultracentrifugation

UCLA – University of California, Los Angeles

V – Voltage

Vif – Viral Infectivity Factor

VLDL – Very Low Density Lipoproteins

Vpr – Viral protein R

wk – Week

WD – Western Diet

WHR – Waist-to-hip ratio

yr – Year

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Kelesidis T, Reddy ST, Huynh D, Meriwether D, Fogelman AM, Navab M, Yang OO. Effects of lipid-probe interactions in biochemical fluorometric methods that assess HDL redox activity. Lipids Health Dis 2012; 11:87. No permissions required. This work was supported by the RO1 grant HL095132 and by the Public Health Services grant HL-30568, the UCLA AIDS Institute and the UCLA Center for AIDS Research (AI28697). We would like to thank Dr Judith Currier for providing funding support through the NIH National Heart, Lung, and Blood Institute (RO1 HL095132). Partial funding for laboratory work was provided by the University of Washington's CVD and Metabolic Complications of HIV/AIDS Data Coordinating Center (5R01HL095126)

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3) Manuscript that is in preparation for submission:

Kelesidis T, Currier JS, Huynh D, Roberts CK, Fogelman AM, Navab M, Reddy ST, Yang OO. A High Throughput Biochemical Fluorometric Method for Measuring HDL Redox Activity. This work was supported by the RO1 grant HL095132, by the Public Health Services grant HL-30568, the UCLA AIDS Institute and the UCLA Center for AIDS Research (AI28697) Partial funding for laboratory work was provided by the University of Washington's CVD and Metabolic Complications of HIV/ AIDS Data Coordinating Center (5R01HL095126).

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1) Published manuscript:

Kelesidis T, Kendall MA, Yang OO, Hodis H, Currier JS. Perturbations of circulating levels of RANKL-osteoprotegerin axis in relation to lipids and progression of atherosclerosis in HIV- infected and -uninfected adults: ACTG NWCS 332/A5078 Study. AIDS Res Hum. Retroviruses 2013; 29:938-48. It is partially reprinted here with permission from Mary Ann Liebert, Inc. This work was supported by the RO1 grant HL095132, by the Public Health

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2) Published manuscript:

Kelesidis T, Yang OO, Kendall MA, Hodis HN, Currier JS. Dysfunctional HDL and progression of atherosclerosis in HIV-1-infected and -uninfected adults. Lipids Health Dis 2013; 12:23. No permissions required. Financial support was as above.

3) Submitted Manuscript:

Zanni MV*, Kelesidis T*, Fitzgerald ML, Lo J, Abbara S, Marmarelis E, Hernandez N, Yang OO, Currier JS, Grinspoon S. HDL Redox Activity is Increased in HIV- Infected Men in Association with Indices of Macrophage Activation and Cardiometabolic Risk. (*: co-first authors). Part of this manuscript is presented in this dissertation with permission from the authors: Zanni MV, Grinspoon S. This work was conducted with the support of a Medical Research Investigator Training (MeRIT) award from the Harvard Catalyst / The Harvard Clinical and Translational Science Center (National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health Award 8KL2TR000168-05. This work was also conducted with research funding from Bristol Myers Squibb to Dr. Grinspoon and with the support of National Institutes of Health Grants RO1HL112661 to Dr. Fitzgerald, K23 HL092792 to Dr. Lo, K24 DK064545 and RO1HL095123 to Dr. Grinspoon, M01-RR-01066, M01-RR-01066 and 1 UL1 RR 025758-01 to the Harvard Clinical and Translational Science Center from the National Center for Research Resources, and P30DK040561 to the Nutrition Obesity Research Center at Harvard. This work was also supported by the National Institute of Allergy and Infectious Diseases (AI068634 and AI056933), and UCLA AIDS Institute and the UCLA Center for

AIDS Research (AI28697). Partial funding for laboratory work was also provided by the University of Washington's CVD and Metabolic Complications of HIV/AIDS Data Coordinating Center (5R01HL095126).

4) Manuscript in Preparation:

Kelesidis T, Currier JS, Kelesidis I, Tseng CH, Yang OO. Dysfunctional HDL is associated with markers of immune activation in HIV-1 infected subjects with suppressed viremia. Financial support was as above.

5) Oral Presentations in International Conferences:

a) Kelesidis T, Kendall MA, Yang OO, Currier JS. The interplay of the osteoprotegerin/RANKL axis and dysfunctional HDL in HIV-positive adults: ACTG NWCS 332/A5078 study. Presented at XIX International AIDS Conference 2012, Washington, DC June 2012 [Abstract THAB0201].

b) Kelesidis T, Flores M, Tseng CH, Currier JS, Yang OO. HIV-infected adults with suppressed viremia on antiretroviral therapy have dysfunctional HDL that is associated with T cell activation. Abstract 662; Presented at IDWeek 2012, San Diego, CA October 2012

c) Zanni MV*, Kelesidis T*, Fitzgerald ML, Lo J, Abbara S, Marmarelis E, Hernandez N, Yang OO, Currier JS, Grinspoon S. HDL Redox Activity is Elevated in HIV+ Subjects in Association with Immune Activation and Parameters of Cardiometabolic Risk (Abstract SAT 723-745). Presented at Endo 2013 June 15-18,San Fransisco, CA. 2013
(*: co-first authors)

Chapter 7 contains work performed by Theodoros Kelesidis and Otto Yang in collaboration with Reddy ST, Huynh D, Otoniel Martinez-Maza, Fogelman AM, Navab M, Roberts CK, Currier JS and is a version of a manuscript that is in preparation for submission:

Kelesidis et al. Dysfunctional HDL directly upregulates T cell activation in HIV-1 infection.

This work was supported by the National Institute of Allergy and Infectious Diseases (AI068634 and AI056933), the UCLA AIDS Institute and the UCLA Center for AIDS Research (AI28697). Partial funding for laboratory work was provided by the University of Washington's CVD and Metabolic Complications of HIV/AIDS Data Coordinating Center (5R01HL095126).

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C. Selected Peer-Reviewed Publications (Of 75 [27 Original] Peer-Reviewed Publications, most relevant to current dissertation)

1. Kelesidis T et al: HIV-1 infected patients with suppressed plasma viremia on treatment have pro-inflammatory HDL. Lipids Health Dis 2011; 10:35.
2. Kelesidis T, et al: A biochemical fluorometric method for assessing the oxidative properties of HDL. J Lipid Res 2011; 52:2341-2351.
3. Kelesidis T, et al. Effects of Lipid-Probe Interactions in Biochemical Fluorometric Methods that Assess HDL Redox Activity. Lipids Health Dis. 2012; 11:87.
4. Kelesidis T, et al. Biomarkers of Microbial Translocation and Macrophage Activation: Association With Progression of Subclinical Atherosclerosis in HIV-1 Infection. J Infect Dis 2012; 206:1558-1567.
5. Kelesidis T et al. Dysfunctional HDL and progression of atherosclerosis in HIV-1-infected and -uninfected adults. Lipids Health Dis. 2013 Mar 5; 12:23.
6. Kelesidis T, et al. Perturbations of circulating levels of RANKL-osteoprotegerin axis in relation to lipids and progression of atherosclerosis in HIV-infected and -uninfected adults: AIDS Res Hum Retroviruses. 2013 Jun;29(6):938-48
7. Roberts CK, Katiraie M, Croymans DM, Yang OO, Kelesidis T. Untrained Young Men Have Dysfunctional HDL Compared to Strength Trained Men Irrespective of Overweight/Obesity Status. J Appl Physiol.[in press].

D. Selected Oral Presentations (Of 10 Original in International Conferences, most relevant to current dissertation). Examples are listed in the Acknowledgments section.

Chapter 1

Introduction

1.1 Overview

Human Immunodeficiency virus (HIV)-associated cardiovascular disease (CVD) is a growing clinical problem, which likely results from chronic inflammation/immune activation associated with oxidized lipids. Although High Density Lipoprotein (HDL) is considered protective in atherogenesis, oxidized HDL is dysfunctional and pro-atherogenic. Lipids play key roles in both viral replication and T cell biology, and thus oxidized HDL likely has multiple roles in the pathogenesis of HIV-1 infection. The overall goal of this research is to examine the interactions between oxidized HDL, HIV-1, immune activation, and atherogenesis. We hypothesize that a vicious cycle of HIV-1-induced immune activation, inflammation, production of oxidized HDL, and further immune activation explains the increased rate of atherosclerotic disease in HIV-1 infection.

Because this dissertation focuses primarily on the interplay between immunity, HIV-1, atherogenesis and lipids, the biology of the different components of these interactions will be presented in the Introduction of this dissertation. The first subsection **(1.2)** describes the human immune system, the second subsection **(1.3)** the process of atherosclerosis, the third subsection **(1.4)** the interplay between lipids and HIV-1, the fourth subsection **(1.5)** the role of systemic inflammation, immune activation and abnormal lipids in cardiovascular disease associated with HIV-1 infection, the fifth subsection **(1.6)** the HIV-associated chronic immune activation. Finally, in subsection **1.7** I outline the importance of the research presented in this dissertation and that establishing dysfunctional HDL as a novel mechanistic link between HIV-1, immune activation and atherosclerosis may initiate further studies to explore the efficacy of novel therapeutic interventions (such as HDL mimetic peptides) that might improve the prognosis of HIV infected patients.

1.2 The Human Immune System

The human immune system involves two major components: the innate immune system and the adaptive immune system.[1] Innate immunity is a defense system that identifies “non-self ” invasive agents[2] and provides adaptive immunity with information about the specific response that must be elicited.[3,4] By contrast, adaptive immunity produces an amplified pathogen-specific response. Following infection, immunological memory is formed, which can ensure a more rapid and effective response upon a second encounter with a pathogen. Because this dissertation focuses primarily on activation of the immune system (immune activation) that is important for both HIV-1 immunopathogenesis and cardiovascular disease special attention will be paid to the biology of macrophages (innate immunity) and T lymphocytes (adaptive immunity). The term “immune activation” will be used to indicate activation of both innate and adaptive immunity whereas the term “macrophage activation” and “T cell activation” will be used to indicate activation of the macrophages and T lymphocytes, respectively. The first sub-section **(1.2.1)** describes the macrophages whereas the next section **(1.2.2)** describes the lymphocytes.

1.2.1 The macrophages

Human macrophages display great anatomical and functional diversity.[5] Adult tissue macrophages are defined as end cells of the mononuclear phagocytic lineage derived from circulating monocytes that originate in the bone marrow. However, this definition is inadequate as macrophages have several origins.[6] Other functional classifications of macrophages refer to inflammatory state[7,8] but these classifications cannot represent the high transcriptional diversity of macrophages.[6] Macrophages are a very diverse set of cells that constantly shift their functional state in response to changes in tissue physiology or environmental challenges resulting in a causal association of macrophages with disease states, such as atherosclerosis, obesity and cancer.

1.2.2 The T lymphocytes

The adaptive immune system is composed of humoral and cell-mediated immune components. The extracellular spaces are protected by the humoral response, in which antibodies produced by B cells can prevent the spread of infection by binding to extracellular microorganisms and invoking a directed immune response.[9] On the other hand, pathogens which replicate inside cells, and cannot be detected by antibodies are destroyed by the cell-mediated response, which involves the actions of various subpopulations of T lymphocytes. Cell-mediated responses depend on the direct interaction between T lymphocytes and infected cells, which present antigens that the T lymphocytes recognize.

There are two general classes of T lymphocytes: $CD4^+$ and $CD8^+$ T lymphocytes. Upon antigen recognition, naïve $CD4^+$ T lymphocytes generally mature into helper T cells, which produce various cytokines that can activate and regulate either the humoral or the cell-mediated response. On the other hand, most naïve $CD8^+$ T lymphocytes mature into cytotoxic T lymphocytes upon activation, which eliminate cells that have been invaded by intracellular organisms. In all cases, T lymphocytes recognize their targets by detecting peptide fragments (epitopes) derived from the foreign proteins of the invading pathogen. These epitopes are first captured by major histocompatibility complex (MHC) molecules in the host cells, and are then displayed on the cell surface.[10-12]

1.3 The process of atherosclerosis

Atherosclerosis is the most frequent cause of death in industrialized societies. It is a complex disease of the arterial wall that involves both innate and adaptive immunity as well as lipoproteins and is characterized by the formation of atheromas (atherosclerotic plaques) that can lead to ischemia, thrombosis, myocardial infarction, or stroke.[13,14] Because this dissertation focuses primarily on the role of innate and adaptive immunity and lipoproteins, in HIV-1 immunopathogenesis and cardiovascular disease, the contribution of innate **(subsection 1.3.1)**, adaptive immunity **(subsection 1.3.2)** and lipoproteins **(subsection 1.3.3)** in atherogenesis will be reviewed separately.

1.3.1 Innate immunity and atherosclerosis

Inflammation is considered a physiological response to tissue stress, injury, and infection.[15] It is not completely understood how inflammatory events that occur in atherosclerosis contribute to the formation and evolution of atherosclerotic lesions. It is known that low-density lipoproteins (LDL) particles, when retained and oxidized in the arterial wall, are the main inducers of the pathological process.[16] It is assumed that macrophage activation is a key event in atherogenesis and that macrophages in the arterial wall, ingest modified LDL particles, become lipid-laden foam cells and initiate inflammation.[17]

Oxidation reactions and phospholipid peroxidation generate identical oxidation-specific epitopes within cell membranes and lipoproteins that alert innate immunity for the presence of dangerous oxidized waste.[18,19] Oxidized LDL (ox-LDL) represent “modified self ” that has immunogenic properties, can activate receptors of innate immunity that identify these lipoproteins called scavenger receptors (SRs) and can promote effector responses that lead to atherogenesis.[20] Thus, although scavenger receptors are used by cells mainly to take up modified (oxidized or acetylated) LDL,[21-23] CD36,[23] scavenger receptor class B1 (SR-B1),[24] and lectin-like oxidized LDL receptor-1 (LOX-1)[24,25] also bind other modified

lipoproteins such as oxidized High density lipoprotein (HDL). A new area of research has emerged with the discovery that SRs are also expressed on lymphocytes where they cooperate with signaling pattern recognition receptors (PRRs) to regulate their function. Thus, SRs represent a highly specialized tool of innate immunity to control tissue homeostasis, pathogen elimination, and other functions, rather than act as specific receptors for modified LDL internalization.[26-29] However, the mechanisms of atherogenesis are numerous and have not been fully elucidated. Among these mechanisms, activation of the scavenger receptors (SRs) and the NF-kappaB (NF-kB) pathway in macrophages and endothelial cells are major mechanisms of atherogenesis that also mediate macrophage and T cell activation and the interplay of lipids with immunity and are thus the focus of study in this dissertation.

1.3.2 Adaptive immunity and atherosclerosis

Although T cells have been identified in atherosclerotic lesions, it is unclear whether plaque T-cell activity is specific against a common antigen.[12,30] Different autoantigens are considered to be the activators of a specific T-cell response in the lesions, and among them ox-LDL epitopes are the main candidate.[12,30] The presence of autoreactive T cells specific for heat shock proteins[31] autoantigens (such as DNA antigens) accumulated in the atherosclerotic lesion[32] local loss of Treg cells' function[33] the generation of a pathologically specific Th2 response against modified lipoproteins or against an unidentified specific antigen in the lesions[34] have all been suggested to contribute to atherogenesis. Studies of atherosclerosis in hyperlipidemic mice demonstrate that adaptive immunity profoundly affects lesion formation.[35] Atherosclerosis is the result of a complex outcome of the balance between subsets of immune cells and their products[36] and much progress has been made in understanding this balance through studies of murine models (reviewed in [36]).

Over many decades, it was assumed that the adaptive immune response was exclusively directed against antigenic peptides presented by MHC class I and II molecules.

However, the discovery of CD1 proteins, a group of molecules that are specialized to bind and present lipid-containing antigens to restricted T cells, has opened a new dimension in our understanding of how immunity works.[37] It is also accepted that apoE lipoproteins serve as a depot of lipid antigens that can be released to stimulate immunity both locally and distant from the site of infection.[38] Finally, although scavenger receptors are mainly expressed on macrophages and endothelial cells,[24,25] oxidized lipids and lipoproteins can activate T lymphocytes *in vitro*, and directly increase expression of scavenger receptors such as LOX-1.[39,40] Thus, lipid antigens and lipoproteins can directly interact with adaptive immunity that plays a major role in atherogenesis.

1.3.3. Modified lipoproteins and atherosclerosis

Modified LDL has proatherogenic effects and normal high density lipoprotein (HDL) has antiatherogenic effects. Inflammation and imbalance of lipid metabolism are tightly interrelated.[41] As outlined above modified (oxidized or acetylated) LDL has a major role in the pathogenesis of atherogenesis.[21-23] Normal high density lipoprotein (HDL) protects against LDL-induced cytotoxicity[42] and has been observed to prevent LDL oxidation by cultured endothelial cells[43] and the induction of human aortic endothelial cells and smooth muscle cells to produce monocyte chemoattractant-1 (MCP-1) by LDL.[44] Normal HDL and its components such as apolipoprotein (apo) A-I can remove or inhibit the activity of lipids in LDL, which are required for oxidation such as hydroperoxyoctadecadienoic acid (HPODE) and hydroperoxyeicosatetraenoic acid (HPETE).[45] These atheroprotective effects of normal HDL are secondary to HDL-associated enzymes such as paraoxonase[46] and platelet activating factor acetylhydrolase[47] which can prevent or destroy the formation of the LDL-derived oxidized phospholipids.

Roles of HDL in atherogenesis are not simply quantitative, but are highly qualitative. While normal HDL counteracts oxidized lipids and protects against cardiovascular disease (CVD), HDL can become oxidized and dysfunctional in the setting of chronic inflammatory

diseases.[48,49] Van Lenten et al. reported that the activity of atheroprotective HDL-associated enzymes was reduced in rabbits and humans during the course of an acute phase reaction, resulting in HDL that increased cell-mediated LDL oxidation and increased monocyte chemoattractant-1 (MCP-1) production.[50] HDL from coronary artery disease patients with normal blood lipid levels is defective both in its ability to prevent LDL oxidation and in its ability to inhibit the biologic activity of oxidized lipids.[51] This impaired HDL becomes pro-inflammatory, as characterized by (i) decreased levels and activity of anti-inflammatory, antioxidant factors,[52] (ii) gain of pro-inflammatory proteins,[50] (iii) increased lipid hydroperoxide content,[53] (iv) reduced potential to efflux cholesterol,[49] and (v) diminished ability to prevent LDL oxidation.[54,55] Dysfunctional HDL contains a significantly altered proteome including increased amounts of acute phase proteins.[56-58] This modified, dysfunctional HDL has been associated with increased CVD risk of patients with familial hypercholesterolemia[59] chronic kidney disease [60]various inflammatory conditions such as diabetes,[48,61] metabolic syndrome[62] autoimmune diseases such as systemic lupus erythematosus,[63] rheumatoid arthritis,[63,64] scleroderma,[65] inflammatory bowel disease[66] and leprosy.[67] Thus, dysfunctional HDL has a key role in atherosclerosis.[49]

There is limited understanding of HDL function in humans. Measuring HDL cholesterol levels provides information about the size of the HDL pool, but does not predict HDL composition or function. HDL particles are heterogeneous in shape, density, size, composition and have multiple functional properties such as reverse cholesterol transport (RCT), anti-oxidant, anti-inflammatory, and antithrombotic activities.[49,61] Due to the complexity of the HDL particles, HDL function has not been studied extensively in humans. Currently, HDL functional properties are mostly determined by cell-based assays such as measurement of cholesterol efflux capacity.[68,69] A cell based assay based on the ability of HDL to prevent LDL induced oxidation, monocyte chemotaxis and MCP-1 production in the presence of human arterial wall endothelial cells can be used to determine HDL function.[55] The values obtained with LDL plus the test HDL are divided by the values obtained with LDL alone to give the HDL-inflammatory index (HII). Thus, LDL alone would give an HII of 1.0.

Proinflammatory HDL was defined by an HII value ≥ 1.0 and antiinflammatory HDL was defined by an HII value < 1.0. HDL from human subjects with proinflammatory HDL as assessed by the cell based assay was also less effective in promoting cholesterol efflux from cholesterol loaded human monocyte-macrophages *in vitro* than HDL from human subjects with antiinflammatory HDL and a cholesterol efflux assay can also be used to determine HDL function.[70] A recent study demonstrated that cholesterol efflux capacity from macrophages, a metric of HDL function, has a strong inverse association with measures of atherosclerosis, independently of the HDL cholesterol level.[71] Another study showed that HDL has less anti-inflammatory capacity, as assessed by HDL inflammatory index (HII) - a measure of the ability of HDL to mitigate oxidation of LDL- in the setting of acute coronary syndrome (ACS) compared with controls or subjects with chronic coronary artery disease (CAD).[72] However, these cell-based assays are highly labor-intensive and subject to substantial assay variation.[73] In addition, the optimal method for performing such studies with regard to donor cells, type of acceptor and readout, has yet to be determined and standardization of these methods is needed. Thus, the limitations and labor of these cell-based assays render them inaccessible to many researchers, and difficult to scale up for large-scale clinical trials or routine clinical use.[73]

The isolated redox (pro-oxidant/antioxidant) activity of HDL is associated with HDL function, and thus cell-free assays may allow a direct biochemical assessment of HDL function. Recent interest has focused on the functional consequences of HDL oxidation.[54,74] Oxidation could conceivably contribute to the formation of dysfunctional HDL, proposed to be present in humans with cardiovascular disease.[54,74] Oxidized phospholipids and direct oxidation of HDL may reduce the activity of anti-atherogenic enzymes lecithin-cholesterol acyltransferase (LCAT), paraoxonase (PON1) and platelet-activating factor acetylhydrolase and may reduce the ability of HDL to mitigate monocyte chemotaxis[75] and perform other normal functions such as cholesterol efflux.[76,77] Injection of oxidized phospholipids into atherosclerosis susceptible C57BL/6J mice, but not in atherosclerosis resistant C3H/HeJ mice, resulted in a decrease in PON1 activity.[78] Forte et al. reported that mouse models of

atherosclerosis have elevated plasma levels of oxidized phospholipids, which result in decreased activity of anti-atherogenic enzymes LCAT, paraoxonase and platelet-activating factor acetylhydrolase compared with controls.[79] HDLox has increased content for lipid hydroperoxides [54,80] that are also known to be present in oxidized Low density Lipoprotein (LDLox) and that mediate atherogenesis.[54] Levels of reactive oxygen species (ROS), such as lipid hydroperoxides produced from oxidation of lipoproteins, are significantly associated with the formation of dysfunctional HDL[54,55,74] and the oxidative properties of HDL are closely associated with the function of HDL.[75] A first generation assay detecting ROS using the fluorescent probe DCF (2', 7'-dichlorofluorescein) showed correlation to a measure of the ability of HDL to mitigate cellular effects of oxidation of LDL.[55] However, the oxidative instability of DCF has rendered the DCF assay technically challenging. Thus, robust assays to evaluate the function of HDL in these inflammatory states are needed to supplement the measurement of HDL cholesterol levels in the clinic.

1.4. The interplay between lipids and Human Immunodeficiency virus

Viruses can directly regulate lipid metabolism and vice versa lipids can directly regulate the life cycle of many viruses. Numerous viruses such Hepatitis B virus,[81] Hepatitis C virus (HCV),[82,83] Cytomegalovirus (CMV),[84] West Nile virus, [85] Dengue virus,[86] may directly manipulate and alter cellular cholesterol synthesis and lipid metabolism. On the other hand, certain virions such as HCV associate with lipids and apolipoproteins, and this is thought to alter the physical properties of virions, in addition to greatly enhancing infectivity.[87,88] For example, HCV assembles virions at lipid droplets, and secretion of infectious virus is dependent upon the secretion pathway of lipoproteins.[89,90] HCV and some enteroviruses also depend on phospholipid signaling for replication.[91] Many viruses such as HCV, Dengue virus, enteroviruses also require components of the autophagocytic machinery, which is directly regulated by the lipid metabolism,[92] for efficient viral replication. HDL, which is the ligand for scavenger receptor SR-BI, enhances the process of HCV viral entry whereas oxidized LDL and VLDL block the entry process.[90] Lipoprotein lipase is the key enzyme involved in lipolysis of triglycerides in circulating VLDL and modulates cellular uptake of the HCV virus.[90] Apolipoprotein A-I (apoA-I), the major protein of HDL, is required for replication of hepatitis C virus and apoA-I may be a new possible target for antiviral therapy.[93] The Human Immunodeficiency virus is also highly dependent on lipids to complete its life cycle.

The Human Immunodeficiency Virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS).[94-97] The virus, first identified in 1983, is a member of the genus Lentivirus in the Retroviridae family.[98,99] The basic pathology in AIDS is a loss of $CD4^+$ T lymphocytes and a variety of disorders in immune cell function. The development of this disease results from a lack of control of HIV replication by the host immune system.[100-102] AIDS is defined by the reduction in $CD4^+$ T lymphocytes (<200 cells/μl) or the onset of opportunistic infections and malignancy. Neurologic and gastrointestinal disorders are also commonly found in AIDS patients. There are two types of HIV: HIV-1 and HIV-2. Although both viruses can cause AIDS, HIV-1 has had a greater impact on public health because it is

more virulent, widespread, and infectious than HIV-2.[103] This dissertation, therefore, focuses only on HIV-1.

HIV-1 contains cholesterol binding proteins and can directly interact with lipids. The HIV-1 virion is about 100-120 nm in diameter[104] and contains the envelope and three structural Gag proteins: matrix (MA, p17), CA (p24), and nucleocapsid (NC, p7), which result from the proteolytic cleavage of the Gag precursor p55. The surface of HIV-1 virions is covered with gp120-gp41 heterodimers which are embedded in a lipid bilayer and attached to matrix proteins. The viral capsid is composed of the viral p24 Gag capsid (CA) protein and contains two copies of the single-stranded RNA genome and various viral proteins, including reverse transcriptase (RT, p66), integrase (IN, p32), the NC proteins (p9 and p7), Vif, Nef, and Vpr.[105] Certain viral proteins such as Gag and Nef bind cholesterol and interact with lipids directly through their myristylated moiety.[106,107]

The life cycle of HIV-1 heavily depends on lipid interactions. Lipid rafts in the host cell membrane have key roles in viral replication and contain the outer membrane CD4 and chemokine receptors utilized by the HIV-1 Env to bind and enter target cells, and are important for the assembly of budding virions.[108-110] As mentioned above, the surface of the HIV-1 virion is covered with two envelope proteins, gp120 and gp41, which mediate fusion of the virus with a host cell which is then released into the cell. Shortly after this process, the viral core proteins disintegrate, allowing the RNA genome, reverse transcriptase, integrase and other viral proteins to enter the cell where new virions are formed.[111-113] Nascent virions then bud from the cell surface, taking up the viral envelope proteins. After the virion is released, the viral protease cleaves Gag and Gag-Pol polypeptides into various structural and functional proteins to form mature virions.[105] Recent studies have shown that lipids on the host cell membrane can influence the efficiency of HIV virus-cell fusion and that viral cholesterol-binding proteins in HIV such as gp41 have important roles in virus entry.[106,107] Oxidized lipids can directly increase expression of chemokine receptors that mediate the entry of HIV into a cell[107,114,115] and can also affect HIV infectivity.[116,117] Finally, HIV Nef alters the lipid composition of virions and cells, binds cholesterol *in vitro* and *in vivo*[118] and directly

affects metabolism of cholesterol.[119] Normal HDL may have an antiviral role; amphipathic domains of apoA-I reduce gp41-mediated membrane fusion through direct interactions with virions.[120,121] Viral cholesterol-binding proteins such as Nef directly affect lipid rafts of natural target cells of HIV-1 (macrophages, $CD4^+$ T cells) and these effects may increase HIV-1 infectivity.[106,114,116,118,119] Indeed, HIV-1 Nef mobilizes lipid rafts in macrophages through a pathway that competes with ABCA1-dependent cholesterol efflux, a major function of HDL, [122] and lipid rafts are a major determinant of HIV-1 antiviral responses.[123-129] Oxidized lipoproteins like ox-LDL may directly modify lipid rafts.[130] The effect of HDL oxidation on this process is unknown, but lipid oxidation decreases the stability, alters the orientation of apoA-I on HDL particle[131] and thus could interfere with the normal antiviral activity of HDL. Thus, it is possible that HIV infection induces a vicious cycle of changes in the oxidized lipids that can also further affect the HIV per se as well as the immune system.

The HIV-1-specific $CD8^+$ T lymphocyte (CTL) response is a key arm of immunity that contributes to partial control of viral replication within infected persons and may be regulated by oxidized lipids. HIV-1 is capable of stimulating strong CTL immune responses in infected patients, despite causing profound immunodeficiency. Data from *in vivo* studies that have shown a temporal relationship between the rise of CTL responses and the fall of HIV-1 viremia during the acute phase of infection,[132-135] together with *in vitro* viral suppression experiments and the observations of CTL-mediated escape mutations *in vivo*,[136-138] support the role of CTLs in the control of HIV-1 infection. Certain HLA-I allele types are associated with the rate of progressing towards AIDS after HIV-1 infection.[139,140] Despite the strong induction of CTL responses by HIV-1 during the acute phase of infection, containment of HIV-1 by the immune system eventually fails, and without antiviral retroviral therapy most patients will progress to AIDS. The CTL function is markedly abnormal in most infected persons, [141,142] showing inappropriate activation, loss of functions such as cytokine production and proliferation, exhaustion and abnormal differentiation. A variety of host (CTL effector functions, antigen specificity, and HLA-I restriction) and viral (e. viral escape mutation, downregulation of HLA-I molecules by HIV-1 Nef) factors could affect the quality of

CTL response to effectively control HIV-1.[143] While each of these factors greatly impacts the ability of CTLs to control HIV-1 infection, this dissertation will only focus on the possible role of oxidized lipids as determinants of CTL antiviral efficiency based on the effects of oxidized lipids on adaptive immunity as outlined above and the established immunoregulatory effects[30,39,144-147] of lipid hydroperoxides. Thus abnormalities of CTLs could be driven by oxidized HDL, through direct loss of CTL function or indirect effects via HDL impact on antigen presenting cells.

1.5 The role of systemic inflammation, immune activation and abnormal lipids in cardiovascular disease associated with HIV-1 infection

Increased cardiovascular disease (CVD) in HIV-1 infection is a growing clinical problem. Since the advent of effective antiretroviral therapy (ART), management of the long-term complications of HIV-1 infection has moved to the forefront of care for many patients since ART has extended life expectancy[148] and reduced deaths related to opportunistic infections.[149] However, recent data suggest that CVD represents an increasingly important cause of death among HIV-infected patients.[150,151] Studies show an approximately two-fold increased risk in ischemic heart disease[152,153] and four-fold increased risk in sudden cardiac death[154-156] in the HIV-infected population relative to the general population. However, the relative contributions of viremia, immune activation, ART and inflammation to the increased CVD risk are unclear.[155] Understanding and addressing the causes of this increased CVD risk is an important public health concern for HIV-infected patients.

Chronic systemic inflammation is an essential element of the pathogenesis of atherosclerosis in HIV-1 infection. The role of inflammation in atherosclerosis has been confirmed by the strong association between the most sensitive marker of inflammation, C-reactive protein (CRP) and clinical manifestations of atherosclerosis[157-159] as well as many mechanistic studies.[160,161] In addition, systemic inflammatory disorders are commonly associated with an increased risk of atherosclerosis.[162] Among the many manifestations of inflammation, relevant atherogenic events include induction of expression of adhesion molecules on endothelium[163] and monocytes[164] and secretion of chemokines attracting leukocytes to the vessel wall.[165] Plasma levels of a number of proinflammatory cytokines are increased in HIV-infected individuals even in patients with low residual viremia.[166] Increased levels of the acute-phase response markers have been reported in patients with all-cause mortality during ART interruption in the SMART study.[167] Although the immune response to HIV replication is likely to be a major source of elevated plasma cytokines, HIV infection is also associated with extensive damage to gut mucosa, followed by increased translocation

of microbial components from the gut into the bloodstream such as lipopolysaccharide (LPS).[168] Plasma (LPS)-binding proteins, such as sCD14 are markers of microbial translocation that are elevated in HIV infection[168] and are associated with HIV disease progression.[169] Microbial translocation causes low-grade endotoxemia, which is associated with chronic systemic inflammation and activation of the peripheral T cell compartment[168] and monocytes.[170] Thus, it is possible that increased microbial translocation during chronic HIV infection is linked pathogenetically to the increased risk of CVD in HIV-infected individuals but this remains to be further elucidated.

Residual immune activation in treated HIV-1 may be contributing to atherosclerosis. HIV-1 infection is characterized by a chronic state of immune activation that is an independent predictor of disease progression. Although viremia is suppressed with ART, this immune activation does not normalize with therapy.[171] It is believed that this process is mediated by bowel microbial translocation.[171,172] Despite the strong evidence for associations between immune activation and coronary artery disease (CAD), there is limited information about the activation status of monocytes in individuals with a higher risk of CAD.[173] Monocytes from HIV-infected patients possess many characteristics of activated monocytes, e.g., expression of activation markers CD38, CD69, antigen-presentation molecules HLA-DR which correlates with plasma LPS levels, [170] and spontaneous production of proinflammatory cytokines.[174] Early studies indicated that the proportion of $CD14^+/CD16^+$ monocytes was increased in HIV-infected individuals[175] in comparison with the major $CD14^+$ subset.[176] HIV infection *in vitro* augments expression of β-2 integrins which promote monocyte transendothelial migration, a key event in atherogenesis. [177-179] In an vitro model of HIV-infected, monocyte-derived macrophages HIV-1 reduced emigration of macrophages out of plaques.[180] Thus, HIV-1 may contribute to the increased risk of CAD through macrophage activation and persistence of macrophages within a developing plaque.

Lipid abnormalities induced by antiretrovirals may play a major role in atherogenesis in HIV-1 infection. As outlined above imbalance of lipid metabolism is the key driver of atherosclerosis. The contribution of ART to risk of CAD is illustrated by the detrimental

effects of intermittent therapy, as reported in the SMART study, [181] as well as the effects of specific regimens, especially those containing Protease Inhibitors (PIs) and the nucleoside reverse transcriptase (RT) inhibitor abacavir.[182,183] The PIs are also causally associated with lipodystrophy, hyperlipidemia and insulin resistance, all of which are strong proatherogenic risk factors. Thus, ART may increase CAD risk, but treatment interruption does not mitigate or possibly increases this risk even further.[181]

HIV-1 infection is associated with systemic lipoprotein abnormalities. At a systemic level, disturbances of plasma lipoprotein metabolism associated with HIV infection and immune dysfunction are characterized by lowered levels of LDL and HDL cholesterol[184,185] and increased levels of triglycerides and are consistent with an atherogenic lipoprotein profile.[186] Recently, it was demonstrated that a decline in HDL levels in treatment-naïve patients correlated with the HIV viral load,[187] supporting the direct role of HIV infection in impairment of cholesterol metabolism. HIV-infected patients also have elevated activity of cholesteryl ester transfer protein, resulting in increased transfer of cholesteryl esters from antiatherogenic HDL to LDL.[188] The mechanisms underlying how HIV infection of monocytes and T cells induces systemic effects on lipoprotein metabolism are not known.

HIV-1 can directly regulate atherogenesis through direct effects on cellular elements of lipid metabolism. On the cellular level, HIV may block reverse cholesterol transport effectively from macrophages by the suppressing ABCA1-dependent pathway and causes accumulation of cholesterol in macrophages.[189] Down-regulation of ABCA1 is mediated by the HIV protein Nef and may be part of the virus' strategy to increase cholesterol content of plasma membrane lipid rafts and assembling virions, resulting in increased viral production and increased infectivity of produced virions.[122,189,190] Examination of atheromatous plaques from HIV-infected individuals showed increased plaque lipid content compared with uninfected donors.[191] Thus, HIV infection impairs reverse cholesterol transport, diminishing cells' ability to remove excessive cholesterol.

1.6 HIV-associated chronic immune activation.

Systemic chronic immune activation is considered as the driving force of $CD4^+$ T-cell depletion and acquired immunodeficiency syndrome (AIDS). Indeed, more recent studies support this: (i) successfully ART-treated patients with undetectable viremia still show higher levels of T-cell activation than healthy controls, with the extent of this residual immune activation being associated with increased morbidity and mortality;[192] (ii) the levels of activated $CD8^+$ T cells were shown to be more closely associated with a shorter survival than viral load or $CD4^+$ T cells;[193] (iii) comparative studies on HIV-2-infected individuals are consistent with a direct causal relationship between $CD4^+$ T-cell depletion and immune activation but not with the virus replication rate;[194] (iv) a few HIV-1-infected individuals with high viremia do not progress to AIDS;[195,196] and (v) elite controllers of HIV, i.e. individuals who spontaneously control viral replication to below detection levels, show levels of T-cell activation higher than healthy donors and even higher than ART-suppressed individuals.[197] Most of the cytokines involved in chronic immune activation and inflammation during HIV-1 infections can be produced by multiple cell types including lymphocytes, macrophages, neutrophils, or natural killer T (NKT) cells.[198]

T cell activation is a major component of immune activation and expression of CD38 is one of the most predictive markers of abnormal immune activation during HIV-1 infection. The first descriptions of HIV-associated immune activation were based on analyses of blood samples collected from chronically HIV-infected individuals. These analyses revealed many parameters of T-cell activation and inflammation quantified in the blood that have been shown to predict the levels of disease progression independently of $CD4^+$ T-cell counts and viral load.[198] Increased numbers of HLA-DR^+, $CD38^+$ $CD8^+$ T lymphocytes were associated with a fall in $CD4^+$ T-cell counts and the development of AIDS.[199] The expressions of the activation markers CD38 and HLA-DR are also increased on HIV-specific $CD8^+$ T cells and correlate with HIV viral load.[200] Although increased numbers of HLA-DR^+, $CD38^+$ $CD8^+$ T-cells were associated with disease progression in HIV-1 infection[199] it is less stronger

marker for the risk of chronic HIV disease progression to AIDS and death compared to elevated CD38 antigen expression on $CD8^+$ T-cells.[201]

Monocyte/macrophage (M/M) activation is also a major component of immune activation. Recent data point toward an association between M/M activation and disease progression. In viremic, untreated patients, the numbers of pro-inflammatory $CD14^+CD16^+$ monocytes are increased.[176,202] In SIV-infected macaques, massive turnover of monocytes predicted progression toward AIDS better than viral load.[203] Less is known on HIV-2, but sCD14, a marker of monocyte activation and an indirect marker of microbial translocation, is also negatively correlated with $CD4^+$ T-cell counts in HIV-2-infected individuals.[204] Soluble CD163 is a M/M-specific scavenger receptor that is also an important marker of macrophage activation and HIV activity in early and chronic infection prior to and after ART.[205,206]

The mechanisms that contribute to chronic immune activation during HIV infection are unclear. Given the complexity of the interaction between HIV and the host immune system, there are multiple mechanisms by which HIV infection may induce immune activation including HIV-1 replication, increased production of pro-inflammatory molecules, microbial translocation and increased homeostatic proliferation in response to $CD4^+$ T-cell depletion.[198] It is possible that several of the proposed mechanisms synergistically contribute to cause abnormal chronic immune activation and the relative contribution of the different mechanisms changes significantly in different phases of HIV infection, in different subsets of HIV-infected individuals, and in naive versus ART-treated patients.

Oxidized lipids could also contribute to immune activation associated with HIV-1 infection. Oxidized lipids may be a significant mediator in the interplay between inflammation, immune activation and atherosclerosis.[207] Inflammation increases oxidized lipids that mediate atherogenesis and which have also diverse and important regulatory functions within both the innate[144] and adaptive immune systems.[39] The effects of the oxidized lipids on the immune system may be mediated though multiple mechanisms including changes in activity of phospholipase A2,[208] changes in levels of receptors for oxidized lipids,[208,209] chemokine receptors expressed on immune cells,[114] changes in

autophagy[210] and changes in lipid rafts.[211] However, these mechanisms of oxidized lipids have not been studied in HIV infection. In view of the established immunoregulatory effects of lipid hydroperoxides[30,39,144-147] that may oxidize HDL and make it dysfunctional as outlined above and the major effects of both normal[30,212] and modified HDL[67,213,214] in regulation of innate immunity, this dissertation will examine whether dysfunctional HDL may also contribute to HIV-1 induced immune activation.

The NF-kB pathway is a major player in the intersection of HIV-1, lipids, immunity and atherogenesis. It is known that activation of immunity induces the NF-κB-dependent production of cytokines and other proinflammatory molecules in a variety of cell types.[212,215] Previous *in vitro* studies have shown that oxidized lipoproteins can directly upregulate production of the receptor activator of nuclear factor-kB ligand (RANKL), a soluble product of activation of the NF-kB pathway, from T cells through scavenger receptors[39,216] and that oxidized HDL can also upregulate the NF-kB pathway.[217] RANLK activates its receptor, receptor activator of the NF-kB (RANK), which is largely expressed by osteoblasts, T and B cells, dendritic cells and fibroblasts and has important immunological functions including the regulation of T-cell growth and dendritic cell functions.[218,219] Osteoprotegerin (OPG), a TNF receptor superfamily member and RANKL decoy receptor, is produced by many tissues including vascular, bone, hematopoietic and immune cells, prevents RANKL from binding to RANK, moderating RANKL-induced osteoclast formation and activity. [218,219] The decisive roles played by RANKL/OPG axis in regulating bone metabolism, the immune system and cardiovascular disease in patients not infected with HIV-1 have been recently reviewed [220-222] and are also summarized in **Figure 1.1** and in **Table 1.1**. Lymphocytes influence the basal production of bone-sparing OPG, however, under inflammatory conditions activated lymphocytes become a significant additional source of RANKL. [221] Thus, elevated RANKL levels in chronic inflammatory conditions can be caused by more intensive synthesis of RANKL and/or a non-sufficient upregulation of OPG synthesis which in turn leads to increased RANKL/OPG ratio. An increased RANKL/OPG ratio has been associated with bone resorption and atherogenesis.[223] Data from both *in vitro* and *in vivo* studies reviewed in

this manuscript and also summarized in **Figure 1.2**[224-237] provide evidence for the significant role of RANKL/OPG in HIV infection that may affect the immune system, and the risk for bone and cardiovascular disease. Thus, it is possible that RANK-L the NFκB pathway may mediate the interplay of immune cells and oxidized lipids and may contribute to accelerated atherosclerosis in patients with HIV. Further studies are needed to elucidate the role of OPG/RANKL axis in HIV-1 infection that may open a window to novel strategies to forestall an epidemic of future bone or cardiovascular disease in this population.

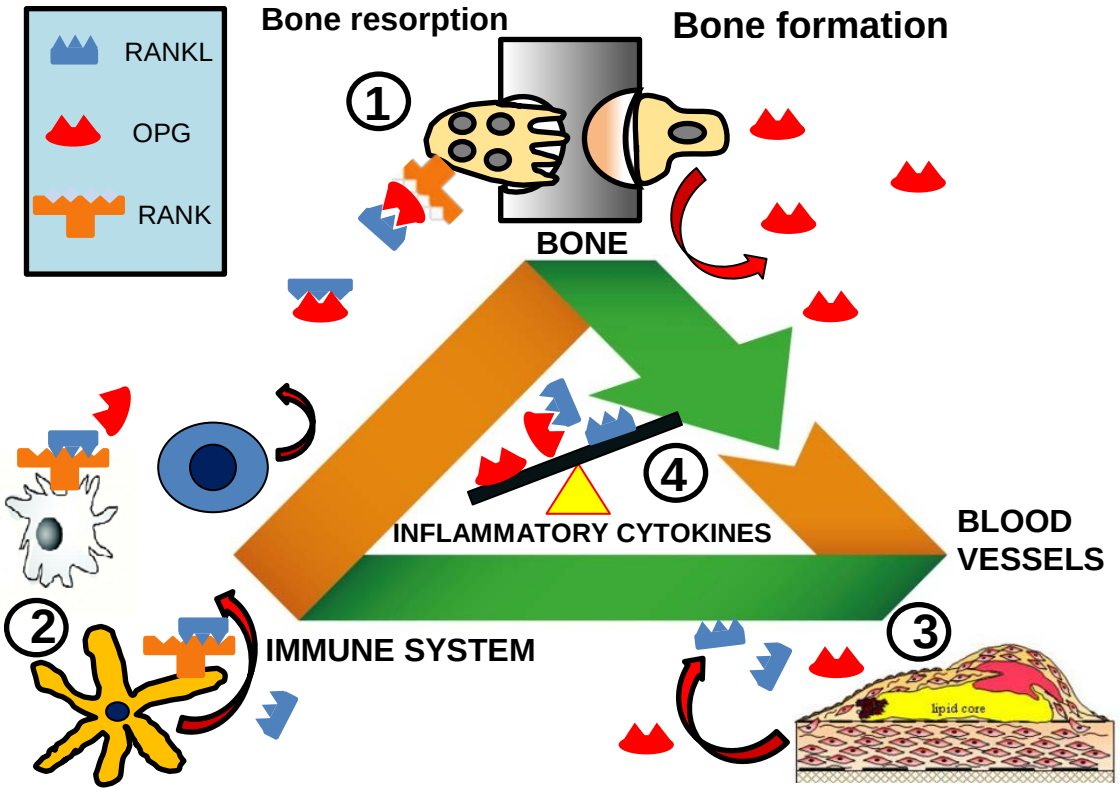

Figure 1.1: The OPG/RANKL system plays critical role in regulating bone metabolism the immune system and cardiovascular disease. 1. Activation of the RANKL–RANK pathway is essential for normal osteoclast differentiation and activation whereas OPG has also been identified as a regulator of bone formation. In the presence of proinflammatory cytokines RANKL expression is induced in osteoblasts, activated T cells, synovial fibroblasts and bone marrow stromal cells, and subsequently binds to its specific membrane-bound receptor RANK and promotes osteoclast differentiation, activation and survival. Conversely, OPG expression is induced in multiple tissues including bone, liver, dendritic cells and activated lymphocytes by factors that block bone catabolism and promote anabolic effects. OPG binds and neutralizes RANKL, leading to a block in osteoclastogenesis and decreased survival of pre-existing osteoclasts. Thus, the OPG/RANKL system regulates the cross-talk between osteoblasts and osteoclasts which determines the bone density and is recognized as one of the major mechanisms of development of osteoporosis. **2.** On the other hand changes in the OPG/RANKL system have also been associated with changes in the immune system (macrophages, dendritic cells, lymphocytes) which are reviewed in Table 3. These immune cells are involved in systemic inflammation, osteoclastogenesis and atherogenesis

3. OPG is found not only in bone, but also in the blood vasculature where its presence has been associated with calcification, and inflammation. In the presence of proinflammatory cytokines, expression of OPG is upregulated in endothelial cells which in turn increases the expression of endothelial cell adhesion molecules helping in the transmigration of lymphocytes and monocytes into the arterial wall. The inflammatory cells upregulate expression of RANKL and formation of vascular smooth cells; RANKL along with OPG and increased RANKL/OPG ratio increase activity of matrix metalloproteinases, leading to increased atherogenesis. **4.** Increased ratio of circulating RANKL/OPG in the setting of systemic inflammation promotes osteoclastogenesis and atherogenesis. Abbreviations: HIV: Human Immunodeficiency Virus, OPG: osteoprotegerin, RANK: Receptor activator of nuclear factor kappa-B, RANKL: Receptor activator of nuclear factor kappa-B ligand

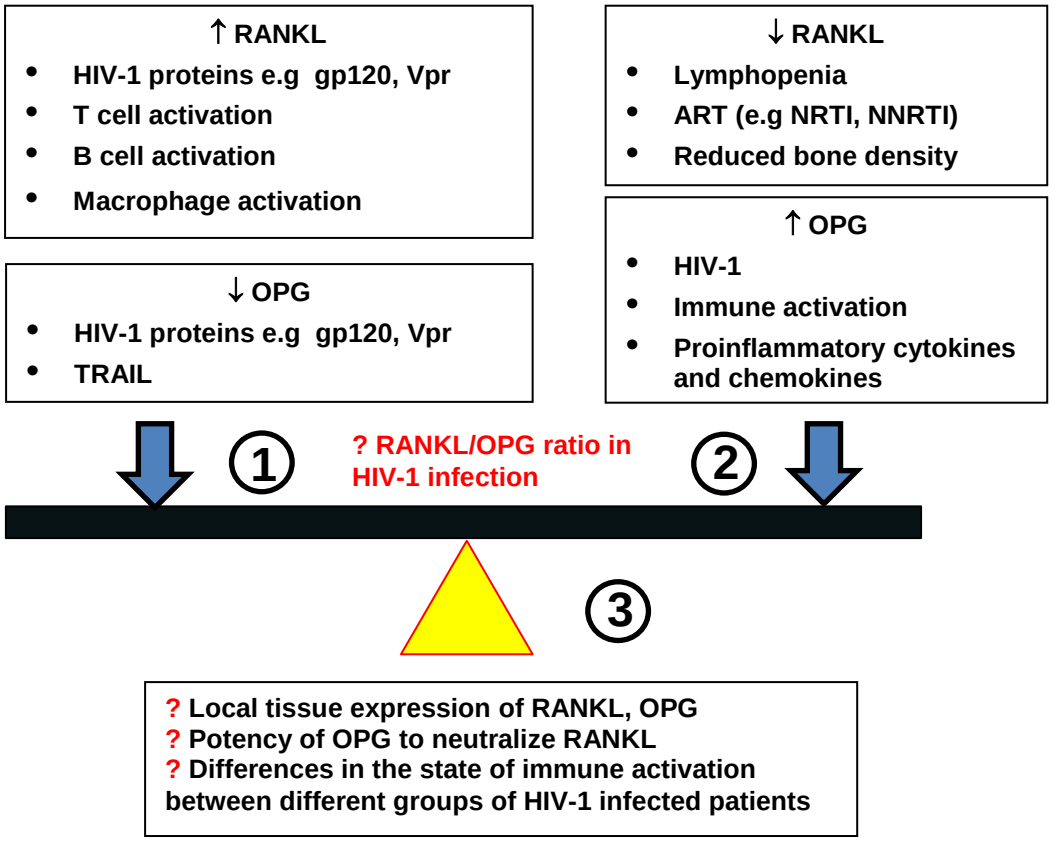

Figure 1.2: HIV-1 infection can affect the OPG/RANKL system through multiple mechanisms. 1. Mechanisms in HIV-1 infection that upregulate the ratio of RANKL/OPG include: a) HIV-1 viral proteins such as gp120 can directly upregulate expression of RANKL in lymphocytes b) HIV-1 infection has been associated with activation of macrophages, B and T cells which can upregulate expression of RANKL c) HIV-1 infection also induces the production of proinflammatory cytokines and chemokines that increase circulating levels of RANKL such as interleukins 1&6, INF-γ and TNF-α d) HIV-1 infected subjects have increased circulating levels of TRAIL which binds OPG and reduces circulating levels of OPG e) Antiretrovirals such as protease inhibitors (PIs) may increase RANKL and reduce OPG circulating levels f) HIV-induced bacterial translocation from the gastrointestinal mucosa ↑ LPS that ↑ osteoclast production by ↑osteoblast production of RANKL, IL-1, and TNFα **2.** On the other hand, mechanisms in HIV-1 infection that downregulate the ratio of RANKL/OPG include: a) HIV-1 can directly upregulate expression of OPG in tissues b) Immune activation can lead to increased production of OPG by lymphocytes and macrophages c) Cytokines upregulated during HIV-1 infection such as TNF-a and RANKL

can also lead to increased circulating OPG levels d) Lymphopenia present during HIV-1 infection may cause reduced production of RANKL by lymphocytes e) Antiretrovirals reduce the upregulation of the RANKL/OPG induced by HIV-1 per se and certain ART such as NRTIs or NNRTIs can reduce circulating levels of RANKL f) Reduced bone density and unfavorable bone microarchitecture may lead to reduced RANKL levels **3.** The relative balance of the factors described in 1) and 2) in combination with unknown local tissue expression of RANKL and OPG, unknown potency of OPG to neutralize RANKL and unclear differences in the state of immune activation between different groups of HIV-1 infected patients may lead to variable circulating RANKL/OPG ratio between different HIV-1 infected subjects that is associated with cardiovascular and bone disease and immune dysregulation. Arrows indicate observed direct effects based on *in vitro* studies and/or observations based on *in vivo* studies and do not necessarily imply causation. Abbreviations: ART: antiretroviral therapy, HIV: Human Immunodeficiency Virus, IFN-γ: interferon-gamma, LPS: lipopolysaccharide, NNRTI: non-nucleoside reverse transcriptase inhibitors, NRTI: nucleoside reverse transcriptase inhibitors, OPG: osteoprotegerin, PI: Protease inhibitor, RANKL: Receptor activator of nuclear factor kappa-B ligand, TNF-a: tumor necrosis factor a, TRAIL: TNF-related apoptosis-inducing ligand.

Direct interaction between HIV-1 and the RANKL-OPG system
• HIV proteins Gp120, Vpr and Tat ↑ RANKL → ↑ binding to RANK →↑ TRAF6 and MKKs → ↑ osteoclast gene differentiation and amplification[224,226]
• Vpr ↑production of RANKL by acting in synergy with glucocoticoid[226,227]
• HIV envelope protein gp120 ↑ RANKL secretion
• RANKL ↑HIV replication
• HIV and ART can cause osteopenia, bone matrix effects, change bone metabolism and elicit bone structure alterations → abnormal RANKL, OPG production[226-234]
• HIV can impair bone homeostasis by gp120/cell membrane interaction inducing apoptosis→ abnormal RANKL, OPG production[224-226,230,232]
• Possible direct HIV-1 infection of osteoblasts → abnormal RANKL, OPG production
• HIV tat protein activates TRAIL which binds OPG[235-237]
Indirect interaction between HIV-1-RANKL-OPG system through innate immunity
• HIV-induced mucosal damage leads to systemic bacterial translocation and ↑ LPS → ↑ osteoclast production by ↑osteoblast production of RANKL, IL-1, and TNFα
• PBMC from HIV-1 seropositive patients produce ↑ RANKL[238]
• RANKL ↑ osteoclastic activity in PBMCs of HIV^+ women vs those of HIV^- women [221,238]
Indirect interaction between HIV-1 and the RANKL-OPG system through adaptive immunity
• RANKL may ↑activation and killing of virus-specific cytotoxic $CD8^+$ T cells.[221,239-241]
• HIV infection ↓T-cell costimulation → ↓ OPG[221,242-244]
• HIV infection ↑ activated mature B cells →↑ RANKL[221,242-244]
• HIV infection ↓ resting memory B cells →↓ OPG production[221,242-244]
• HIV-1 infection causes severe B-cell exhaustion→ ↓ OPG → ↑HIV-induced, osteoclast-mediated bone loss[221,242-244]
• HIV ↑ secretion of M-CSF by macrophages → ↑M-CSF a) ↑ CD4/CCR5 receptors b) ↑virus gene expression in macrophages c) ↑ sensitivity of macrophages to HIV d) ↑ RANKL activity e) ↓ OPG levels → ↑osteoclastogenesis[221,224]
• A block in the transition of monocytes to macrophages in HIV-1 Tg rats may lead to a pooling of monocytes and osteoclasts precursors → ↑ HIV-induced bone loss [221,224]
• RANKL ↑ cross-presentation by DC of HIV antigens and anti-HIV memory responses[245]

Table 1.1: Summary of the interplay between RANKL, OPG, and the Immune system in HIV-1 infection. Abbreviations: ART: antiretroviral therapy, DC: dentritic cells, HIV: Human Immunodeficiency Virus, MAPK: Mitogen-activated protein kinase, M-CSF: Macrophage

colony-stimulating factor, NF-kB: nuclear factor kappa-B, OPG: osteoprotegerin, PBMC: peripheral blood mononuclear cell, RANKL: Receptor activator of nuclear factor kappa-B ligand, TRAF6: tumor necrosis factor receptor-associated protein 6.

1.7 Establishing dysfunctional HDL as a novel mechanistic link between HIV-1, immune activation and atherosclerosis may initiate further studies to explore the efficacy of novel therapeutic interventions that can directly affect HDL function that might improve the prognosis of HIV infected patients

Elucidating the interplay between HIV-1, oxidized lipids, immune activation and atherogenesis is a fundamental research question with important clinical applications. Future clinical management of HIV infection will be based on a better understanding of the interplay between the virus, chronic immune activation and inflammation, the activated monocyte and cholesterol metabolism. It is not known whether there is a link among endotoxemia, proinflammatory cytokine production, and CAD risk in individuals on ART who have a suppressed viral load. Interventions affecting cholesterol homeostasis are by far the most effective way to prevent and in some instances, reverse atherosclerosis. Addressing these questions will allow informed trials of targeted therapies, such as use of drugs that may improve cholesterol metabolism, HDL function, or suppress chronic immune activation.

Apolipoprotein A-I (apoA-I) peptide mimetics potentially can reverse HDL dysfunction. Improving HDL function through modification of its lipid and/or protein content maybe a therapeutic target.[246] Apolipoprotein A-1 (apoA-I), the major protein in HDL is a selective target for oxidation by myeloperoxidase, which results in impaired HDL function.[246] HDL/apoA-I mimetic peptides may have the ability to modify the lipid and protein content of HDL and convert dysfunctional HDL to functional HDL[246] and are promising therapeutic agents for reducing CVD risk.[246-248] *In vivo,* they organize into small cholesterol-containing particles enriched in ApoA-I, reducing lipoprotein peroxidation. These peptides are amphipathic helical peptides with a specific number of hydrophobic phenylalanine residues (based on their number they are names as (2-7) F) as the major determinant of activity to bind nonoxidized lipids and prevent LDL-induced MCP-1 production in the cell based

assay.[249,250] These peptides can prevent formation of atherosclerotic lesions *in vivo* in animal models of atherosclerosis.[251] The remarkable difference in the binding affinity of oxidized lipids for L-4F compared with human apoA-I may explain how such small concentrations of an apoA-I mimetic peptide could change the antiinflammatory properties of HDL when the concentration of apoA-I in the same plasma was much greater.[49]

ApoA-I mimetic peptides may be promising therapeutic agents in HIV-1 infection.

The envelope glycoproteins of HIV-1 contain regions that can fold into amphipathic alpha-helixes which can play a role in virus-induced cell fusion of HIV-1.[121] Although apoA-I mimetic peptides do not have sequence homology to apoA-I, these peptides have the capacity to form class A amphipathic helixes similar to those found in apoA-I and mimic lipid binding properties of apoA-I, producing antioxidant and anti-inflammatory effects.[246] ApoA-I and its amphipathic peptide analogues have been found to directly inhibit the infectivity of HIV-1.[121] Although these results have potential implications for HIV biology and therapy, it has not been investigated whether 4F can directly reduce HIV-1 infectivity and immune activation related to HIV-1 and oxidized lipids. ApoA-I peptide mimetics reduce inflammation by modulating the metabolism of phospholipids by gut bacteria which has been recently implicated in atherosclerosis in both mice and humans.[252] The gut and microbial translocation are also important in the pathogenesis of HIV-1 associated immune activation.[168] In view of those data and the pro-inflammatory effects of oxidized lipids, we hypothesize that apoA-I mimetics can reverse the effects of dysfunctional HDL on the leukocytes important for both HIV-1-induced immune activation and atherogenesis.

Preface

The focus of my dissertation is to develop a better understanding of the role of oxidized High Density Lipoprotein (HDL) in the development of life threatening cardiovascular conditions such as atherosclerosis in HIV-1 infected subjects and how oxidized HDL (HDLox) is a central player in the chronic immune activation associated with HIV-1 infection **(Figure 1.3)**. In the setting of chronic inflammation HDL may become oxidized and dysfunctional and lose its protective effects against cardiovascular disease (CVD) such as ability to remove oxidized lipids. I propose that HIV-1-infected persons have dysfunctional HDL that it both results from and contributes to the chronic immune activation that is central to HIV-1 pathogenesis. Better understanding these processes could offer a new therapeutic avenue to address this chronic immune activation and its negative immunologic and cardiovascular impacts.

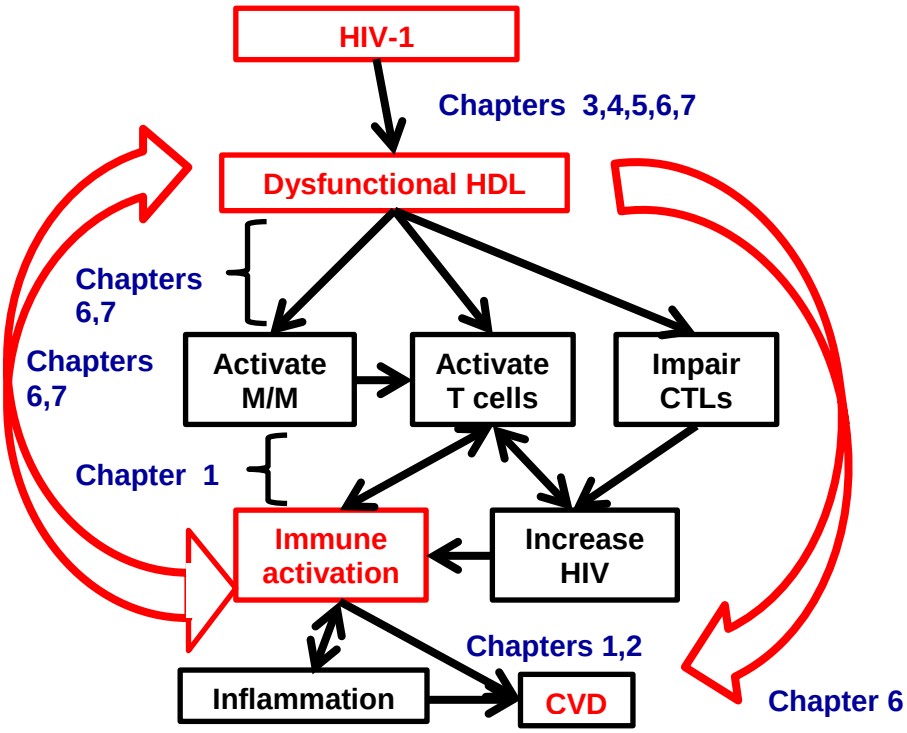

Figure 1.3. Overall hypothesis: Systemic immune activation has a major role in CVD associated with HIV-1 infection **(Chapters 1, 2)**. Systemic inflammation in HIV-1 infection drives the formulation of dysfunctional (oxidized) HDL **(Chapters 3, 4, 5, 6, and 7)** which is independently associated with CVD and markers of immune activation **(Chapter 6)**. Dysfunctional HDL exerts direct immunomodulatory effects **(Chapter 7)** and may reduce antiviral immune responses **(Chapter 7)**. The interplay between lipids, the NF-kB pathway **(Chapters 6, 7)** and scavenger receptors **(Chapter 7)** may explain partially effects of dysfunctional HDL on immunity and atherogenesis **(Chapter 1)** but further mechanistic studies need to elucidate the exact mechanisms **(Chapter 8)**. Overall, the effects of

dysfunctional HDL on immunity and atherogenesis may drive immune activation, systemic inflammation and atherogenesis in HIV-1 infection and may be reversed by the use of HDL mimetic peptides **(Chapters 1, 3, 8)**. Abbreviations: CVD: Cardiovascular disease risk, CTLs: cytotoxic $CD8^+$ T cells, HIV: Human Immunodeficiency Virus, HDL: High Density Lipoprotein, M/M: Monocytes/macrophages

My graduate work on this hypothesis began with the demonstration that residual immune activation in treated HIV-1 infected subjects with suppressed viremia is an independent predictor of CVD in these patients **(Chapter 2)**. Then using a cell based assay in a small pilot study, I demonstrated that HIV-1 infected subjects have dysfunctional HDL and that use of HDL mimetic peptides *in vitro* can improve HDL dysfunction **(Chapter 3)**. I then focused on developing a novel cell free biochemical assay that measures redox properties of HDL as a tool to study HDL function *in vivo* in the setting of large scale studies in humans **(Chapter 4)** to determine whether HIV-1 infected patients have dysfunctional HDL that is independently associated with progression of atherosclerosis. While using this tool I found that this assay had certain biochemical limitations **(Chapter 5, Figure 5.1)** and I went on to develop another novel cell free assay based on immunoaffinity capture of HDL and enzymatic determination of lipid peroxidation of HDL using a well-established fluorochrome **(Chapter 5)**. Using these assays we showed that dysfunctional HDL was independently associated with progression of atherosclerosis and percent non-calcified coronary plaque in HIV-1 infected but not in uninfected controls and that increased HDL redox activity independently associates with indices of macrophage activation and T cell activation **(Chapter 6)**. In view of these *in vivo* observations that support a role of oxidized HDL in immune activation and CVD associated with HIV-1 infection we investigated the direct *in vitro* effects of oxidized HDL on T cell activation and antiviral immune responses and showed that dysfunctional HDL can directly upregulate T cell activation **(Chapter 7)**. Finally, in **Chapter 8**, I draw conclusions from this work, layout goals for further experiments to elucidate the role of oxidized HDL in HIV-1 immunopathogenesis and offer perspectives on the development of new therapeutic strategies such as HDL mimetic peptides that may reduce the CVD and immune activation associated with HIV-1 infection.

Chapter 2

Biomarkers of Microbial Translocation and Macrophage Activation Are Associated With Progression of Subclinical Atherosclerosis in HIV-1 Infection

2.1 Abstract

Background: The relationships between soluble CD14 (sCD14), endotoxin (LPS), and progression of atherosclerosis have not been defined in HIV infection.

Methods: We retrospectively assessed serum sCD14 and LPS levels of 91 subjects in a prospective 3-year study of carotid artery intima-media thickness (CIMT) (ACTG 5078), where subjects were enrolled as risk factor-controlled triads of HIV-uninfected (*n*=36) and HIV-infected individuals with (*n*=29) or without (*n*=26) protease inhibitor (PI) based therapy for ≥ 2 years. The primary endpoint was the yearly rate of change of CIMT (ΔIMT).

Results: In multivariate analysis of the HIV-infected subjects, each 1 µg/ml above the mean of baseline serum sCD14 corresponded to an additional 1.52 µm/yr (95%CI: 0.07, 2.98; *p*=0.04) in the ΔIMT. Every 100 pg/ml above the mean of baseline serum LPS corresponded to an additional 0.49 µm/yr (95% CI: 0.18, 0.81; *p*=0.003) in the ΔIMT. However, in univariate analysis in the HIV-uninfected group sCD14 (p=0.33) and LPS (p=0.27) were not associated with higher ΔIMT. HIV infection was not associated with changes in baseline serum LPS and sCD14 (p>0.1).

Conclusions: Our data are among the first to suggest that serum biomarkers of microbial translocation (LPS) and macrophage activation (sCD14) predict subclinical atherosclerosis progression in HIV-infected persons.

2.2 Introduction

With improved survival, cardiovascular disease (CVD) has become an increasingly important cause of morbidity and mortality among persons infected with Human Immunodeficiency Virus Type 1 (HIV), [150] underscoring the need to better understand the contributors to CVD risk. Evidence from clinical studies supports a link between endotoxemia and the development of atherosclerosis in humans.[253,254] HIV pathogenesis is characterized by a chronic state of immune activation, which is an independent predictor of disease progression in untreated persons.[193] Although bacterial translocation from the gut has been implicated as possible cause of this immune activation, [170,193] its role in the progression of atherosclerosis in HIV-infected persons on antiretroviral therapy remains largely unknown. Previous studies have shown increased serum lipopolysaccharide (LPS) levels in persons with viremia compared to healthy volunteers.[168,255] LPS is thought to induce systemic immune activation by binding to $CD14^+$ monocytes and macrophages, which consequently produce soluble CD14 (sCD14).[256-258] Thus, levels of LPS and sCD14 have been used as biomarkers of bacterial translocation and macrophage activation, respectively, but have not been previously studied in of the context of atherosclerosis in HIV infection.[170]

Measurement of carotid artery intima–media thickness (CIMT) using non-invasive B-mode ultrasound is well documented as a measure of subclinical atherosclerosis, [259-261] and we used the yearly rate of change in CIMT as the primary endpoint for this study. Serum sCD14 and LPS levels were determined on cryopreserved samples, and these biomarkers were compared between HIV-infected individuals and HIV-uninfected individuals. The effects of HIV infection on levels of these serum biomarkers over time were evaluated. Finally, we examined the association between baseline serum sCD14 and LPS with the progression of atherosclerosis as evaluated by the yearly rate of change in CIMT in each of these groups.

2.3 Results

Baseline characteristics of subjects. Baseline data from the 91 subjects in this study have been published.[262] In the 55 HIV-infected subjects, the median blood $CD4^+$ T-cell count was 488 cells/mm^3 and 84% had HIV plasma viremia <50 copies/ml (maximum 379 copies/ml). There were no major differences in anthropometric characteristics between the study groups. In addition, the HIV-infected subjects in this cohort had a higher rate of metabolic abnormalities and levels of sCD14 **(Table 2.1)** compared to the HIV-uninfected subjects.[262]

Characteristic	HIV (N=55)	not HIV (N=36)	P-value
Baseline CIMT (µm)	715.0 (640.0-771.0)	684.0 (639.5-758.0)	0.48*
ΔCIMT (µm/yr)	8.6 (-2.3-14.0)	8.6 (3.9-14.4)	0.81**
Baseline sCD14 (µg/ml)	2.34(1.65-3.02)	1.19 (0.95-1.79)	<0.001*
Week 96/144 sCD14 (µg/ml)	2.20 (1.83- 2.92)	1.33 (1.01-1.78)	<0.001*
ΔsCD14 (µg/ml/yr)	0.07 (-0.15-0.27)	-0.01(-0.13-0.13)	0.35**
Baseline LPS (pg/ml)	439.43 (207.08- 719.46)	496.57 (246.43-1,040.34)	0.26*
Week 96/144 LPS (pg/ml)	559.75 (191.29-1,372.51)	613.46 (293.34-2,376.63)	0.46*
ΔLPS (pg/ml/yr)	50.72 (-54.03-284.87)	39.24 (-67.39-459.00)	0.95, **

Table 2.1: Summary of CIMT, sCD14, and LPS Results

*Wilcoxon p-value for group differences: HIV versus not HIV.

**Wilcoxon p-value for matched group differences within each visit week: pairings assessed were HIV versus not HIV (N=32 matched pairings).

Δ indicates yearly rate of change in the measured parameter

Factors associated with change in CIMT over time. We determined the factors that were significantly ($p < 0.05$) associated with progression of atherosclerosis, defined as the yearly rate of change in CIMT (Table 3). Those associated with higher yearly rate of change in CIMT in univariate analysis within the HIV-infected subjects included higher baseline serum sCD14 and LPS, baseline serum HDL ≥ 35 mg/dl, lower baseline BMI, and lower yearly rate of change in serum sCD14. In multivariate analysis within the HIV-infected subjects, the yearly rate of change in CIMT increased by 1.52 um/yr (95% CI= (0.07, 2.98); p=0.04) for every 1 ug/ml increase in wk 0 sCD14 and increased by 0.49 um/yr (CI= (0.18, 0.81); p=0.003) for every 100 pg/ml increase wk 0 LPS, after controlling for wk 0 HDL

(Figure 2.1). However, in univariate analysis in the HIV-uninfected group, none of the above parameters, including sCD14 (p=0.33) and LPS (p=0.27) were associated with yearly rate of change in CIMT. Overall, these findings indicated that baseline serum biomarkers of microbial translocation and macrophage activation (LPS and sCD14) correlate with progression of subclinical atherosclerosis.

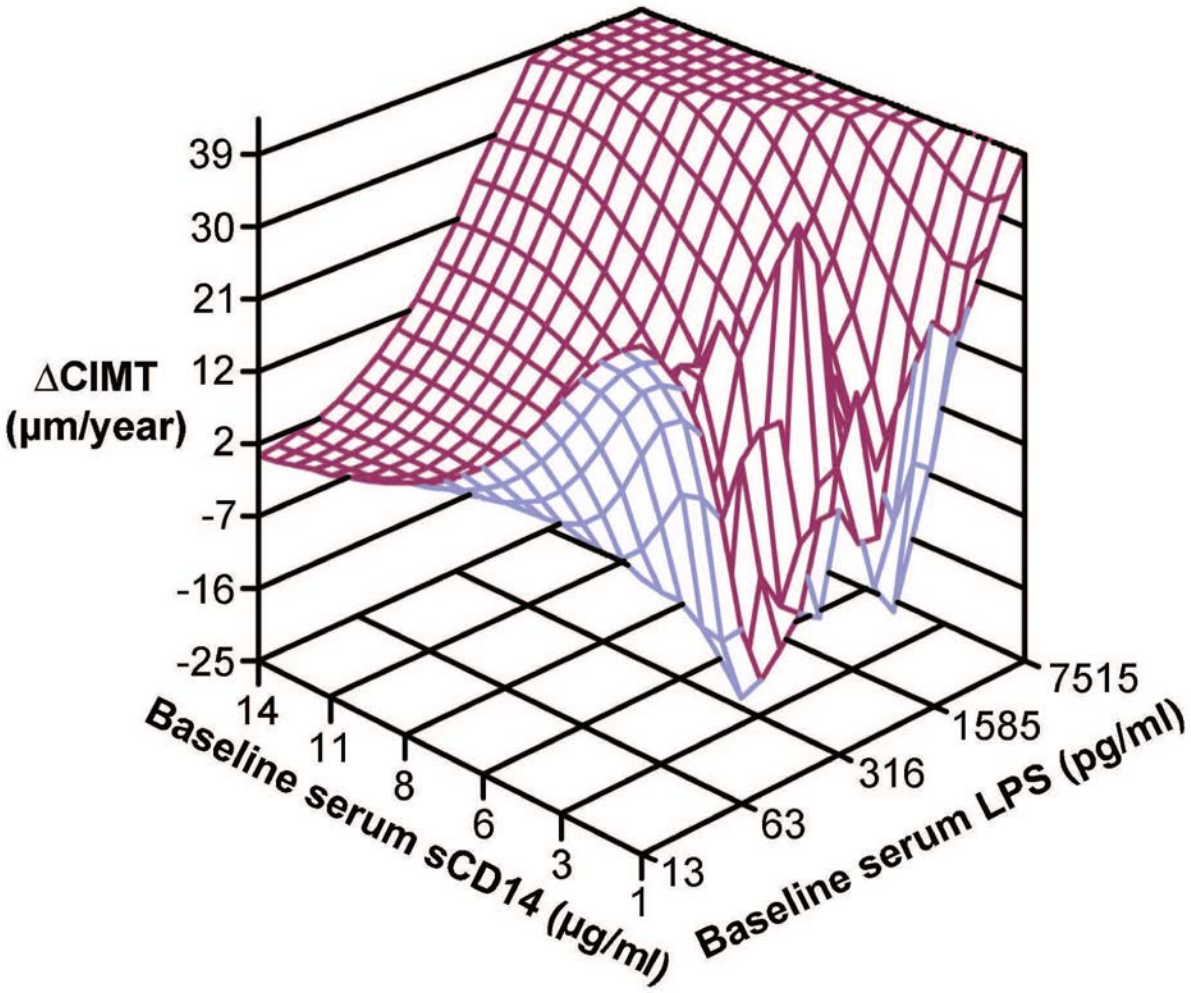

Figure 2.1. 3-D surface plot illustrating the relationship of the Yearly Rate of Change in CIMT (μm/year) (z axis) with baseline levels of sCD14 (μg/ml) (x axis) and baseline levels of LPS (pg/ml) (y axis) for the 55 HIV-infected subjects in the study.

2.4 Discussion

These data are the first to demonstrate that the serum sCD14 and LPS biomarkers of inflammation are associated with the progression of subclinical atherosclerosis, providing a potentially unifying etiology for the observed increased risk of cardiovascular disease in HIV-infected individuals.[263,264]

Atherosclerosis is an inflammatory process in which activated macrophages [41] and T-cells play important roles.[265-268] Lipopolysaccharide (LPS) is a potent stimulant of monocyte/macrophages, which express high levels of the key endotoxin receptor, the TLR-4/CD14 complex [41] that may be associated with increased risk for cardiovascular disease in humans.[254] LPS has also been proposed to be a central mediator of HIV-induced chronic immune activation that is believed to play a pivotal role in progression of HIV infection.[268,269]

Thus, serum soluble CD14 (sCD14), a biomarker of monocyte response to LPS, has been previously described as a plasma marker of atherosclerosis in HIV-uninfected persons.[270] Although elevated serum sCD14 is an independent predictor of mortality in HIV infection,[271] to our knowledge there are no data on the factors affecting serum sCD14 levels and the yearly rate of change in serum sCD14 in HIV-infected and HIV-uninfected subjects.

HIV infection may produce a phenotype of lipid-rich inflamed atherosclerotic plaques that may be vulnerable to rupture, potentially through up-regulated monocyte/macrophage activation.[272] However, correlates of macrophage phenotype and cardiovascular disease are poorly characterized [41] in individuals on antiretroviral therapy (ART) who have suppressed viremia.

In this sub-study, of A5078[259,260] we found that baseline serum sCD14 and LPS levels were positively associated with the yearly rate of change in CIMT in both the overall study population and HIV-infected subjects only. However, the strength of predicting subclinical atherosclerosis progression in HIV-infected persons was greater for traditional risk factors of atherosclerosis such as HDL-C compared to LPS or sCD14.

Consistent with our results, Burdo *et al* demonstrated that HIV-infected subjects with few traditional risk factors can have noncalcified disease that may be related to

monocyte/macrophage activation as measured by serum sCD163.[205] Although these data suggested that monocyte/macrophage activation may contribute to subclinical atherosclerosis, our prospective study with longitudinal measurements of CIMT up to 3 years is the first, to our knowledge, to demonstrate that monocyte/macrophage activation may contribute to progression of atherosclerosis in HIV-infected subjects with low viremia independent of traditional risk factors. Our results extend prior data that showed endothelial dysfunction among HIV-infected subjects with long-standing low viremia.[273]

We demonstrated that baseline levels of serum LPS are associated with progression of atherosclerosis (yearly rate of change in CIMT) in HIV-infected persons. Previous cross-sectional studies have shown that LPS is not associated with development of subclinical atherosclerosis in HIV-infected subjects, [205] and that LPS is not an independent predictor of mortality in HIV infection, [271] in contrast to sCD14. However, the effects of chronically elevated microbial translocation into the bloodstream on the cytokine milieu and activation of monocytes/macrophages and vascular endothelium in the setting of HIV infection are not reversed by antiretroviral therapy.[274] Indeed, treatment of mice with LPS induces development of early atheroma formation.[275] Thus, our results suggest that it is possible that increased microbial translocation during chronic HIV infection is linked pathogenetically to progression of atherosclerosis in HIV-infected individuals.

Limitations of our study include the non-randomized design, the variable duration of follow-up in this cohort and the small sample size especially for the HIV uninfected group. Finally, our study was not designed to detect the effects of the various antiretroviral drugs.

Together, these data lend insight into the dynamic process by which cardiovascular disease develops among HIV-infected individuals despite low or undetectable plasma viremia, and suggest that monocyte/macrophage activation and bacterial translocation contribute to progression of atherosclerosis in these subjects.

As the long-term survival of the HIV-infected population continues to improve, our results emphasize the importance for further studies on the role of different modalities to limit microbial translocation in an effort to reduce modifiable cardiovascular risk factors.

2.5 Materials and Methods

Study Design

A5078 was a prospective, matched cohort study that was designed to investigate the roles of PI therapy and HIV infection on the progression of atherosclerosis; the study design and primary results have been previously published.[259,260] The study enrolled 133 individuals in 45 triads (groups of three individuals matched by age, sex, race/ethnicity, smoking status, blood pressure, and menopause status); detailed inclusion and exclusion criteria have previously been described.[259,260] Each triad consisted of one individual from each of the following groups: 1) HIV-infected individuals with continuous use of PI-based therapy for ≥ 2 years; 2) HIV-infected individuals on antiretroviral therapy without prior PI use; and 3) HIV-uninfected individuals. Atherosclerosis, measured as CIMT, was determined at weeks 0, 24, 48, 72, 96, and 144 in the three groups as previously described. [259-261] Using 182 cryopreserved samples from 91 subjects in this prospective study, serum sCD14 and LPS were measured on two specimens from weeks 0, 96 (if there was no week 144 specimen), and 144. Among the 91 subjects, there were 41 triads represented, of which 15 were complete. We investigated whether there are associations between PI-based treatment, HIV infection, CIMT, serum sCD14, and serum LPS.

Data collection

Right distal common carotid artery far wall intima-media thickness was measured at baseline and longitudinally. Baseline variables of fasting blood glucose, blood lipids, cardiovascular disease-related measurements, blood $CD4^+$ T-cell counts, plasma HIV RNA levels, and serum metabolic markers had been previously been determined.[259,260] Metabolic syndrome was defined by National Cholesterol Education Program (NCEP) criteria.[276] Serum sCD14 levels were quantified by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's protocol (“Quantikine” ELISA, R&D Systems). The limit

of detection of this assay was 125 pg/mL and the intra- and interassay variability were 3.5 ± 1.37% and 5.0 ± 2.8% respectively. Serum lipopolysaccharide (LPS) levels were measured using the PyroGene™ Recombinant Factor C Assay Limulus assay (Lonza), as previously described.[271] The limit of detection of this assay was 0.01 EU/ml and the intra- and interassay variability were 5.2 ± 3.37% and 10.1 ± 3.4% respectively.

Statistical methods

Subjects were matched within triads. A variation on the Wilcoxon signed-rank test was used to compare the two HIV-infected subjects with the HIV-uninfected control subject in each triad. Mixed models linear regression analyses with triad as a random effect were used to evaluate whether baseline measurements of serum sCD14, serum LPS, and CIMT were associated with baseline covariates. Associations between the yearly rate of change in CIMT and baseline covariates were examined using a repeated measures analysis. Covariates univariately associated with the outcome ($p < 0.05$) were examined together using the backward elimination method to build the final multivariate model. SAS version 9.2 PROC MIXED was used for all regression analyses.

Chapter 3

HIV-1 Infected Patients with Suppressed Plasma Viremia on Treatment Have Pro-inflammatory HDL

3. 1 Abstract

Background: The role of pro-inflammatory lipids in systemic immune activation in HIV infection remains largely unknown. We hypothesized that HIV-1-infected persons on antiretroviral therapy would have pro-inflammatory high density lipoprotein (HDL), and that an apoA-1 mimetic peptide might reverse the inflammatory properties of HDL in these persons.

Methods: Plasma was obtained from 10 HIV-1-infected individuals on combination antiretroviral therapy with suppressed viremia and was incubated with the apoA-I mimetic peptide L-4F or sham-treated prior to isolation of HDL. The HDL that was isolated from each sample was tested for its ability to inhibit LDL-induced MCP-1 production in cultures of human aortic endothelial cells.

Results: We found in a small pilot study of HIV-1-infected individuals with suppressed viremia on combination antiretroviral therapy that oxidative stress and inflammation in HIV-1 are associated with a marked reduction of HDL antioxidant/anti-inflammatory activities. *In vitro*, these abnormalities were significantly improved by treatment with the apoA-1 mimetic peptide, 4F.

Conclusions: These preliminary observations suggest that the anti-inflammatory properties of HDL are defective in HIV-1-infected persons despite treatment that is considered to be virologically successful.

3.2 Introduction

With improved antiretroviral therapies and survival among patients with HIV-1, cardiovascular disease (CVD) has become an increasingly important cause of morbidity and mortality.[155] The magnitude of the increased CVD risk among persons with HIV-1 infection is unclear, however, as are the relative contributions of viremia, immune activation, antiretroviral therapy (ART), conventional cardiovascular risk factors (such as age and hypertension), and changes in systemic inflammation. Understanding the relative contributions of host, virus to risk of CVD in HIV-1 infection will aid in the development of strategies for prevention and treatment.

Inflammation has increasingly been recognized to be pivotal in the initiation and perpetuation of arterial injury leading to atherosclerosis and its complications.[277] Pro-inflammatory high density lipoprotein (HDL) may play a role in this process; higher serum levels of pro-inflammatory HDL are associated with the increased rates of CVD in certain chronic inflammatory conditions such as rheumatoid arthritis, and type II diabetes mellitus. [63] While immune activation is a hallmark of HIV-1 infection, it is not known if HIV-1 infection affects levels of pro-inflammatory HDL, or if increases in HDL cholesterol observed after initiation of ART are neutral, atheroprotective or atherogenic in nature.[167,278-280]

With regards to pro-inflammatory lipids, apoA-1 mimetic peptides have been shown to remove oxidation products from lipoproteins and cell membranes and restore structure and function of low density lipoprotein (LDL) and HDL in a wide range of experimental inflammatory conditions in animals.[281] In view of the systemic immune activation that is not entirely reversed despite effective ART[281,282] and which could therefore be conducive to generating pro-inflammatory lipids, we hypothesized that HIV-1-infected persons on ART would have pro-inflammatory HDL, and that an apoA-1 mimetic peptide might reverse the inflammatory properties of HDL in these persons.

3.2 Result

All subjects were men with an average age of 43 ± 14 years (± SD, range 21 - 63) on combination ART. Viral load and $CD4^+$ T cell counts were available on nine subjects; these had plasma HIV-1 RNA values <50 copies/mL and mean blood $CD4^+$ T cell counts of 595 ± 166 cells/mm^3 (range 367 - 966). Mean HII values for HDL in the sham treated plasma were 2.16 ± 0.63 (range 1.01 - 2.73) while mean HII values for HDL in the L-4F treated plasma were significantly lower ($p<0.0001$) at 0.89 ± 0.16 (range 0.64 - 1.19). These values were compared to previously reported mean values from healthy controls (0.43 ± 0.05 and 0.20 ± 0.04 for sham and 4F treatment respectively **(Figure 3.1)**.[281] Thus, high-density lipoprotein isolated from plasma of persons with ART treated HIV-1 infection was highly pro-inflammatory, and was markedly less pro-inflammatory after treatment of plasma with 4F *in vitro* **(Figure 3.1)**. Both the high baseline HII and reduction of the inflammatory index after 4F treatment were observed in all 10 samples, suggesting an overall pro-inflammatory lipid profile that was partially reversible by 4F.

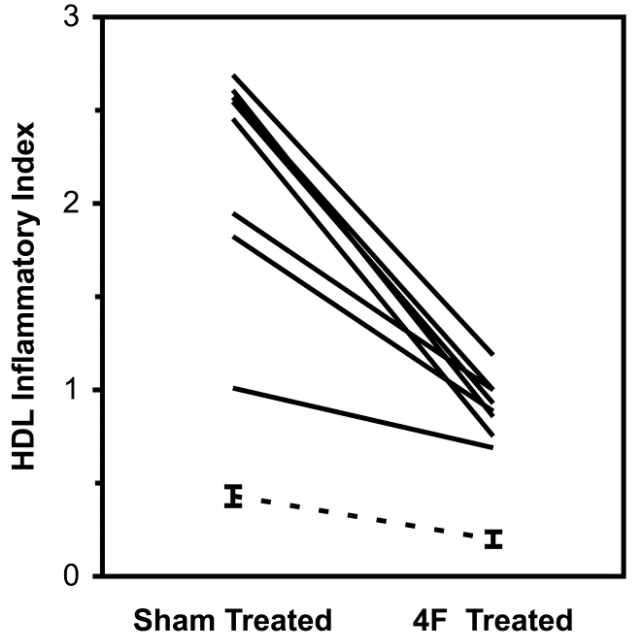

Figure 3.1: HDL inflammatory index from plasma of patients with HIV (sham-treated versus L-4F- treated). The dotted line indicates previously reported values from healthy controls (0.43 ± 0.05 and 0.20 ± 0.04 for sham and 4F treatment respectively). In this assay, anti-inflammatory HDL is defined as HDL inflammatory index values <1.0 and proinflammatory HDL is defined as HDL inflammatory index values >1.0.

3.3 Discussion

Classically, HDL is believed to play an important role in mitigating oxidative stress and inflammation by uptake, processing and disposal of oxidized lipids.[246,277] However, systemic inflammation has been shown to lower this antioxidant and anti-inflammatory activity by transforming HDL to a pro-oxidant, pro-inflammatory acute-phase HDL that enhances the tendency of LDL to induce monocyte chemotaxis.[277] It has been demonstrated that mice that are genetically susceptible to develop atherosclerosis have augmented pro-inflammatory HDL. [246,281] HDL, therefore, not only removes excess LDL-derived cholesterol from peripheral tissues, but also has a major role in mitigating LDL-induced inflammation.

In our small cohort, all HIV-1-infected persons showed a marked reduction of HDL anti-inflammatory activity as evidenced by impaired HDL mediated inhibition of LDL-induced monocyte chemotactic activity. The observed defect cannot be attributed to low plasma HDL level in these patients, as equal amounts of isolated HDL were assayed; thus the observed HDL dysfunction must be due primarily to qualitative abnormalities. Oxidative stress and inflammation are nearly constant features of HIV-1 infection and the prevailing inflammatory state likely contributes to the observed reduction of HDL anti-inflammatory function in this population. This could lead to a vicious cycle in which the underlying inflammation and oxidative stress induce HDL dysfunction, which results in further inflammation.

Treatment of plasma samples from the HIV-1-infected subjects with the potent apoA-1 mimetic peptide 4F results in a marked increase in HDL anti-inflammatory activity. 4F is an 18-amino acid apoA-1 mimetic peptide, and its anti-inflammatory effects are mediated by its ability to preferentially remove oxidation products from lipoproteins and cell membranes, thereby restoring HDL and LDL function and structure, improving cell function, and attenuating inflammation.[246] ApoA-1 mimetic peptides have been shown to reduce atherosclerosis and attenuate inflammation in experimental animals without significantly changing plasma lipid levels.[246] 4F treatment in this study generally failed to return the pro-

inflammatory activity of HDL to the low levels that we have previously reported in healthy controls.[281]

These preliminary observations suggest that the anti-inflammatory properties of HDL are defective in HIV-1-infected persons despite treatment that is considered to be virologically successful, and that standard clinical lipid profile testing may not be an adequate measurement of the risk for CVD in these individuals. In this small pilot study we did not assess the possible association of proinflammatory HDL with other markers of immune activation and inflammation and the possible effect of 4F on these markers. An ongoing prospective study will aim at addressing the possible association of proinflammatory HDL with other markers of inflammation, immune activation and progression of atherosclerosis in patients with HIV infection. This study will also evaluate the effect of different types of antiretroviral treatment regimens versus HIV infection itself on the HDL inflammatory index.

In conclusion, to our knowledge this is the first demonstration that HIV-1 infection is associated with a marked reduction of HDL antioxidant/anti-inflammatory activities. *In vitro*, these abnormalities were significantly improved by treatment with the apoA-1 mimetic peptide, 4F.

3.4 Materials and Methods

Patients

Plasma was obtained from 10 HIV-1-infected individuals on combination antiretroviral therapy with suppressed viremia (below 50 copies of RNA/ml). Informed consent was obtained from all subjects.

Determination of proinflammatory HDL

Plasma was incubated with the apoA-I mimetic peptide L-4F at a concentration of 1 μg/mL for 15 minutes at 37ºC, or sham-treated prior to FPLC fractionation. HDL-containing FPLC fractions were tested for their ability to inhibit LDL-induced MCP-1 production in cultures of human aortic endothelial cells as previously described.[281] Results were normalized as a ratio to the effect of control LDL without HDL to yield the HDL inflammatory index (HII). More specifically HII is calculated from the ratio LDL-induced monocyte chemotactic activity (as determined by MCP-1 production in cultures of human aortic endothelial cells) in the presence of HDL/ LDL-induced monocyte chemotactic activity in cultures of human aortic endothelial cells in the absence of HDL.[281] Data were analyzed with Student's t-test using SPSS 13.5 software (Chicago, IL, USA) and expressed as mean ± SD; P-values less than 0.05 were considered significant.

Chapter 4

A Biochemical Fluorometric Method for Assessing the Oxidative Properties of HDL

4.1 Abstract

Most current assays of HDL inflammatory properties are cell-based. We have developed a fluorometric biochemical assay based on the oxidation of dihydrorhodamine 123 (DHR) by HDL. This cell-free assay assesses the oxidative potential of HDL by measuring increasing fluorescence due to DHR oxidation over time. The assay distinguishes the oxidative potential of HDL taken from different persons, and the results are reproducible. Direct comparison of this measurement correlated well with results obtained using a validated cell-based assay (r^2=0.62, $p<0.001$). This new fluorometric method offers an inexpensive, accurate, and rapid means for determining the oxidative properties of HDL that is applicable to large scale clinical studies.

4.2 Introduction

There is a continuing search for new biomarkers of increased risk for atherosclerotic disease. High-density lipoproteins (HDL) are “Janus-like” lipoproteins with the capacity to be anti-inflammatory in the basal state and proinflammatory during acute-phase responses.[283] Normally functioning anti-inflammatory HDL (aHDL) removes reactive oxygen species (ROS) from low-density lipoproteins (LDL), reducing both the oxidation of LDL and the recruitment of inflammatory mediators that are conducive to atherosclerosis.[45,51] However, pro-inflammatory HDL (pHDL) is defective in this function;[49] this HDL can be pronounced in some persons, including those with chronic inflammatory conditions that predispose to atherosclerosis.[80,284] Thus, although plasma HDL cholesterol levels are inversely related to risk across large populations, HDL function rather than absolute level may be a more accurate indicator for risk of developing atherosclerosis.[49]

Currently, HDL inflammatory properties are most often determined by cell-based assays.[285] However, the limitations and labor of these cell-based assays render them inaccessible to many researchers, and difficult to scale up for large-scale clinical trials or routine clinical use. Previously a cell-free assay that measures pHDL activity by testing the effect of HDL on the production of reactive oxygen species after oxidation and conversion of dichlorodihydrofluorescein diacetate (DCF-DA) to fluorescent DCF (2',7'-dichlorofluorescein) was developed to provide an alternative to cell-based assays.[55] This was based on our demonstration that levels of reactive oxygen species (such as lipid hydroperoxides produced from oxidation of lipoproteins) are significantly associated with the monocyte chemotactic activity and inflammatory properties of HDL that are measured by a cell-based assay.[55] Thus this measurement of reactive oxygen species after HDL exposure as reflected by the increase DCF fluorescence reflects the oxidative properties of different types of HDL that vary in their capacity to engage in redox cycling, which correlates with HDL inflammatory index (HII). [49] This approach promised to allow a direct biochemical assessment of pHDL activity without relying on a biologic readout. Although this assay yielded results that correlated to the cell-based assay, it did not achieve widespread usage due to i) the

oxidative instability of DCF-DA and the resulting tendency for minor variations in experimental conditions to cause inconsistency of the assay and ii) the sensitivity of the assay to any degree of hemolysis.

Here we describe an approach to quantify the oxidative activity of pHDL in a cell-free, biochemical assay. The products of redox cycling are detected as time-dependent oxidation of the fluorogenic probe dihydrorhodamine 123 (DHR) to fluorescent rhodamine 123.[286] The rate of DHR oxidation in the presence of HDL reflects the oxidative/antioxidative activity of HDL. This assay provides a readout that is highly correlated to a validated cell-based assay, [80] yet is highly reproducible, and amenable to high-throughput implementation.

4.3 Results

HDL samples reduce the oxidation rate of DHR consistently by differing amounts. To assess whether the oxidation rate of DHR is affected by the properties of HDL, HDL samples were tested for their effect on DHR oxidation rate. Alone, DHR exposed to air became oxidized (and therefore fluorescent) at a linear rate between 10 to 50 minutes **(Figure 4.1)**. The rate of oxidation of DHR was significantly less with added HDL, and the two HDL samples (an example of aHDL compared to pHDL) showed clearly different effects in this regard **(Figure 4.1A)**. Furthermore, when the amount of added HDL was varied, there was a clear dose-dependence in the oxidative effects on DHR that was linear in the range of 2.5 to 15 µg (cholesterol) of added HDL per well in the assay **(Figure 4.1C)**.

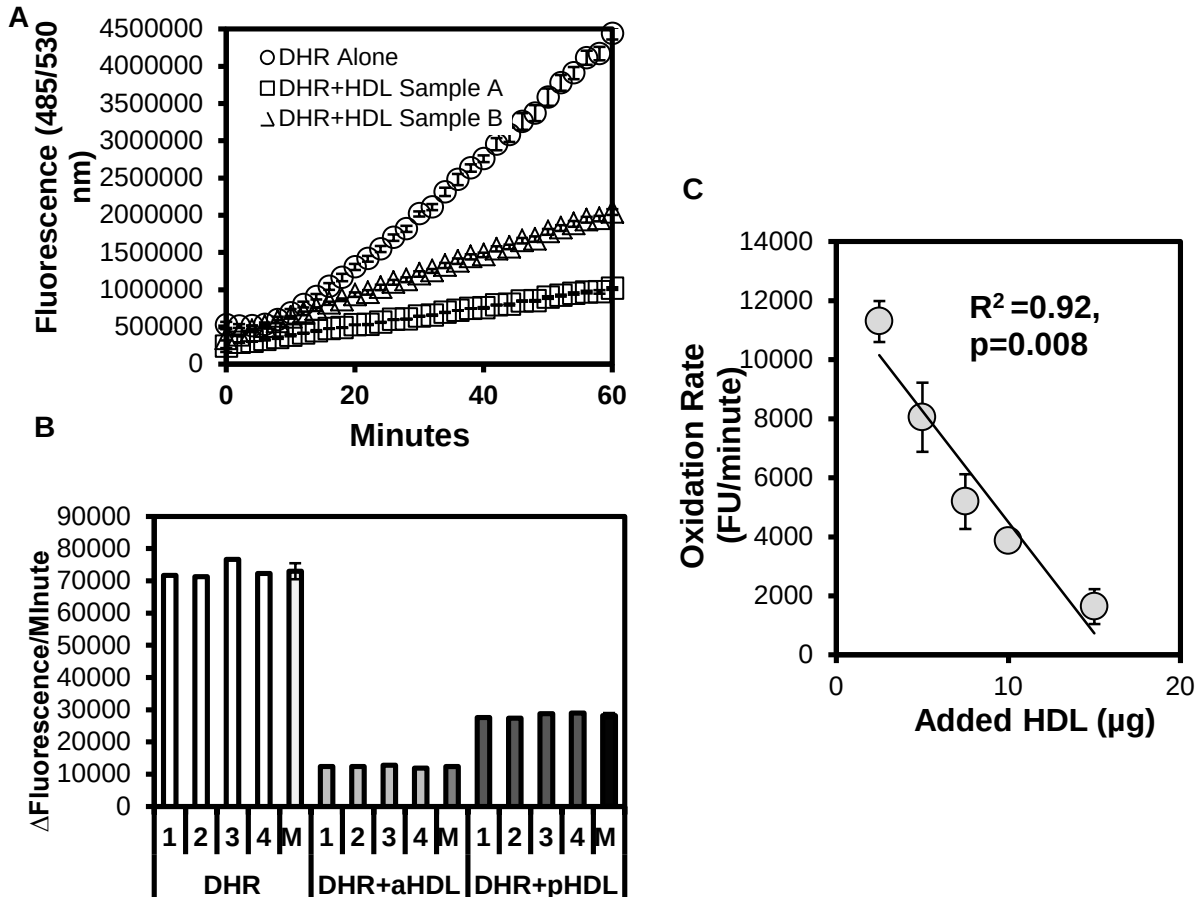

Figure 4.1. Spontaneous oxidation of DHR and effect of added HDL. In a 96 well flat bottom plate (flat bottom), 50 µM DHR was added to each well alone or with 5 µg (cholesterol) of FPLC-purified HDL from a donor with anti-inflammatory HDL (aHDL) and from a donor with pro-inflammatory HDL (pHDL) in a total volume of 175 µl (saline with 150 mM NaCl and HEPES 20 mM pH 7.4), each in quadruplicates. Spontaneous air-oxidation of

DHR at 37˚C was then followed in two-minute intervals using a fluorescence microplate reader set to detect 485/538 nm excitation/emission. **A.** The means and standard deviations of the quadruplicate fluorescence measurements are plotted over time. **B**. The rates of change in fluorescence between 20 and 50 minutes (calculated by linear regression) are plotted for the quadruplicates, as well as means/standard deviations. **C.** FPLC-purified HDL was added in varying concentrations (cholesterol) to 50 µM DHR in 175 µl in a 96 well flat bottom plate and the rate of change in fluorescence was measured. The rates of change in fluorescence (means and standard deviations) are plotted against the amounts of added HDL. DHR with no added HDL demonstrated a rate of 25,342 ± 2,619 fluorescence units/minute (not plotted).

Lipid-probe interactions explain the reduction in the oxidation rate of DHR after addition of HDL. We observed a significant reduction in fluorescence signal and oxidation rate of DHR after addition of HDL **(Figure 4.1)**. To determine whether this reduction is caused by antioxidant effect of HDL or nonspecific lipid-probe interactions we tested the effect of addition of different lipids with known oxidative properties on the oxidation rate of DHR. We found that addition of LDL, HDL **(Figure 4.1)** and other lipids that are known not to be antioxidant such as oxidized L-*α*-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (oxPAPC) leads to significant decrease in oxidation signal of DHR. To further investigate the dose dependent interaction between DHR and lipoproteins, increasing concentrations of lipoproteins (HDL and LDL) were incubated with 50 uM of DHR. **Figure 4.1C** shows that increasing concentrations of lipoproteins resulted in statistically significant decrease in fluorescence for each concentration of lipoprotein used. There was an inverse significant correlation observed between the quantity of lipoprotein added and DHR fluorescence and oxidation rate (**Figure 4.1C**). These results are consistent with lipid-probe interactions and possible fluorescence quenching as explanation of reduction in the readout of DHR after addition of lipids.

Differences in fluorescence after addition of same amount of different HDL samples correspond to real differences in the degree of oxidation of DHR. In order to ensure that the differences we observed with respect to fluorescence corresponded to real differences in the degree of oxidation of DHR, we subjected samples of HDL-CAD and HDL-Non CAD that had been combined with DHR in the manner of the fluorometric assay to LC/MS/MS analysis in order to determine the amount of DHR remaining in each **(Figure 4.2)**. Regardless of

whether the HDLs were isolated by sequential ultracentrifugation (UC) **(Figure 4.2a)** or by fast performance liquid chromatography (FPLC) **(Figure 4.2b)**, HDL from healthy non CAD donors contained significantly more DHR than HDL from CAD-patient donors after an initial 2 hour incubation with 50 uM DHR ($p = 0.001$, $n=3$ for each pair). As the amount of DHR remaining in the samples corresponds in this setting to the degree of oxidation of DHR, it follows that HDL from non CAD donors significantly inhibited the oxidation of DHR compared to HDL from CAD patients. In separate experiments, these same pairs of HDL were shown to exhibit significant differences in both the rate of increase of fluorescent signal and in fluorescent signal after two hours incubation, during the standard fluorometric assay. Both UC and FPLC isolated HDL-CAD exhibited a significantly greater rate of increase in fluorescent signal and significantly more final fluorescence than the respective HDL-non CAD samples.[75] Finally, the dose dependent interaction between DHR and HDL was confirmed by LC/MS/MS.[75]

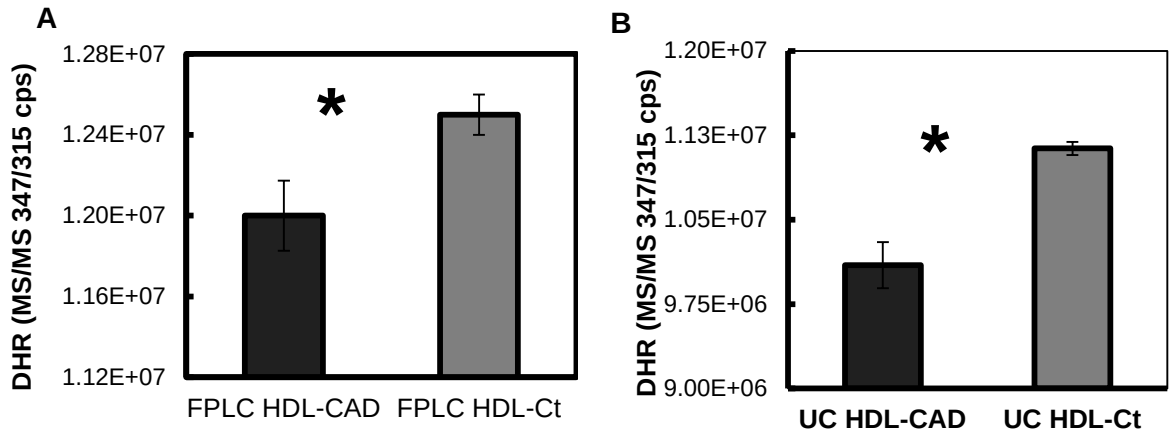

Figure 4.2. HDL from healthy donors significantly inhibits the oxidation of DHR compared to HDL from CAD patients, as determined by LC/MS/MS. HDL was isolated from the serum of healthy and CAD-patient donors by either sequential ultra-centrifugation (UC) or FPLC. Control (non-CAD) and CAD HDL pairs (HDL-CAD and HDL-Ct), one for each method of isolation, were combined in triplicate with DHR and incubated in the dark for 2 hrs. Each sample contained 2.5 ug HDL and 50 uM DHR in 175 total uL HEPES-buffered saline. After the incubation, urate was added (0.025 mM final concentration) to slow the further oxidation of DHR. The amount of DHR remaining in each sample was then determined by LC/MS/MS. For both the HDL-CAD and HDL-Ct pair isolated by UC (Figure 2a) and the pair isolated by FPLC (Figure 2b), the samples of the HDL-Ct contained significantly more DHR than the samples of the HDL-CAD (* $p = 0.001$, $n=3$).

The DHR-based assay of HDL oxidation function yields highly reproducible measurements despite fluorescence quenching. To counteract the effect of fluorescence quenching between HDL and DHR, relative differences in the oxidation rate of DHR for a specific amount of HDL (2.5 ug or 5 ug) were studied between different samples. To assess whether the assay is reproducible so that results of different experiments are comparable, six HDL samples were assessed in four independent experiments. Again, all HDL samples reduced the oxidation rate of DHR with low intra-assay variability between the quadruplicates, which ranged from 5.8 to 7.6% (mean 6.6%). Between independent replicates of the experiment, inter-assay variability for each of the samples ranged from 5.0 to 11.9% (mean 8.6%). These results indicated that the assay can reliably measure HDL oxidative function.

Results from the DHR assay correlate with a validated cell-based assay. To compare the results of this DHR assay to those obtained using a validated cell-based assay[285] 30 HDL samples were assessed using both assays **(Figure 4.3)**. Comparing DHR oxidation rate to the HDL inflammatory index, there was a strong positive correlation (r^2 = 0.62, p<0.001).

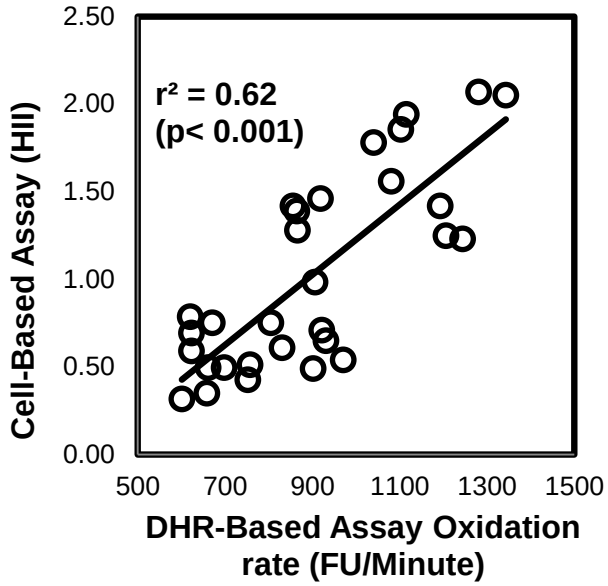

Figure 4.3. Correlation of DHR method with cell-based method. Thirty samples of FPLC-purified HDL were assessed for their ability to inhibit DHR oxidation as shown in Figure 4.1, and their HDL inflammatory index was determined in a cell-based assay as

described in Materials and Methods. The values from each assay are plotted against each other

Oxidized HDL is proinflammatory HDL. To determine whether there is an association between oxidative and inflammatory properties of HDL, HDL was oxidized as described in Materials and Methods and inflammatory properties were determined by a validated cell-based assay **(Figure 4.4)**.[285] Antiinflammatory HDL from healthy volunteers became proinflammatory (pHDL) after oxidation **(Figure 4.4)**. Thus, the DHR assay determines functional, oxidative properties of HDL that are associated with inflammatory properties of HDL.

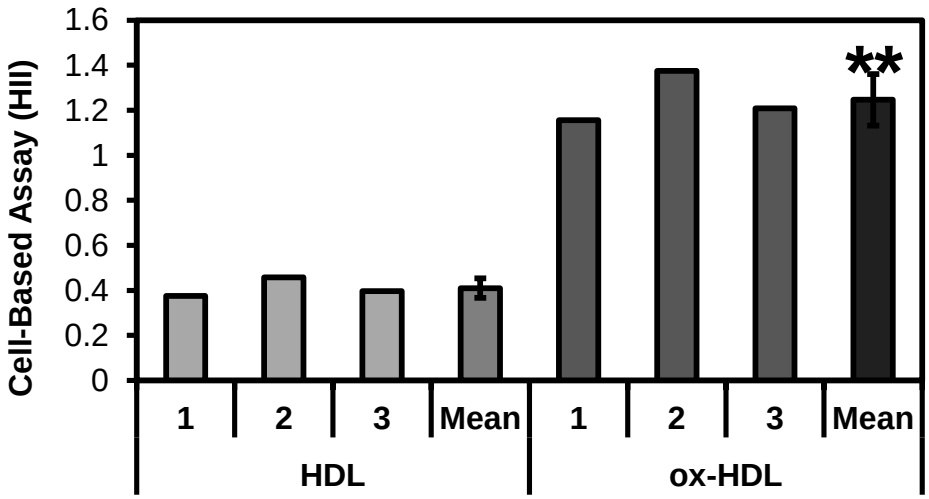

Figure 4.4. Oxidized HDL has inflammatory properties. The HDL inflammatory index was determined for three samples of FPLC-purified HDL from healthy volunteers in a cell-based assay as described in Materials and Methods. The same HDL samples were oxidized as described in Materials and Methods (oxHDL) and the HDL inflammatory index was determined. The oxidized HDL had significantly higher HDL inflammatory index (p=0.002, paired t-test).

The DHR assay can detect dysfunctional HDL in different conditions. To further validate the new method we used the DHR assay to detect dysfunctional HDL in conditions in which dysfunctional HDL is known to be present such as established animal models of atherosclerosis,[57] rheumatoid arthritis [64] and Human Immunodeficiency virus infection (HIV).[75] The DHR assay could detect established effect of statins on functional properties of HDL in animal models of atherosclerosis such as LDLR-/- **(Figure 4.5A)** and ApoE-/- mice **(Figure 4.5B)**. In addition, the functional properties of HDL in patients with RA as determined by a previously validated cell-free assay using DCF correlated significantly with

the functional properties of HDL as determined by the oxidation slope of DHR **(Figure 4.6)**. Finally, the DHR assay could detect dysfunctional HDL in HIV infection **(Figure 4.7)** even when the HDL from these patients was mixed with HDL from non-HIV patients at various ratios **(Figure 4.8)**.

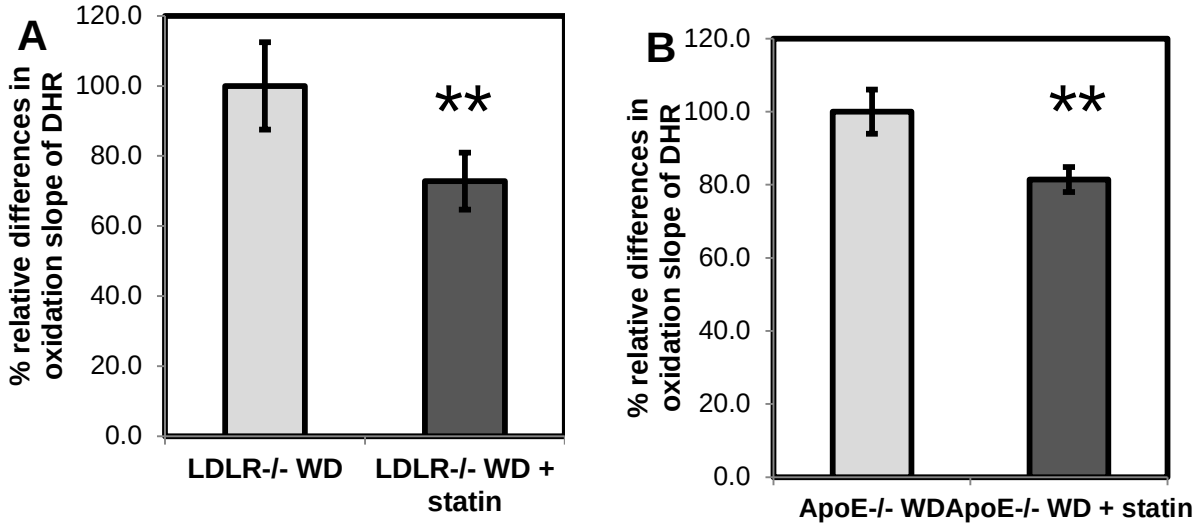

Figure 4.5. The DHR assay can detect established effect of statins on functional properties of HDL in animal models of atherosclerosis. A. Using FPLC, HDL was isolated from 3 pooled plasma samples from LDLR-/- mice on Western Diet (LDLR-/- WD) for 2 weeks and from 3 pooled plasma samples from LDLR-/- mice on Western Diet for 2 weeks which were also treated with pravastatin 12.5 ug/ml for 2 weeks. Each plasma sample was pooled from 4 mice (12 mice in total). Oxidation of DHR was assessed as described in Figure 1, using 2.5µg (cholesterol) of added HDL. The oxidation slope of DHR in the presence of HDL from LDLR-/- WD + statin was normalized to the oxidation slope of DHR in the presence of HDL from LDLR-/- WD and the % relative differences are shown. The data shown represent the average of measurements from 3 independent experiments. There was a statistically significant reduction in the oxidation slope of DHR in the presence of HDL isolated from LDLR-/- WD + statin mice compared to the oxidation slope of DHR in the presence of HDL isolated from LDLR-/- WD mice ($p=0.01$) B. Using FPLC, HDL was isolated from 3 pooled plasma samples from ApoE-/- female mice on Western Diet (LDLR-/- WD) for 2 weeks and from 3 pooled plasma samples from ApoE-/- female mice on Western Diet for 2 weeks which were also treated with pravastatin 12.5 ug/ml for 2 weeks. Each plasma sample was pooled from 4 mice (12 mice in total). Oxidation of DHR was assessed as described in Figure 1, using 2.5µg (cholesterol) of added HDL. The oxidation slope of DHR in the presence of HDL from ApoE-/- WD + statin was normalized to the oxidation slope of DHR in the presence of HDL from ApoE-/- WD and the % relative differences are shown. The data shown represent the average of measurements from 3 independent experiments. There was a statistically significant reduction in the oxidation slope of DHR in the presence of HDL isolated from ApoE-/- WD + statin mice compared to the oxidation slope of DHR in the presence of HDL isolated from ApoE-/- WD mice ($p=0.01$).

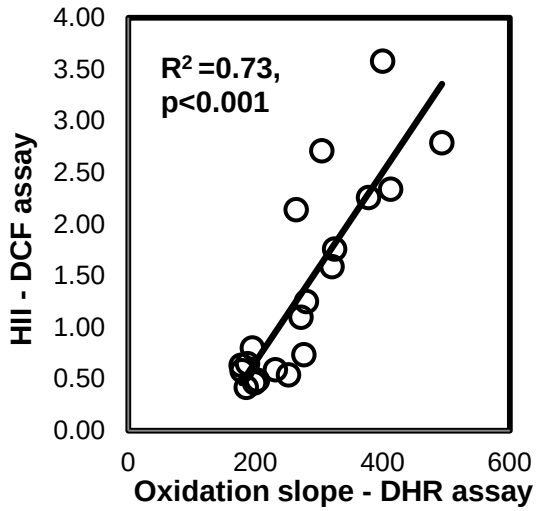

Figure 4.6. Correlation of DHR method with previous cell-free method. Twenty samples (10 from healthy volunteers and 10 from patients with rheumatoid arthritis) of HDL isolated using precipitation with dextran sulfate were assessed for their ability to inhibit DHR oxidation as shown in Figure 1, and their HDL inflammatory index was determined in a cell-free assay using dihydrodichlorofluorescein (DCFH) as described in Materials and Methods. The values from each assay are plotted against each other.

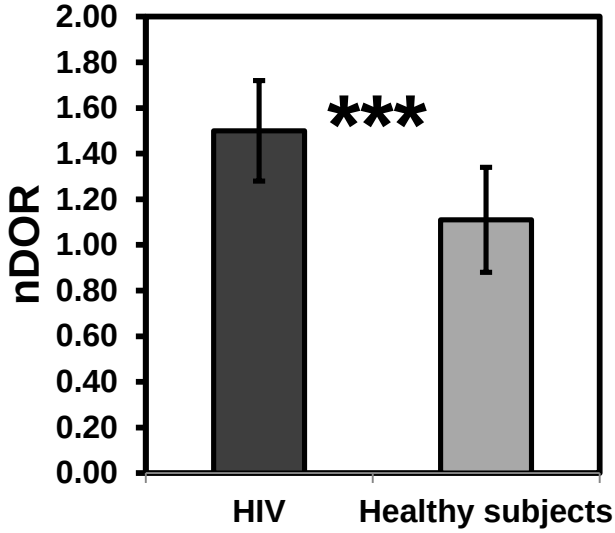

Figure 4.7. HIV-1 infected subjects have dysfunctional HDL.
HDL was isolated from serum of 50 patients with HIV infection and 50 healthy subjects, using ultracentrifugation as described in Methods. Oxidation of DHR in the presence of these 100 different HDL samples was assessed as described in Methods, using 2.5µg (cholesterol) of added HDL. The slope of oxidation of each sample were normalized as ratios to the DOR of a control pool of plasma HDL from 50 healthy control subjects and the normalized DOR (nDOR) is shown on the y axis. The data (means of quadruplicates) from three independent experiments are plotted. The mean nDOR value from HIV-infected subjects was significantly ($p<0.001$) higher (1.50± 0.25) compared to the mean nDOR value from healthy subjects (1.11±0.23) when HDL was purified using ultracentrifugation.

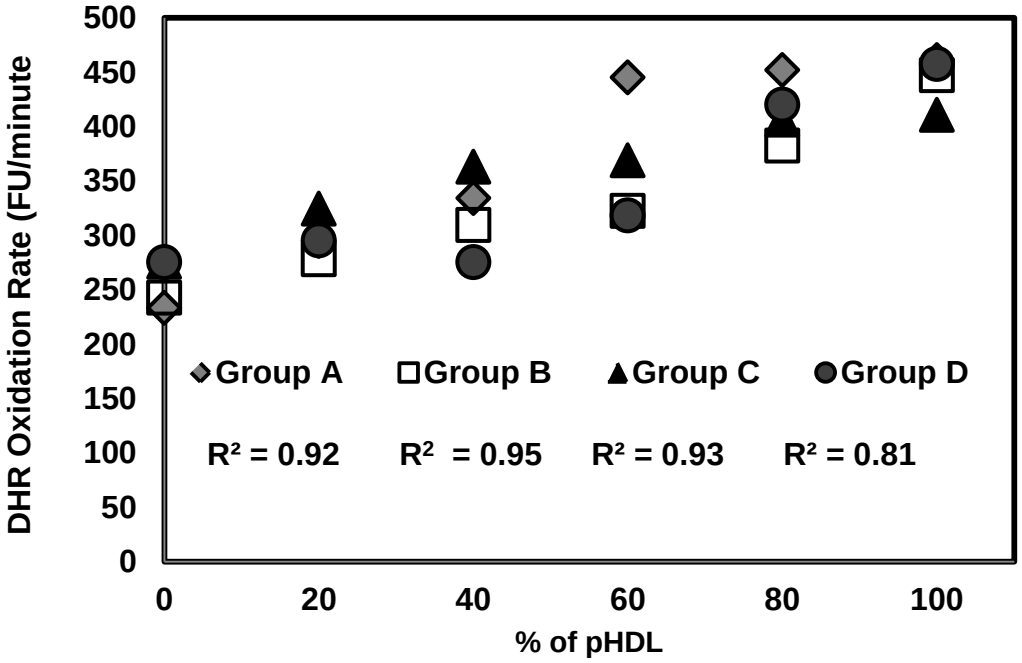

Figure 4.8. The novel assay can detect dysfunctional HDL in HIV Infection. HDL was isolated from plasma of 4 patients with Human Immunodeficiency Virus Infection (HIV) who were not treated with antiretroviral therapy and 4 healthy volunteers (non-HIV) using polyethylene glycol (PEG). The HDL from each patient with HIV (pHDL) was mixed with HDL from non-HIV patient at certain ratios so that the % of pHDL in each reaction was 0, 20, 40, 60, 80, 100% and the total amount of added cholesterol in each reaction was stable (2.5 ug of cholesterol). Oxidation of DHR in the presence of four different pairs of HDL (Group A, B, C, D) was assessed using 2.5µg (cholesterol) of added HDL. The correlation coefficient (R^2) of the DHR oxidation slope with the % of pHDL in each reaction is shown for each group. The statistically significant correlation in all four pairs (R^2 >0.8, $p<0.01$) demonstrates the ability of the DHR assay to detect pHDL in HIV infection even when the HDL from these patients was mixed with HDL from non-HIV patients at various ratios.

4.4 Discussion

Growing evidence suggests that HDL varies significantly in its phenotype and influence on cardiovascular disease risk through varying pro- or anti-inflammatory effects.[48,54] Atherosclerosis begins when LDL is trapped in the arterial wall and seeded with reactive oxygen species (ROS), [44] resulting in oxidized LDL that attracts monocytes and induces localized inflammation.[45,51] Properly functioning HDL removes ROS from LDL, antagonizing this process. HDL, however, can be "chameleon-like" in that it can be pro-inflammatory under certain clinical conditions such as acute phase responses[283] and various pathogenic states such as diabetes. Measuring the functional status of HDL, rather than HDL cholesterol concentration, may be more informative in predicting cardiovascular disease. Unfortunately, the best assays for HDL function have been cell-based assays, [285] which are technically prohibitive for many researchers and logistically difficult for large scale studies. The function of HDL has been determined in previous assays based on the capacity of HDL to prevent the formation or to inactivate oxidized phospholipids produced by LDL.[55]

Recent interest has focused on the functional consequences of HDL oxidation. Oxidation could conceivably contribute to the formation of dysfunctional HDL, proposed to be present in humans with cardiovascular disease.[55] One potentially important pathway for generating dysfunctional HDL via oxidation involves myeloperoxidase that mediates conversion of protein tyrosine residues to 3-chlorotyrosine, and methionine residues to methionine sulfoxide (MetO).[287] In addition, MetO can also be formed from exposure of HDL's major protein, apolipoprotein A-I (apoA-I) to H_2O_2 [288] or lipid hydroperoxide,[289,290] the latter generated during the oxidation of HDL lipids. Reactive oxygen species (ROS) such as 1*e*-oxidants (i.e., hydroxyl radical ions) and 2*e*-oxidants (i.e. H_2O_2 and peroxynitrite) have been shown to oxidize tyrosine and methionine residues[291] which can have dramatic consequences on the functions of apoA-I/HDL, including reverse cholesterol transport.[292]

Dihydrorhodamine 123 (DHR) may be oxidized by a variety of oxidants, including hypochlorous acid generated by myeloperoxidases, peroxynitrite anion formed by oxidation of nitric oxide and hydrogen peroxide in the presence of peroxidases.[286,293] Although DHR

has mostly been used to determine oxidation of various molecules in intracellular matrix [293] previous studies have demonstrated that oxidation rate of DHR can be used to quantify redox activity in extracellular matrix such as plasma.[286] The oxidation of HDL is the result of oxidation of both the lipid and protein component. During air oxidation of the HEPES-saline-lipoprotein-DHR solution both 1*e*-oxidants and 2*e*-oxidants are generated.[286] 1*e*-oxidants, preferentially react with the lipoprotein's lipids and this causes lipid peroxidation with the resulting accumulation of hydroperoxides of phospholipids and cholesteryl-esters[294] that then oxidize apoA-I's methionine to MetO.[289,290,295] The measurement of the slope of changes in fluorescence of DHR over time in response to addition of different types of HDL corresponds to the rate of formation of lipid hydroperoxide products and ROS. Thus, the oxidation rate of DHR can be used to quantify the intrinsic ability of HDL to get oxidized which has been shown to affect HDL function.[292] Thus, in the current study, we demonstrate a novel cell-free assay that biochemically examines the oxidative ability of HDL, providing a functional measurement that correlates well with a validated cell-based assay. We utilize DHR as a substrate similar to a previously reported assay to measure the redox potential of iron in plasma.[286] Using the new method, we demonstrate that addition of normal HDL led to significantly smaller increase in the fluorescent signal of DHR compared to pHDL.

In the previous cell free assay we demonstrated lipid probe interactions between the fluorochrome DCFH with various lipids.[55] To our knowledge, the interactions of lipids with DHR have not been previously tested. We found that addition of lipids, including prooxidant lipids contained in lipoproteins, lead to significant decrease in oxidation signal of DHR and thus the decrease in the oxidation rate of DHR after addition of HDL was caused by lipid probe interactions. However, we show that our DHR-based assay of HDL oxidation yields highly reproducible measurements despite fluorescence quenching. In addition, the inverse correlation between HDL concentration and oxidation rate of DHR allows quantification of functional properties of HDL using very low concentrations of HDL (1.25 ug per sample). Thus, using this assay we were able to quantify relative differences in the oxidative properties of different HDL samples that correlate with their functional properties.

This DHR-based cell-free assay improves upon the prior dichlorofluorescein-diacetate (DCFH-DA)-based cell-free assay.[55] While also measuring the ability of HDL to inhibit oxidation, this earlier assay had several technical challenges that have limited its utility. It was designed to mimic the cell-based assay and test the effect of HDL in combination with LDL. This introduced additional variability, because different preparations of LDL can vary in their oxidative activity, and lipid-probe interactions become less consistent with multiple lipids. Mixing LDL and HDL with DHR in our assay, we observed inconsistent results that appeared to be caused by fluorescence quenching (data not shown), compared to the highly consistent results seen with HDL alone. Another technical challenge was the instability of DCFH-DA, which needed to be dissolved in methanol for activation to the active molecule DCFH, and protected from room air because DCFH is unstable and prone to auto-oxidation.[293,296-299] In contrast, DHR is relatively stable, [293,296-299] and oxidizes at a predictable rate when exposed to room air. Furthermore, conversion of DCFH-DA to DCFH can be mediated by esterases that are carried over variably during the lipid purification process, adding another variable that is difficult to control, while DHR requires no activation and is not prone to this effect.[293,296-299] Another point of improvement is the kinetic approach for measuring oxidation rate during a linear phase, which lends greater precision compared to a single endpoint measurement.

We also demonstrate that although different methods of HDL isolation affect the oxidation rate of DHR when HDL is added, ultracentrifugation, FPLC, and isolation of HDL using precipitation with dextran sulfate or polyethylene glycol can be used to determine functional properties of HDL using our DHR-based cell-free assay. The use of polyethylene glycol may allow HDL isolation and use of this method in large scale studies. The oxidation rate of HDL was higher in samples isolated using ultracentrifugation. Removal of much of the albumin bound to the HDL particle during the process of ultracentrifugation may alter the association of ROS associated with lipoproteins.[300,301]

This novel DHR-based assay offers an attractive alternative to current cell-based assays. Because it measures a biochemical rather than biologic phenomenon, it may be

more precise. The inter-assay variability of about 8 to 10% compares favorably with the cell-based assay, which has variability of >15%.[285]

The correlation of HDL effects on DHR oxidation rate to the biologic readout of HDL in a cell-based assay is consistent with a proposed mechanism whereby HDL exerts its effects through modulating ROS, [55] and suggests that the assay accurately reflects the balance of aHDL and pHDL. Further support for the biological relevance of this measurement is the finding that oxidation of DHR was higher with LDL than with any of the HDL samples tested and was significantly reduced after addition of HDL from statin treated mice with atherosclerosis compared to after addition of HDL from non-statin treated mice . Indeed, treatment of these mice with statins has been shown to reduce inflammatory properties of HDL[302] and we demonstrate that the DHR assay can detect the favorable effect of statins on functional properties of HDL. Finally, the DHR assay can detect dysfunctional HDL in patients with RA and HIV infection confirming our previous results.[302]

The fact that our assay quantifies oxidation of HDL by a range of different oxidants increases its applicability to biological samples, at least in the context of cardiovascular diseases. This is because various oxidants contribute to oxidative modifications taking place in the affected arterial wall during atherogenesis.[303] Thus, while “oxidized HDL” is not a chemically defined term, the oxidation rate of DHR corresponds to the intrinsic oxidative features of modified lipoproteins. However, we exclusively used *in vitro* generated oxidized forms of HDL, whereas the oxidative modifications occurring to HDL in the diseased artery wall are conceivably more complex.[303] An additional potential limitation of the present assay is that HDL is subject to continuous remodeling *in vivo*. This includes dissociation of apoA-I from the lipoprotein particle, a process that could be increased by oxidation.[304] Clearly, future studies are required to assess the utility of the assay described for clinical studies.

In conclusion, this new assay offers a rapid method for measuring the properties of HDL which inhibit oxidation. It yields results that correlate well with a validated cell-based assay. This new technical approach may offer a convenient tool for studies of the role of HDL functional phenotype in the development of atherosclerosis *in vivo*.

4.5 Materials and Methods

Subjects.

Blood samples were collected from patients with coronary artery disease (CAD) or equivalent as defined by the National Cholesterol Education Program Adult Treatment Panel III criteria [276] and were collected from patients referred to the cardiac catheterization laboratory at the University of California, Los Angeles. After signing a consent form approved by the Human Research Subject Protection Committee of the University of California, Los Angeles, the patient donated a fasting blood sample collected in a heparinized tube. HDL and LDL were isolated from blood samples from patients who had angiographically documented coronary atherosclerosis despite normal total cholesterol (200 mg/dl), LDL cholesterol (130 mg/dl), HDL cholesterol (males, >45 mg/dl; females, >50 mg/dl), and triglycerides (< 150 mg/dl), who were not receiving hypolipidemic medications and who were not diabetic, and who were not active smokers. All the patients had at least a 50% narrowing of at least one coronary artery. HIV-infected subjects had HIV-1 RNA >= 10 000 copies/ml, were not receiving antiretroviral therapy, had no documented coronary atherosclerosis and normal total cholesterol (200 mg/dl), LDL cholesterol (130 mg/dl), HDL cholesterol (males, >45 mg/dl; females, >50 mg/dl), and triglycerides (< 150 mg/dl), were not receiving hypolipidemic medications and were not diabetic. Rheumatoid arthritis (RA) patients were recruited from the rheumatology offices at the University of California, Los Angeles (UCLA) via flyers posted in the offices and in the UCLA Medical Center. Normal volunteers were recruited to age and sex match the patients under a protocol approved by the Human Research Subject Protection Committee of the University of California, Los Angeles.

Mice

ApoE and LDLR null mice originally purchased from the Jackson Laboratories on a C57BL/6J background were obtained from the breeding colony of the Department of Laboratory and Animal Medicine at the David Geffen School of Medicine at UCLA as previously described.[302] The mice were maintained on a Western diet (Teklad, Harlan,

catalog # TD88137) for 2 weeks and a group of mice was also treated with pravastatin at 12 ug /ml drinking water, or approximately 50 ug per day for two weeks. All experiments were performed using protocols approved by the Animal Research Committee at UCLA.

Reagents

Dihydrorhodamine 123 (DHR) and Dihydrodichlorofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR). DHR was prepared as stock of 50 mM in dimethyl sulfoxide (DMSO) and DCFH as previously described. [64] Iron-free HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered saline (HBS, HEPES 20 mM, NaCl 150 mM, pH 7.4) was prepared as previously.[286] The DHR stock was diluted 1:1000 in HEPES saline solution to prepare a working solution of 50 µM. Purified Apolipoprotein A-I from human plasma was obtained from Sigma-Aldrich (St. Louis, MO). Pravastatin sodium (Lot No. M000301, Catalog number P6801) was purchased from LKT Laboratories, Inc.

HDL and LDL purification

HDL and LDL were isolated from cryopreserved human plasma (with or without added sucrose) by ultracentrifugation, fast performance liquid chromatography (FPLC), or dextran sulfate and precipitation with polyethylene glycol. These were aliquoted and stored as previously described.[305-307] HDL cholesterol was quantified using a standard colorimetric assay (Thermo DMA Co., San Jose, CA, USA) as previously described.[55] HDL cholesterol was oxidized by use of copper ions (OxHDL) as previously described.[308]

Other assays of HDL function.

The HDL-inflammatory index was determined for each subject's HDL as described previously.[285] In this assay a value >1.0 is considered pro-inflammatory and a value <1.0 is considered anti-inflammatory.

The DCF-based cell-free assay was performed as previously described.[55]

DHR-based cell-free assay of HDL function

Quadruplicates of HDL (5µg of cholesterol unless otherwise specified were added to 96-well plates (polypropylene, flat bottom, black, Fisher Scientific, USA). HBS was added to each well to a final volume of 150 µl, followed by addition of 25 µl of the working solution of 50µM DHR, for a total volume of 175 µl (final DHR concentration of 7 µM). Immediately following DHR addition, the plate was protected from light, placed in the fluorescence plate reader and fluorescence of each well was assessed at two minute intervals for an hour with either a DTX 800/880 Multimode Detector (Beckman Coulter, California, USA) or Synergy 2 Multi-Mode Microplate Reader (Biotek, Vermont, USA), using a 485/538 nm excitation/emission filter pair with the photomultiplier sensitivity set at medium. Both readers gave equivalent readings (correlation coefficient of 0.99). Using Microsoft Excel software the oxidation rate was calculated for each well as the slope for the linear regression of fluorescence intensity between 10 to 50 minutes, expressed as FU minute^{-1} (fluorescence units per minute). HDL oxidative function was calculated as the mean of quadruplicates for the wells containing the HDL sample.

LC/MS/MS analysis

LC/MS/MS was performed using a 4000 QTRAP quadruple mass spectrometer (Applied Biosystems) equipped with an electrospray ionization source. The HPLC system utilized an Agilent 1200 series LC pump equipped with a thermostatted autosampler (Agilent Technologies). Chromatography was performed using a Luna C-18(2) column (3 µm particle, 150 × 3.0mm; Phenomenex) with a security guard cartridge (C-18; Phenomenex) at 40^{o}C. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The autosampler was set at 4^{o}C. The injection volume was 20 µl, and the flow rate was controlled at 0.4mL/min. The data acquisitions and instrument control were accomplished using Analyst 1.4.2 software (Applied Biosystems). Detection was accomplished by using the multiple reaction monitoring (MRM) mode. The parameter

settings used were: ion spray voltage= 5500 V; curtain gas=22 (nitrogen); ion source gas 1=34; ion source gas 2 =25; ion source gas 2 temperature=250^{o}C. Collision energy and collision cell exit potential were optimized to obtain optimum sensitivity. The transition monitored was mass-to-charge ratio (m/z): m/z 347.050→315.1 for DHR.

LC/MS/MS experiments

HDL from control and CAD-patient donors was combined with DHR, and the amount of DHR remaining in the samples after two hour incubation was determined by LC/MS/MS. First, UC and FPLC isolated HDLs were combined in triplicate with DHR. Each sample contained 2.5 ug HDL and 50 uM DHR in a final volume of 175 uL HEPES-buffered saline. The samples were incubated in the dark for two hours. Urate was then added (final concentration 25 uM) to slow the further oxidation of DHR.[298] The samples were transferred in minimal light to autosampler vials (Fisher scientific, USA) for LC/MS/MS analysis. The samples were ordered such that HDL-control samples were measured last, in order to ensure that any additional air oxidation of DHR did not result in a false positive as regards the difference between HDL-CAD and HDL-Ct with respect to DHR. In a separate experiment, UC-isolated HDL from a healthy donor was serially diluted, in triplicate, from 10 to 0.625 ug cholesterol. The dilutions were combined with DHR and incubated in the dark for two hours as above. After incubation, urate was added; the samples were transferred to autosampler vials; and the amount of DHR remaining was determined by LC/MS/MS.

Statistical analysis

Statistical analysis was performed using Excel (Microsoft Corporation, Seattle, WA). Group means were compared using the Student's *t*-test for unpaired variates with $p < 0.05$ considered to be statistically significant. Correlation coefficients between variables were calculated using least squares linear regression.

Chapter 5

Limitations of Biochemical Assays of HDL Function and an Improved High Throughput Biochemical Fluorometric Method for Measuring HDL Redox Activity

5.1 Abstract

Most current assays of HDL functional properties are cell-based. In a cell-free assay of HDL function described in Chapter 4, an immediate reduction in the fluorescence signal is observed after addition of HDL to dihydrorhodamine 123 (DHR), due to fluorescence quenching from lipid-probe interactions. Understanding this process is important for interpretation of the results of fluorescence-based cell-free assays that measure oxidative properties of lipids. We found that the DHR assay of HDL function has limitations such as prominent lipid-probe biochemical interactions, when cryopreserved apoB depleted serum is used. To overcome these limitations, we have developed a fluorometric biochemical assay that measures reliably the endogenous HDL redox activity (HRA) of a specific amount of HDL cholesterol based on the oxidation of the fluorochrome Amplex Red while taking into consideration lipid-probe biochemical interactions, method of HDL isolation, freeze-thaw, cryopreservation and matrix effects. Direct comparison of this measurement correlated well with results obtained using previously validated cell-based ($r=0.47$, $p<0.001$) and cell-free assays ($r=0.46$, $p<0.001$). This assay could detect dysfunctional HDL in conditions in which dysfunctional HDL is known to be present such as established animal models of atherosclerosis, and Human Immunodeficiency virus infection (HIV). HDL immunoaffinity capture allows isolation of HDL in 96 well plates and in combination with the use of Amplex red the endogenous HRA is measured in situ. HRA as assessed by this assay correlated significantly with measures of cardiovascular disease such as carotid intima media thickness ($r=0.35$, $p<0.01$) and subendocardial viability ratio ($r=-0.21$, $p=0.05$) and physiological parameters such as metabolic and anthropometric parameters ($p<0.05$). The new assay could also detect previously established favorable effects of exercise on HDL function. This new fluorometric method offers an accurate, reproducible and rapid means for determining HDL function that is suitable for high throughput implementation and is applicable to large scale clinical studies.

5.2 Introduction

There is a continuing search for new biomarkers of increased risk for atherosclerotic disease. Manipulation of HDL has great potential in reducing cardiovascular risk but genetic and other studies in humans indicate the complexity in the relationship of HDL to atherosclerosis.[49,61] Indeed, lower HDL values are not uniformly associated with excess cardiovascular risk while higher HDL levels may not always confer a protective benefit. [49,61] Measuring HDL cholesterol levels provides information about the size of the HDL pool, but does not predict HDL composition or function. Thus, HDL function rather than absolute level may be a more accurate indicator for risk of developing atherosclerosis.[49,61]

Due to the complexity of the HDL particles, measurement of HDL function has not been studied extensively in humans.[71] Robust assays to evaluate the function of HDL are needed to supplement the measurement of HDL cholesterol levels in the clinic. Currently, HDL functional properties are most often determined by cell-based assays such as measurement of cholesterol efflux capacity.[68,69] However, the limitations and labor of these cell-based assays render them inaccessible to many researchers, and difficult to scale up for large-scale clinical trials or routine clinical use.[73]

We have previously developed a cell-free assay that measures HDL function by testing the effect of HDL on the production of reactive oxygen species (ROS) after oxidation and conversion of dichlorodihydrofluorescein diacetate (DCF-DA) to fluorescent DCF (2',7'-dichlorofluorescein) to provide an alternative to cell-based assays.[55] Although this assay has been used in many studies in humans to study HDL function, [56,71,309,310] it has not found widespread usage due to the oxidative instability of DCF-DA and technical difficulties in getting consistent results. Due to the limitations of the DCF-based assay, we recently developed an alternative biochemical assay to quantify the HDL redox activity (HRA) that measures the products of redox cycling as the rate of oxidation of the fluorogenic probe dihydrorhodamine-123 (DHR) to fluorescent rhodamine 123.[75] The relative stability of DHR in contrast to DCF, the kinetic approach of the DHR assay and the ability of the DHR assay to detect dysfunctional HDL in different inflammatory conditions,[75] offered an attractive

alternative to current cell-based assays. We identified factors that are important for lipid-probe interactions and that may increase experimental variability in fluorescence-based assays that quantify oxidative properties of lipoproteins.[311] Factors related to the lipoprotein (type and concentration of lipoprotein, presence of apolipoproteins, purification method of lipoprotein, HDL-LDL interactions), fluorescent probe (type and concentration of probe) and environmental factors (type and concentration of diluent, temperature, pH, matrix effects) can all affect the fluorescence signal and lipid-probe interactions **(Figure 5.1)**.

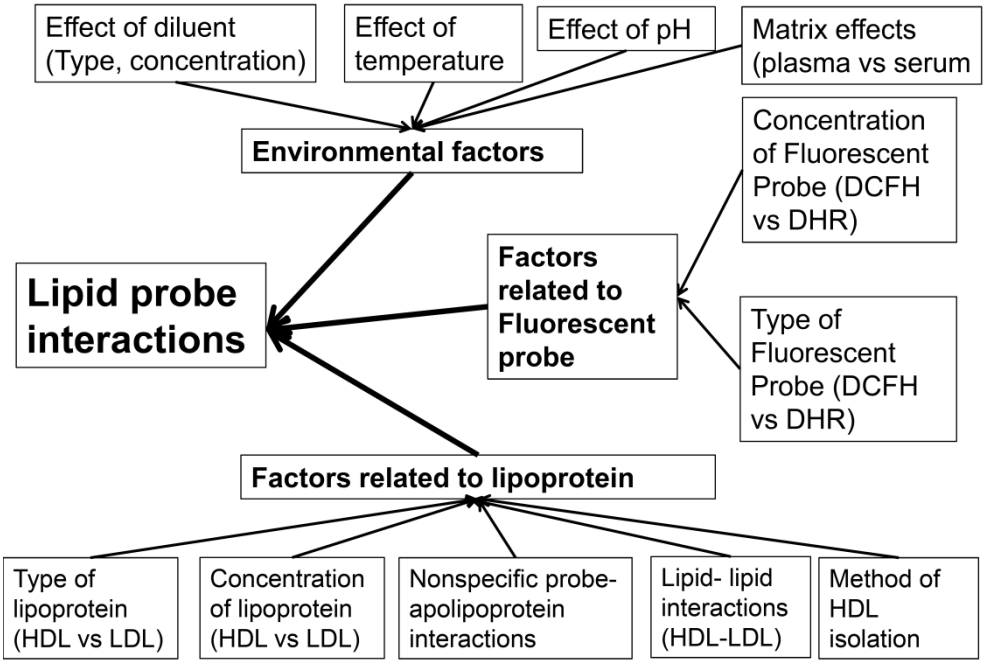

Figure 5.1 Summary of factors that affect lipid-probe interactions in biochemical assays of HDL functions.

Although in the DHR assay, the endogenous HRA is assessed in terms of the capacity of a specific amount of HDL to engage in *in vitro* redox cycling[75] and HRA is measured as relative differences in the oxidation rate of DHR between different samples, the exact underlying biochemical mechanism of the DHR-based assay remains to be determined. In addition, the complexity of the matrix-lipid-probe-ROS interactions in the setting of systemic inflammation may complicate interpretation of the results, [311] and further optimization of these fluorescence based assays is needed.

Herein we describe an alternative approach to quantify the HRA, a measure of HDL function, in a cell-free biochemical assay. Amplex Red reagent in the absence of cholesterol oxidase and in the presence of horseradish peroxidase (HRP) specifically quantifies the endogenous lipid hydroperoxides of a specific amount of HDL cholesterol. We demonstrate that this approach in combination with immunoaffinity capture of HDL for HDL isolation may overcome many confounding factors that affected the readout in the previously described cell free assays of HDL function.[311] Thus, this assay may be used in large scales studies as a tool to determine the role of HDL functional phenotype in the development of atherosclerosis *in vivo*.

5.3 Results

Amplex Red can specifically measure lipid peroxidation of a specific amount of HDL.

Amplex Red in the presence of the enzyme cholesterol oxidase has been reliably used to quantify cholesterol content of HDL based on lipid peroxidation of HDL.[312,313] The biochemical reaction of the Amplex Red Reagent with the OH radical in the presence of horseradish peroxidase (HRP) to produce highly fluorescent resorufin and measure peroxides is well established.[312,313] In the absence of cholesterol oxidase and in the presence of horseradish peroxidase (HRP) Amplex Red specifically quantifies the endogenous lipid hydroperoxides of a specific amount of HDL cholesterol **(Figure 5.2)**.Using this modification (no cholesterol oxidase) for the same amount of HDL cholesterol differences in the rate of lipid peroxidation between different samples (as detected by the Amplex Red reagent) may correspond to differences in HDL redox activity **(Figure 5.2)**.

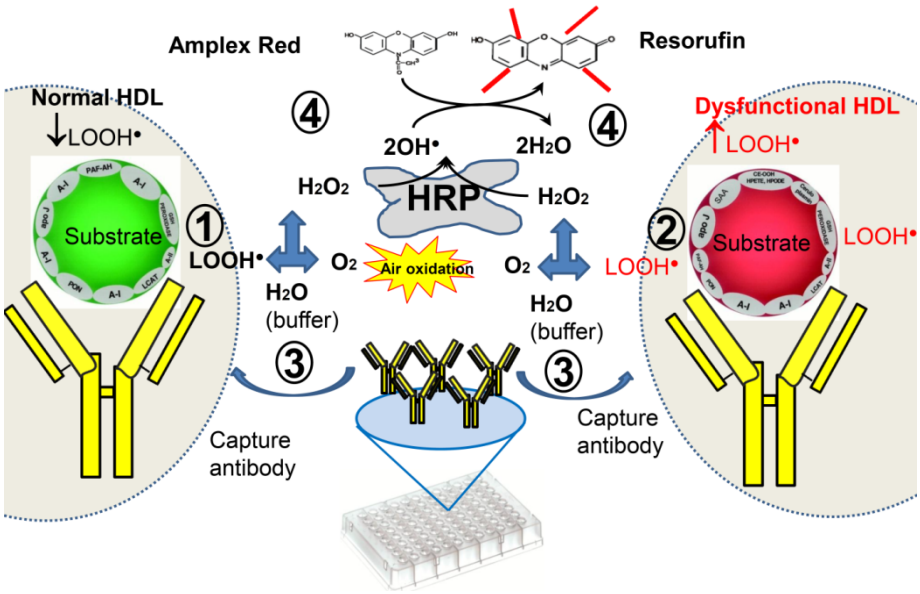

Figure 5.2: Principle of the Amplex Red assay of HDL function. 1. The acute-phase (AP) reaction favors the formation of dysfunctional HDL. In the basal state, HDL contains apoA-I and apoJ as well as 4 enzymes, paraoxonase (PON) and platelet-activating factor acetylhydrolase (PAF-AH), lecithin: cholesterol acyltransferase (LCAT) and plasma reduced glutathione selenoperoxidase (GSH peroxidase) that can prevent the formation of or inactivate the LDL-derived oxidized phospholipids found in oxidized LDL. As a result, in the basal state, HDL may be considered anti-oxidant. **2.** As previously published[53] during the acute-phase reaction, A-I may be displaced by the pro-oxidant acute-phase reactant Serum amyloid A (SAA). Another pro-oxidant acute-phase reactant, ceruloplasmin, associates with HDL as does the anti-oxidant acute phase reactant apoJ. PON, PAF-AH, and LCAT decrease in HDL during the acute-phase reaction, and the lipid hydroperoxides (LOOH) 5-hydroperoxyeicosatetraenoic acid (HPETE), hydroperoxyoctadecadienoic acid (HPODE),

and cholesteryl linoleate hydroperoxide (CE-OOH) increase in HDL. The net effect of the changes in HDL during the acute-phase reaction is the production of pro-oxidant, HDL particles (AP-HDL or dysfunctional HDL). **3.** HDL can be isolated using different methods such as ultracentrifugation, PEG precipitation and immunoaffinity capture (shown). Using immunoaffinity capture of HDL and commercially available antibodies against total human HDL, HDL is isolated from a specific volume (e.g 100 ul) of either a) non EDTA plasma b) serum c) apoB depleted serum **4.** Amplex® Red (N-acetyl-3, 7-dihydroxyphenoxazine) reagent is a colorless substrate that reacts with hydrogen peroxide (H_2O_2) and more specifically with the OH radical in the presence of horseradish peroxidase (HRP) with a 1:1 stoichiometry to produce highly fluorescent resorufin (excitation/emission maxima=570/585 nm). This highly stable, sensitive and specific fluorogenic substrate for HRP has been widely used to develop a variety of fluorogenic assays for enzymes that produce hydrogen peroxide. For example Amplex Red reagent coupled with the enzymes cholesterol oxidase and HRP permit the ultrasensitive quantitation of HDL cholesterol based on lipid peroxidation. Resorufin is produced by the reaction of the Amplex® Red reagent with H_2O_2 produced from the cholesterol oxidase-catalyzed oxidation of cholesterol. In the absence of cholesterol oxidase, the "endogenous" hydroperoxide content of a specific amount of HDL cholesterol can be quantified in the presence of HRP and Amplex Red. High hydroperoxide content of a specific amount of HDL cholesterol has previously been shown to be significantly associated with abnormal HDL function. The background production of hydroxyradicals as a result of air oxidation of the buffer (based on the readout of the blank well that contains Amplex Red reagent and buffer) is subtracted from the fluorescent readout of each well.

Determination of rate of production of resorufin was performed by measuring the slope of fluorescence increase during the first 60 minutes when the rate of oxidation is linear. These differences could be demonstrated using both purified HDL isolated by ultracentrifugation (data not shown) and apoB depleted serum **(Figure 5.3)**, a matrix that is characterized by more prominent lipid probe interactions.[311]

Lipid probe interactions are still present when using Amplex Red in fluorescent assays of HDL function. Since use of fluorescent assays of HDL function may allow study of the role of the functional phenotype of HDL in the setting of large scale studies[75] especially with the use of a robust, reproducible, high throughput method to extract HDL from patient serum such as PEG precipitation[72], we determined the (apo) B-depleted serum-Amplex Red interactions in patients with inflammatory conditions such as HIV versus healthy subjects. Alone, Amplex Red exposed to air became oxidized (and therefore fluorescent) at a linear rate within 60 minutes **(Figure 5.3)**. The rate of oxidation of Amplex Red was significantly

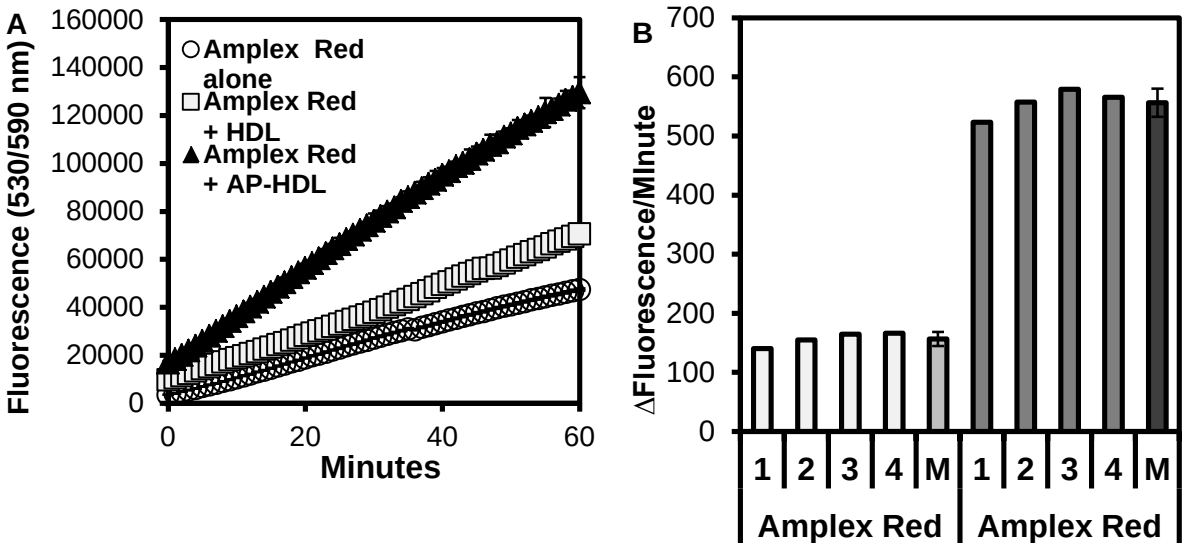

Figure 5.3. Oxidation of Amplex Red and effect of added HDL. In a 96 well flat bottom, 50 ul of 1X reaction buffer (0.5 M potassium phosphate, pH 7.4, 0.25 M NaCl, 25 mM cholic acid, 0.5% Triton® X-100) was added to each well alone or with 5 µg (cholesterol) of apoB depleted serum (as determined by a cholesterol assay) from a donor with anti-inflammatory HDL (HDL) and from a donor with acute phase HDL (AP-HDL), each in quadruplicates. 50 ul of HRP was then added to all wells followed by incubation at 37C for 60 min. 50 ul of Amplex Red Reagent (final concentration 300 µM) was then added to each well for a total volume of 150µl. Resorufin is produced by the reaction of the Amplex® Red reagent with H_2O_2 and the rate of production of resorufin was followed at 37˚C in one-minute intervals using a fluorescence microplate reader set to detect 530/590 nm excitation/emission. **A.** The means and standard deviations of the quadruplicate fluorescence measurements are plotted over time. **B.** The rates of change in fluorescence between 0 and 60 minutes (calculated by linear regression) are plotted for the quadruplicates, as well as means/standard deviations. The background fluorescence of the blank well (no HDL) was subtracted from the readout of each well for each timepoint.

less with added HDL, and different HDL samples (dysfunctional HDL compared to normal) showed clearly different effects in this regard **(Figure 5.3)**. Furthermore, when the amount of added HDL was varied, there was a clear dose dependence in the oxidation rate of Amplex red that was linear in the range of 1.25-10 µg (cholesterol) of added HDL per well in the assay (data not shown). Thus, apoB depleted serum-probe interactions were also present with Amplex Red similarly to the previously used fluorescent probes.[311]

Use of Amplex Red in combination with HRP may overcome lipid probe interactions when measuring lipid peroxidation of HDL. To determine whether the reduction in the fluorescence signal of Amplex Red after addition of HDL can be minimized or abolished by adding an enzyme that specifically catalyzes the lipid peroxidation and the oxidation of

Amplex red, we tested the effect of addition of different concentrations of HRP on the oxidation rate of Amplex Red in the presence of a specific amount of HDL cholesterol. In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin and this reaction has been used to detect as little as 10 picomoles of H_2O_2 in a 100 µL volume (50 nM) or 1×10^{-5} U/mL of HRP.[314-316] Increasing concentrations of HRP 1-10 U/ml increased the oxidation rate of Amplex Red in the setting of lipid peroxidation of specific amount of HDL, compared to when Amplex Red was used without any HRP. The Amplex Red assay could linearly quantify the content of hydroperoxides associated with a specific amount of HDL cholesterol when ≤ 10 ug of HDL is added, with low interassay and intra-assay experimental variability (< 11%) irrespective of the used method of HDL isolation (data not shown). Thus, the relative differences in HRA between different samples (dysfunctional HDL versus normal HDL) can be quantified with the use of Amplex Red and HRP with minimal lipid-probe interactions.

The Amplex Red assay can detect dysfunctional HDL *in vivo*. To further validate the new method we used the Amplex Red assay to detect dysfunctional HDL in conditions in which dysfunctional HDL is known to be present such as established animal models of atherosclerosis,[57,75] and Human Immunodeficiency virus infection (HIV).[75] The Amplex Red assay could detect established effect of statins on functional properties of HDL in animal models of atherosclerosis such as LDLR-/- **(Figure 5.4A)** and ApoE-/- mice **(Figure 5.4B)**.

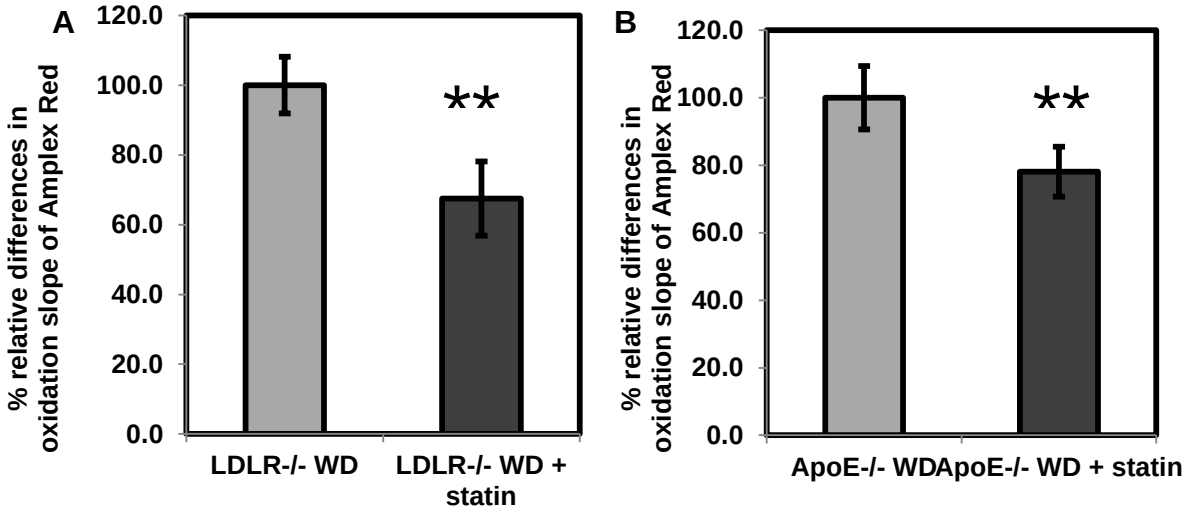

Figure 5.4 The Amplex red assay of HDL function can detect established effect of statins on functional properties of HDL in animal models of atherosclerosis. **A:** By using FPLC, HDL was isolated from three pooled plasma samples from LDLR$^{-/-}$ mice on Western diet (LDLR$^{-/-}$ WD) for two weeks and from three pooled plasma samples from LDLR$^{-/-}$ mice on Western diet for two weeks that were also treated with pravastatin 12.5 µg/ml for two weeks. Each sample was pooled from 4 mice (12 mice in total). Oxidation of Amplex Red was assessed as in Figure 5.3, using 2.5 µg (cholesterol) of added HDL. The oxidation slope of Amplex Red in the presence of HDL from LDLR$^{-/-}$WD+ statin was normalized to the oxidation slope in the presence of HDL from LDLR$^{-/-}$ WD, and the % relative differences are shown. The data represent the average of measurements from 3 independent experiments. There was a statistically significant reduction in the oxidation slope of Amplex Red in the presence of HDL isolated from LDLR$^{-/-}$ WD+ statin mice compared with the oxidation slope of DHR in the presence of HDL isolated from LDLR$^{-/-}$ WD mice (** P = 0.01) **B:** By using FPLC, HDL was isolated from three pooled plasma samples from ApoE $^{-/-}$ female mice on Western diet (ApoE $^{-/-}$ WD) for two weeks and from three pooled plasma samples from ApoE $^{-/-}$ female mice on Western diet for two weeks that were also treated with pravastatin 12.5 µg/ml for two weeks. Each plasma sample was pooled from 4 mice (12 mice in total). Oxidation of Amplex Red was assessed as in A. There was a statistically significant reduction in the oxidation slope of Amplex Red in the presence of HDL isolated from ApoE $^{-/-}$ WD + statin mice compared with the oxidation slope of Amplex Red in the presence of HDL isolated from ApoE $^{-/-}$ WD mice (** P = 0.01).

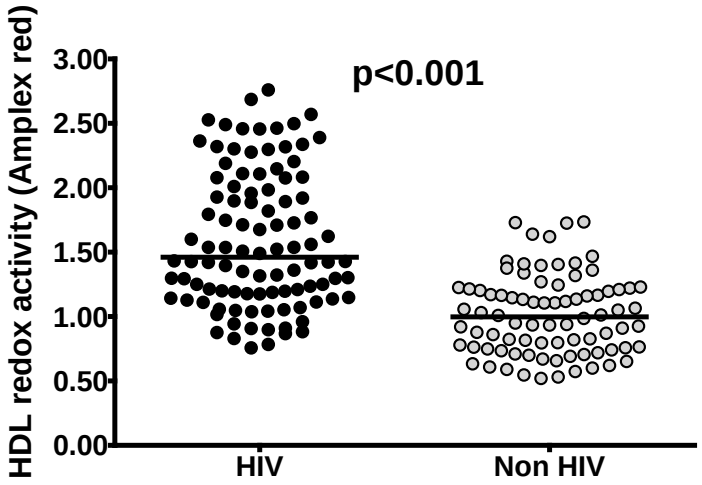

Figure 5.5 The Amplex Red Assay of HDL function can detect acute phase HDL *in vivo* in subjects previously shown to have dysfunctional HDL. ApoB depleted serum was isolated by PEG precipitation from 50 healthy subjects and 100 patients with HIV infection and that have previously been shown to have acute phase HDL.[311] The HIV-infected subjects had significantly higher HRA (1.59±0.53) compared to the uninfected subjects 1.01±0.31) (p<0.001)

In addition, using samples from a previous cohort of HIV patients known to have dysfunctional HDL,[75] the Amplex Red assay confirmed that these patients had higher HRA compared to healthy controls **(Figure 5.5)**. Thus, using this methodology we demonstrate

that HDL from patients with dysfunctional HDL has a higher rate of lipid peroxidation of a specific amount of HDL (HRA) compared to HDL from healthy patients.

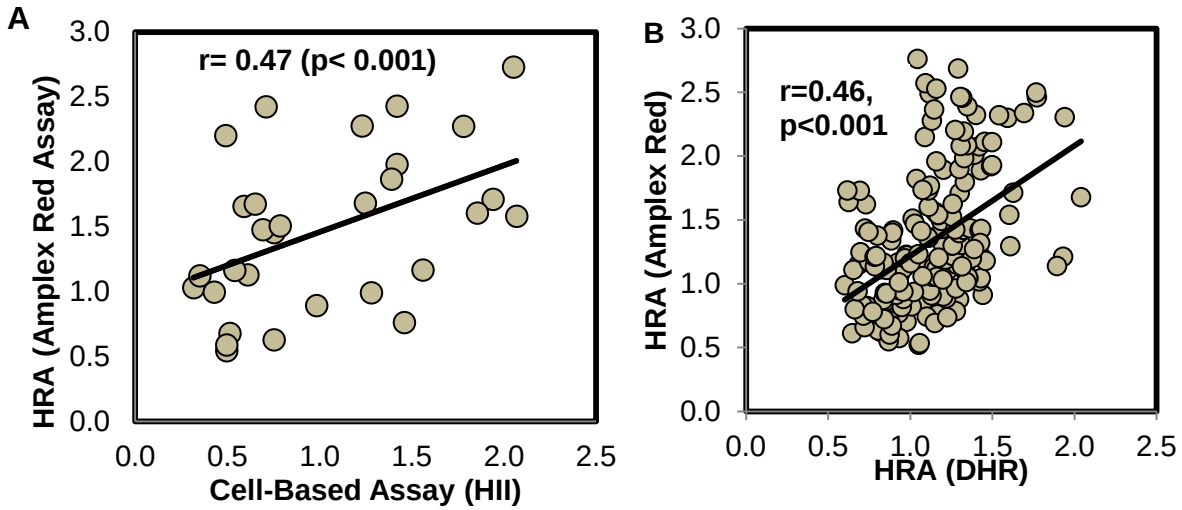

Figure 5.6. The readout from the Amplex Red Assay of HDL function correlates significantly to the readout of a previously validated cell based and cell free assays of HDL function. A. Thirty samples of FPLC-purified HDL were assessed for their HDL redox activity (HRA) using the Amplex Red assay, and their HDL inflammatory index was determined in a cell-based assay as described in Materials and Methods. The values from each assay are plotted against each other. **B.** ApoB depleted serum was isolated by PEG precipitation from 50 healthy subjects and 100 patients with HIV infection that have previously been shown to have acute phase HDL.[311] HDL redox activity (HRA) was determined with the Amplex Red assay and with the dihydrorhodamine (DHR) assay as described in Methods. Non-cryopreserved apoB depleted serum was used for the DHR assay and the readout was normalized by the readout of a pooled control. The values from each assay are plotted against each other.

Results from the Amplex Red assay correlate with previously validated cell-based and cell-free assays. To compare the results of the Amplex Red assay to those obtained using a validated cell-based assay[75,285] 30 HDL samples were assessed using both assays **(Figure 5.6A)**. Comparing HRA as measured with Amplex Red to the HDL inflammatory index, there was a strong positive correlation ($r = 0.47$, $p<0.001$). Moreover, to compare the results of the Amplex Red assay to those obtained using a validated cell-free assay[75] 60 HDL samples were assessed using both assays **(Figure 5.6B)**. HRA as measured with Amplex Red correlated significantly to the HRA as measured with DHR ($r = 0.46$, $p<0.001$).

The immunoaffinity capture of HDL can be used in the Amplex Red assay of HDL function to isolate HDL and minimize albumin contamination. Although PEG precipitation is a

reproducible, high throughput method to extract HDL from patient serum,[72] a major issue with this method is contamination of the HDL with other plasma proteins, especially albumin. Albumin represents a very abundant and important circulating antioxidant that binds ROS,[317] removes lipid peroxidation products[318] and can also significantly interfere with the fluorescent readout in biochemical assays of HDL function.[311] Although albumin contamination of HDL can be minimized using ultracentrifugation to isolate HDL this method is not high throughput and does not allow use of this assay in large scale studies. We have previously used immunoaffinity capture of HDL to study changes in the proteome associated with dysfunctional HDL.[56] Commercially available total HDL ELISA kits can be used to capture HDL in 96-well plates when a specific volume of blood, purified HDL or apo-B depleted serum is added. For this purpose we have validated and used two commercially available kits (Kit A: Genway, San Diego, CA; Kit B: Biotang Inc, Waltham, MA). The immunogen is total human HDL derived from pooled plasma from healthy donors and the antibody is chicken (kit A) and mouse (kit B) anti-HDL. The sensitivity of detection of total HDL is 1.5 ng/ml for both kits. According to the manufacturer's instructions, by immunoelectrophoresis and ELISA the antibodies in these kits react specifically with human HDL and not with other human serum proteins including human IgG and human serum albumin (package insert, personal communication). Indeed, using immunoaffinity capture of HDL and 2 different methods to detect albumin content, we showed that the HDL captured on 96-well plates is largely free of albumin (data not shown).

Amplex Red can reliably measure lipid peroxidation of a specific amount of HDL isolated using immunoaffinity capture of HDL. We used Amplex Red in the presence of HRP and in combination with immunoaffinity capture of HDL (for HDL isolation) to specifically quantify the rate of lipid peroxidation (HRA) of a specific amount of HDL cholesterol. Using this methodology with different matrices we demonstrate that HDL from patients with dysfunctional HDL has a higher rate of lipid peroxidation of a specific amount of HDL compared to HDL from healthy patients **(Figure 5.7)**. However, since the proteome of dysfunctional HDL has not been fully elucidated, [57,58] it is possible that dysfunctional HDL

may bind less efficiently to different commercially available HDL antibodies and the total amount of HDL protein may not be directly compared between different subjects.

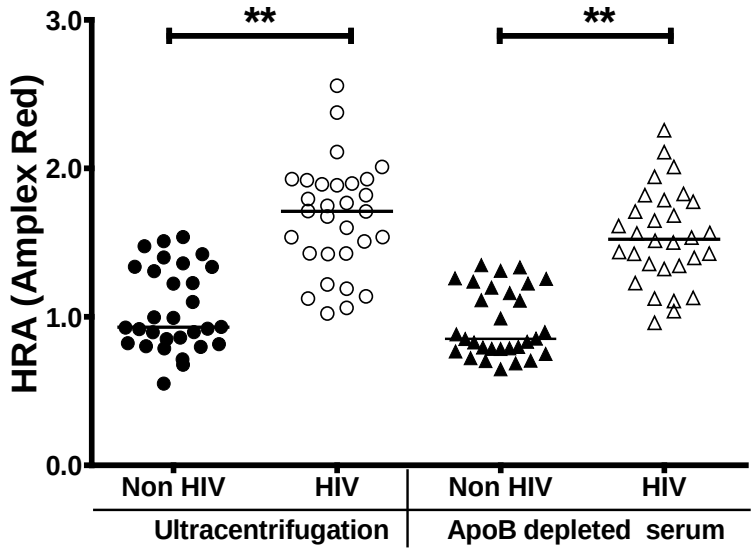

Figure 5.7 The Amplex Red Assay of HDL function in combination with immunoaffinity capture of HDL can detect acute phase HDL *in vivo* in subjects previously shown to have dysfunctional HDL. HDL was isolated using immunoaffinity capture as described in Methods from 30 healthy subjects and 30 patients with HIV infection that have previously been shown to have acute phase HDL.[311] The following different matrices were added in 96 well plates for immunoaffinity capture of HDL: a) purified HDL isolated by ultracentrifugation (5 ug of HDL cholesterol as determined by cholesterol assay), b) apo-B depleted serum isolated by PEG precipitation (5 ug of HDL cholesterol as determined by cholesterol assay). In the latter method, the fluorescent readout (that corresponds to HRA) was normalized to the HDL cholesterol concentration (measured by the clinical lab). The Medians are shown.

Thus, to determine whether the approach of immunoaffinity capture of HDL depends on the quality of commercially available HDL antibodies, we used 2 different commercially available HDL kits with the Amplex Red assay (kit A and B) to measure HRA in 60 samples. We found comparable results (data not shown) which indicate that the detected differences in HRA are real and not artificial secondary to variations in binding of HDL to the antibody. Thus, the suggested method may allow high throughput isolation of HDL and in situ detection of HRA that is associated with HDL function.

The HRA as measured with the novel assay can be used as a marker of biologic processes in humans. To further validate the utility of the new assay to measure HRA as a marker of HDL function and other physiological processes in humans, HRA was measured

blindly in a previously well-established cohort of 90 subjects that looked into the effect of exercise on metabolic and other physiological parameters. Using the samples from this study we found that the Amplex Red assay of HDL function can detect previously established favorable effects of exercise on HDL function **(Figure 5.8)**.[319]

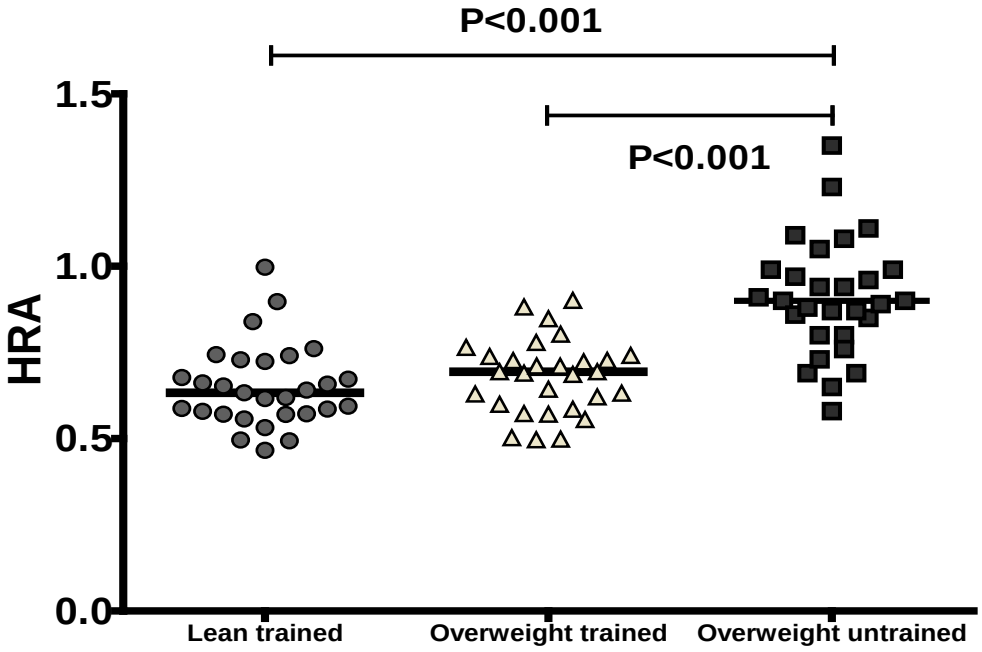

Figure 5.8. The Amplex Red assay of HDL function can detect previously established favorable effects of exercise on HDL function. HRA was measured in a previous cohort of 90 humans looking into the effect of exercise on metabolic and other physiological parameters. In this study high-intensity resistance training (RT) improved central and brachial blood pressures in the overweight untrained (OU) group, while having no effect on major indices of arterial stiffness in overweight/obese young men, without weight loss (unpublished data). Using the samples from this study we found that HRA was significantly lower in both trained groups compared to the untrained group (LT vs. OU: 0.65±0.12 vs. 0.91±0.17, p=<0.001; OT vs. OU: 0.68±0.11vs. 0.91±0.17, p=0.003), and LT and OT were not significantly different (p=0.12).

Additionally, we assessed associations of HRA with indices of vascular and metabolic disease **(Figure 5.9)**. HRA (adjusted for HDL cholesterol concentration as outlined above) had significant negative associations with the subendocardial viability ratio (r= -0.21, p=0.05), relative strength (r= -0.45, p<0.001), homeostatic model assessment of insulin resistance (r= -0.25, p=0.02), HDL (r= -0.92, p=<0.00001), adiponectin (r= -0.29, p<0.01) and significant positive associations with anthropometric parameters of obesity such as BMI

(r=0.50, p=<0.0001), waist circumference (r=0.59, p=<0.0001), trunk fat (r=0.56, p=<0.0001), total fat mass (r=0.55, p=<0.0001) total fat mass (r=0.55, p=<0.0001), C-reactive protein (CRP) (r=0.28, p=<0.001), fasting glucose (r= 0.23, p=0.03), fasting insulin (r= 0.21, p=0.04), amylin (r= 0.23, p=0.04), leptin (r= 0.48, p<0.001), oxidized LDL (r= 0.36, p<0.001), triglycerides (r= 0.36, p<0.001). These associations remained significant in multivariate analysis after adjusting for metabolic and anthropometric parameters (unpublished data). These pilot data demonstrate our ability to measure HRA and associate cardiovascular and metabolic risk phenotypes with these measures. Larger case control studies will further define the utility of this assay as a marker of biologic processes and diseases in humans.

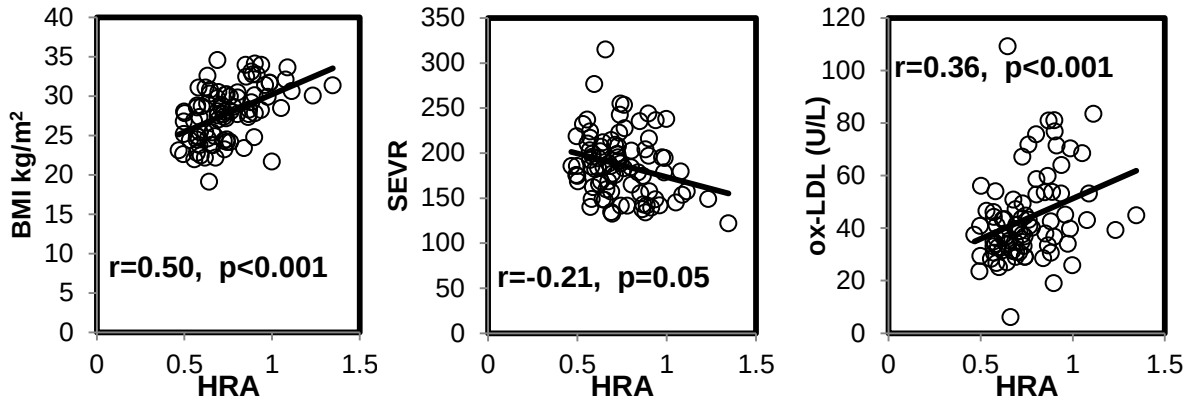

Figure 5.9. The HRA as measured with the novel assay is significantly associated with numerous anthropometric, metabolic and physiological parameters in humans. HRA was measured in a previous cohort of 100 humans looking into the effect of exercise on metabolic and other physiological parameters. The values from HRA for each subject are plotted against representative physiological parameters such as Body Mass Index (BMI), subendocardial viability ratio (SEVR), a noninvasive measure of subendocardial perfusion, C reactive protein (CRP) and oxidized Low Density Lipoprotein (ox-LDL).

5.4 Discussion

Growing evidence suggests that HDL varies significantly in its phenotype and influence on cardiovascular disease risk.[61,74] Previous work has suggested dysfunctional HDL to be pronounced in chronic inflammatory conditions that predispose to atherosclerosis.[74,283] This impaired (dysfunctional) HDL, is characterized by (i) decreased levels and activity of anti-inflammatory, antioxidant factors; [49,61] (ii) gain of pro-inflammatory proteins; [52] (iii) increased lipid hydroperoxide (LOOH) content;[50] (iv) reduced potential to efflux cholesterol; [53] and (v) diminished ability to prevent LDL oxidation.[320] Measuring the functional status of HDL, rather than HDL cholesterol concentration, may be more informative in predicting cardiovascular disease.[70] Unfortunately, the most widely used assays for HDL function have been cell-based assays[61] which are highly labor-intensive and subject to substantial assay variation[68,69] since the optimal method for performing such studies with regard to donor cells, type of acceptor, type of readout, has yet to be determined. Thus, cell based assays of HDL function are technically prohibitive for many researchers and for use in large scale studies.

Cell-free assays of HDL function may be more precise than cell-based assays because they measure a biochemical rather than biologic phenomenon. Thus, we previously developed a cell free assay based on the fluorochrome DCF that assessed the HDL inflammatory index (HII), a measure of the capacity of HDL to prevent the formation or to inactivate oxidized phospholipids produced by LDL.[73] This was based on our demonstration that levels of ROS (such as lipid hydroperoxides produced from oxidation of lipoproteins) are significantly associated with inflammatory properties of HDL that are measured by the HII. [55] This assay has been previously used in multiple studies in humans[55] and this measurement of HDL has been associated with other measures of HDL function such protein biomarkers associated with dysfunctional HDL,[57,58,71,309,310] HDL anti-inflammatory function measured as the ability of test HDLs to inhibit LDL-induced monocyte chemotactic activity in human aortic endothelial cell monolayers[310] and measurement of oxidized fatty acids in HDLs.[309,310] It has

also been validated *in vivo* in animal models of atherosclerosis.[309] Furthermore, HDL function measured using this assay has been correlated with systemic inflammation in coronary heart disease (CHD) patients[56] and cardiovascular disease outcome.[72] However, the oxidative instability of DCF-DA, variations in the donor LDL used in the assay and increased lipid-probe interactions of two lipoproteins used in the same biochemical reaction increased experimental variability of this method.[56]

In view of the significance of HDL redox activity for HDL function and the previous demonstration that the oxidation rate of DHR can be used to quantify redox activity in extracellular matrix such as plasma,[292] we developed a biochemical assay of HDL function that is based on DHR and in which the endogenous HRA is assessed in terms of the capacity of a specific amount of HDL cholesterol to engage *in vitro* redox cycling.[286] In contrast to DCFH which is unstable and prone to auto-oxidation, DHR is relatively stable, requires no activation and oxidizes at a predictable rate when exposed to room air.[75] In addition the kinetic approach for measuring oxidation rate during a linear phase, lends greater precision compared to a single endpoint measurement. This assay was validated *in vitro*[75] against an established monocyte chemotaxis assay of HDL function and *in vivo*.[75]

High density lipoprotein is the major carrier of lipid hydroperoxides in human blood plasma[312,313] and dysfunctional HDL had increased endogenous “ROS load” and redox activity.[294] 1*e*-oxidants such as OH^- preferentially react with the lipoprotein's lipids and this causes lipid peroxidation with the resulting accumulation of hydroperoxides of phospholipids and cholesteryl-esters[61,289,290,294,295] that then oxidize apoA-I's methionine to MetO.[294] We have previously shown that increased lipid hydroperoxide (LOOH) content is associated with dysfunctional HDL[321] and that oxidized HDL is dysfunctional HDL.[289,290,295] However, although DHR may measure the capacity of HDL cholesterol to engage *in vitro* redox cycling,[75] DHR is not a substrate for oxidation by H_2O_2 [75] suggesting that lipid hydroperoxides in HDL are not promoting DHR oxidation. Thus the biochemical mechanism of the DHR assay of HDL function remains to be determined. In addition, when we used apoB depleted serum and cryopreserved samples in the setting of larger studies, we found

that lipid-probe interactions that depend on the type and concentration of the lipid and the fluorescent probe, method of HDL isolation, freeze-thaw, different diluents and matrices and changes in pH may complicate interpretation of the results.[322] The limitations of the DHR assay have been previously described in detail.[311] Thus, only fresh apoB depleted serum samples and purified HDL isolated by ultracentrifugation may be used with the DHR assay and this may limit its utility for large scale clinical studies.

Herein we describe an approach to quantify the HDL redox activity, a measure of HDL function, in a novel cell-free, biochemical assay. We looked for a fluorescent probe that would meet the following criteria i) would reliably and specifically quantify the rate of lipid peroxidation of a specific amount of HDL cholesterol ii) enzymatic amplification of the measurement of ROS with this probe would overcome lipid-probe-ROS interactions that may be a limitation of biochemical assays of HDL function[75] iii) would be a reasonable choice to measure ROS accurately based on the known challenges and limitations that most widely used fluorescent probes have for detecting and measuring ROS.[311]

Amplex red is a fluorogenic substrate with low background fluorescence, which reacts with H_2O_2 with a 1:1 stoichiometry to produce fluorescent resorufin.[323] Amplex Red can be oxidized by horseradish peroxidase (HRP) which vastly increases the yield of resorufin[313,324] and this assay is a reliable method to continuously measure the extracellular formation of H_2O_2[313,324] in 96 well plate format.[313,324] We have used the Amplex Red reagent to quantify ROS produced by oxidized lipids in cell culture supernatants.[312,313] Indeed, Amplex Red in the presence of the enzyme cholesterol oxidase has been reliably used to quantify cholesterol content of HDL based on lipid peroxidation of HDL.[325] Using a modification of this assay, we demonstrate that Amplex Red, in the absence of cholesterol oxidase and for the same amount of HDL cholesterol, can detect differences in the rate of lipid peroxidation between different HDL samples that correspond to differences in HDL function.

The products of redox cycling are detected as time-dependent oxidation of the fluorogenic probe Amplex Red that in the presence of horseradish peroxidase (HRP)

specifically quantifies the rate of lipid peroxidation of a specific amount of HDL cholesterol and the rate of reaction of the OH^- with Amplex Red. We demonstrate that this approach in combination with immunoaffinity capture of HDL for HDL isolation may overcome many confounding factors that affected the readout in the previously described cell free assays of HDL function and provides a measurement of HRA that correlates well with previously validated cell-based and cell-free assays of HDL function.

This Amplex Red-based cell-free assay improves upon the prior DHR-based cell-free assay. While also measuring the HRA the biochemistry of the Amplex Red fluorochrome and its ability to detect ROS and lipid hydroperoxides is well established. In the absence of cholesterol oxidase the Amplex Red detects the intrinsic hydroperoxide content of a specific amount of HDL cholesterol. Moreover, the use of immunoaffinity capture may allow HDL isolation and use of this method in large scale studies and removal of much of the albumin bound to the HDL particle that may alter the association of ROS with lipoproteins.[75] Finally, the inter-assay variability of <15% compares favorably with cell-based assays of HDL function, which have variability of >15%.[317]

In addition using animal models of atherosclerosis and 2 different human studies we show that this assay can be used as a marker of cardiovascular disease and biologic processes *in vivo*. The correlation of HRA as measured by the Amplex Red assay to the biologic readout of HDL in a cell-based assay is consistent with a proposed mechanism whereby HDL exerts its effects through modulating ROS,[54,74] and suggests that the assay accurately reflects HDL function. Further support for the biological relevance of this measurement is the finding that for the same amount of HDL cholesterol the HRA was significantly reduced in HDL from statin treated mice with atherosclerosis compared to HDL from non-statin treated mice. Indeed, treatment of these mice with statins has previously been shown to reduce inflammatory properties of HDL[55] and we demonstrate that the Amplex Red assay can detect the favorable effect of statins on functional properties of HDL. Moreover, the Amplex Red assay can detect dysfunctional HDL in patients with HIV infection confirming our previous results.[302] The fact that our assay quantifies HRA that correlates

significantly with cardiovascular disease and other physiological parameters in humans such as obesity and insulin resistance increases its applicability to biological samples, at least in the context of cardiovascular diseases. This is because 1*e*-oxidants (i.e., hydroxyl radical) contribute to oxidative modifications taking place in the affected arterial wall during atherogenesis.[75] Thus, while "oxidized HDL" is not a chemically defined term, the oxidation rate of Amplex Red corresponds to the intrinsic HRA of specific amount of HDL lipoproteins.

Finally, we showed that matrix effects, freeze thaw, sample handling and long term storage of blood specimens at -80C can affect the HRA but the relative differences in HRA between different samples can still be reliably detected with the Amplex Red reagent with either serum or non EDTA plasma, fresh or cryopreserved samples and with up to 2 freeze-thaw cycles. However, the oxidative modifications occurring to HDL in the diseased artery wall are conceivably more complex[303] and HDL is subject to continuous remodeling *in vivo*. This includes dissociation of apoA-I from the lipoprotein particle, a process that could be increased by oxidation.[303] Clearly, future studies are required to assess these various aspects, as well as the utility of the assay described for clinical studies.

In conclusion, this new assay offers a rapid method for measuring the redox properties of HDL. It yields results that correlate well with previously validated cell-based and cell-free assays of HDL function and can be used as a marker of cardiovascular disease and biologic processes in humans. This new technical approach may offer a convenient tool for studies of the role of HDL functional phenotype in the development of atherosclerosis *in vivo*.

5.5 Materials and Methods

Human subjects: Blood samples were collected from patients with coronary artery disease (CAD) or equivalent, HIV-1-infected individuals on combination antiretroviral therapy (ART) with suppressed viremia (below 50 copies of RNA/ml) and healthy subjects were recruited at the University of California, Los Angeles (UCLA) as described in Chapter 4. [285] In the exercise study, 90 young adult males, ages of 18-30 were recruited and categorized into 3 phenotypes based on training status and BMI: lean trained (LT, n=30, ≥4 d/wk resistance training (RT), BMI<25kg/m^2), overweight trained (OT, n=30, ≥4 d/wk RT, BMI >27 kg/m^2) and overweight untrained populations (OU, n=30, no structured exercise regimen, BMI>27 kg/m2). Overweight untrained subjects participated in only light intensity physical activity ≤2 times/wk. All of the study protocols were approved by the UCLA IRB.

Mice, reagents, HDL and LDL were prepared as described in Chapter 4 and as previously.[75,305-307]

Immunoaffinity capture of HDL: We have previously used immunoaffinity capture of HDL to study changes in the proteome associated with dysfunctional HDL.[56] Briefly, 96-well polyvinyl chloride microfilter plates (BD Biosciences) were precoated with 1-5 ug/ml of chicken anti-human HDL antibodies (GenWay Biotech, San Diego, CA) overnight at 4°C. Following incubation of the pre-coated plates with individual plasma samples diluted at 1:10 with 1X PBS, serum at 1:20 dilution, or HDL at 1:2 dilution, the plates were washed thoroughly. Detection of HDL was confirmed by HDL-capturing sandwich enzyme-linked immunosorbent assays (ELISA) as described previously using corresponding primary antibodies to human Apo A-I at 1:2,500 dilution.[56] In addition, total HDL was captured using polyclonal antibodies included in 5 commercially available kits [Genway Inc (kit A); Biotang Inc (kit B), Cusabio Inc (kit C), China; WKEA Inc, China (kit D); Wuhan EIAab.,Ltd; China (kit E)) according to the manufacturer's instructions.

Measurement of HDL cholesterol: HDL cholesterol was quantified using a standard colorimetric assay (Thermo DMA Co., San Jose, CA, USA) as previously described.[75] Samples were also assayed by the UCLA Clinical Laboratory for total

cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), non-HDL cholesterol and triglycerides (TG).

HDL Inflammatory index: The HDL-inflammatory index was determined for each subject's HDL as described previously.[55] In this assay a value >1.0 is considered pro-inflammatory and a value <1.0 is considered anti-inflammatory.

DHR-based cell-free assay of HDL function: The DHR assay was performed as previously described in Chapter 4.

Amplex red assay: *Method A: Use of PEG precipitation for HDL isolation.* ApoB depleted serum was isolated from human plasma using PEG precipitation and then a specific amount of HDL cholesterol (5 ug; quantified using a standard colorimetric assay, Thermo, CA, USA) was added to 96-well plates (polypropylene, flat bottom, black, Fisher Scientific, USA) in quadruplicates with the appropriate volume of 1X Reaction Buffer for a total volume of 50 ul per reaction well. A 2 mM resorufin solution was used to prepare a standard curve to determine the moles of product produced in the Amplex® Red reaction. The appropriate amount of 2 mM resorufin reference standard was diluted into 1X Reaction Buffer to produce resorufin concentrations of 0 to ~20 µM). 1X Reaction Buffer without cholesterol was used as a negative control. A 20 mM H_2O_2 working solution was used as a positive control. A volume of 50 µL was used for each reaction. 50 ul of 300 µM of Amplex Red reagent (Invitrogen) containing 2 U/mL HRP and 0.2 U/mL cholesterol esterase were then added to each microplate well containing the samples and controls. The fluorescence of each well was assessed at one minute intervals over 30 minutes with a Plate Reader (Biotek, Vermont, USA), using a 530/590 nm filter pair. The fluorescence at each timepoint may be expressed: 1) relative to the maximum fluorescence observed over 30 minutes 2) relative to fluorescence of the respective timepoint of a reference control sample 3) as specific concentration of resorufin (uM) extrapolated from the resorufin standard curve at the specific timepoint using 4 parameter logistic regression analysis. The slope of the reaction of the Amplex Red reagent with the endogenous hydroperoxides present in HDL in the absence of cholesterol oxidase, corresponds to the endogenous HRA of each sample

and was calculated over 30 min using the Gen5 2.01 software (Biotek, Vermont, USA). Alternatively, a specific volume (50 ul) of apoB-depleted serum was added in each well in quadruplicates and the mean fluorescence readout (slope) was normalized by the HDL cholesterol concentration of each sample as measured by the clinical lab (mg/dl).

Method B: Use of HDL immunocapture for HDL isolation. The following matrices were used for HDL capture in 96 well plates: 1) specific amount of commercially available purified HDL (Sigma) (5 ug of HDL cholesterol) 2) specific amount of purified HDL isolated by ultracentrifugation (5 ug of HDL cholesterol) 3) specific amount of apo-B depleted serum isolated by PEG precipitation (5 ug of HDL cholesterol) 4) specific volume of plasma or serum (100 ul) 5) specific volume of apo-B depleted serum (100 ul). These matrices were then added into 96 wells and HDL was isolated using immunoaffinity capture of HDL as described above. The Amplex Red assay was performed as described above. When a specific volume (100 ul) of apoB-depleted serum or plasma/serum was added in each well in quadruplicates, the mean fluorescence readout (slope) was normalized by the HDL cholesterol concentration of each sample as measured by the clinical lab (mg/dl).

Data collection: In the exercise study measurements of serum glucose, serum insulin, homeostasis model assessment for insulin resistance (HOMA), oxLDL, high-sensitivity C-reactive protein, body composition parameters, anthropometric parameters, blood pressure (i.e. brachial systolic and diastolic pressure, bSBP, bDBP) maximal strength testing, subendocardial viability ratio (SEVR) were performed as previously described.[326]

Statistical analysis: Statistical analyses were performed with the use of Stata statistical software 12 (StataCorp LP., College Station, TX). Group means were compared using the Student's t-test for unpaired variates with $p < 0.05$ considered to be statistically significant. Correlation coefficients between variables were calculated using least squares linear regression. In the exercise study, post-hoc Pearson correlation analyses were used to determine the relationships between HRA and cardiovascular and metabolic disease risk biomarkers.

Chapter 6

Dysfunctional HDL, Immune activation and Atherosclerosis in HIV-1-Infected and -Uninfected Adults: *In vivo* studies

6.1 Abstract

Background: HIV is associated with immune dysregulation, immune activation, atherosclerosis and low-HDL dyslipidemia. HDL function rather than absolute level may be a more accurate indicator for risk of developing atherosclerosis. In inflammatory milieus, HDL becomes dysfunctional, taking on pro-inflammatory/pro-oxidant properties. Dysfunctional HDL has reduced antioxidant properties and high HDL redox activity (HRA), but it is unknown whether abnormal HDL function is associated with immune activation and progression of atherosclerosis in HIV-1-infected subjects. In this Chapter we investigate this question using three independent studies, novel assays of HDL function that are outlined in Chapters 4 and 5 and we relate HRA among HIV subjects to indices of cardiometabolic risk and immune activation. In addition, oxidized HDL may interact with the NF-kB pathway and a soluble product of activation of this pathway is the receptor activator of the NF-kB ligand (RANKL). The RANKL-osteoprotegerin (OPG) axis has been shown to play a role in the inflammatory process of atherogenesis and may be regulated by changes in levels of cholesterol. However, the interplay between HIV-1 infection, lipids, the RANKL-OPG axis and atherosclerosis is poorly defined.

Methods: We retrospectively measured serum HDL function in 1) 91 subjects from a prospective 3-year study of carotid artery intima-media thickness (CIMT), which enrolled triads of risk factor-matched persons that were HIV-1-uninfected (n=36) or HIV-1+ with (n=29) or without (n=26) protease inhibitor (PI)-based therapy for ≥ 2 years **(study 1)** 2) 102 HIV-infected subjects (95% on antiretroviral therapy) and 41 matched non-HIV controls without clinical cardiovascular disease underwent metabolic phenotyping/flow cytometry and coronary CT angiography **(study 2)**. 3) 150 HIV-1 infected patients with HIV-1 RNA <500 copies/mL on antiretroviral therapy (ART) and 50 matched non-HIV controls **(study 3)**. HDL function was assessed using two biochemical assays that use fluorochromes (dihydrorhodamine-123 or DHR and Amplex Red) and measure HRA in which higher fluorescence readout corresponds to dysfunctional HDL phenotype. The fluorescence readout from each sample was normalized to the readout of HDL from pooled serum of

healthy blood-bank donors. In addition, serum RANKL, OPG, and RANKL/OPG ratio were retrospectively assessed for 91 subjects in study 1 and their associations to the primary outcomes of levels of circulating lipids, HRA and atherosclerosis progression were determined using multivariate regression models. T cell activation (CD38 expression) was determined in study 3 using flow cytometry.

Results: Study 1: In univariate analysis of 55 HIV-1-infected subjects, greater waist circumference and lower serum HDL were significantly associated with higher baseline levels of HRA ($p=0.01$). These subjects had significant increases in levels of HRA over time (3 years) that were associated with white race ($p=0.03$), higher nadir $CD4^+$ count ($p<0.001$). We found that the DHR assay has limitations when using cryopreserved samples and we were unable to detect an association of HRA with progression of atherosclerosis in this small matched cohort study (study 1). However we also used the improved Amplex red Assay that is based on immunoaffinity capture of HDL and specific enzymatic amplification of detection of hydroperoxides associated with HDL and that may minimize biochemical limitations such as lipid probe interactions. Using the Amplex Red assay we found that in multivariate analysis of the HIV-infected subjects, higher baseline HRA was associated with the ΔCIMT increasing by 2.3 mm/yr (95% CI = (0.24, 5.6); $p = 0.03$) but no association between ΔCIMT and HRA was seen in the controls. Serum RANKL and RANKL/OPG ratio were significantly lower in HIV-infected versus -uninfected subjects ($p < 0.01$). Multivariate models for HIV-1+ subjects, but not in uninfected controls, demonstrated that perturbations in serum cholesterol levels were significantly associated ($p < 0.05$) with perturbations in serum levels of RANKL and OPG, and their ratio (RANKL/OPG). **Study 2:** In addition, in the larger Study 2 using the DHR assay we found that HRA was significantly higher in the HIV-infected group versus the non-HIV group (1.4 ± 0.01 versus 1.3 ± 0.01, $p=0.03$) and was independently associated positively with log-transformed levels of the monocyte/macrophage activation marker sCD163 ($r=0.24$, $p=0.02$) and with percent non-calcified plaque among all HIV-infected subjects ($r=0.29$, $p=0.03$). **Study 3:** Higher HRA was associated with increased immune

activation markers such as higher frequency of activated T $CD8^+$ cells (r=0.40; p<0.0001) and #CD38 on $CD8^+$ T cells (r=0.36; p<0.0001).

Conclusions: These data are among the first showing increased HRA among HIV-infected subjects on ART with low cardiovascular risk profile versus matched non-HIV subjects with comparable HDL levels. In HIV-infected subjects but not in uninfected controls, HRA associates with indices of macrophage and T cell activation, soluble products of activation of the NF-kB pathway, parameters of cardiometabolic risk and two different measures of subclinical atherosclerosis a) CIMT and b) percent non-calcified coronary plaque. These *in vivo* observations support a significant role for dysfunctional HDL in the interplay between HIV-1, immune activation and atherosclerosis.

6.2 Introduction

Among HIV-infected patients, antiretroviral therapy (ART) has extended life expectancy[148] and reduced deaths related to opportunistic infections.[149] However, recent data suggest that cardiovascular disease (CVD) represents an increasingly important cause of death among HIV-infected patients.[150,151] Studies show an approximately two-fold increased risk in myocardial infarction (MI) [153] and four-fold increased risk in sudden cardiac death[156] in the HIV-infected population relative to the general population. Understanding and addressing the causes of this increased CVD risk is an important public health concern for HIV-infected patients.

High-density lipoprotein (HDL) is generally accepted to have cardioprotective functions, participating in reverse cholesterol transport and exerting anti-inflammatory/anti-oxidant effects.[49,61] Moreover, low HDL levels track with heightened CVD risk in non-HIV populations.[327,328] HIV-infected patients have a pattern of dyslipidemia, characterized, in part, by low HDL levels.[184] However, heightened CVD risk in HIV persists even after controlling for dyslipidemia, [153] suggesting that circulating lipoprotein levels may not adequately reflect lipoprotein functionality. This inference is supported by pharmacologic trials of cholesterol ester transferase protein inhibitors in non-HIV populations, wherein elevation of HDL levels did not correlate with CVD risk reduction.[329,330] Indeed, basic and clinical studies suggest that HDL functionality may be altered in inflammatory milieus such that this typically anti-inflammatory/anti-oxidant lipoprotein takes on pro-inflammatory and pro-oxidant properties, becomes dysfunctional and may contribute to the risk of coronary disease.[50,63] Thus, HDL function rather than absolute level may be a more accurate indicator for risk of developing atherosclerosis.[49,61] Dysfunctional HDL has increased redox activity[75] and reduced antioxidant properties that may contribute to atherogenesis.[54,70] We have shown in Chapters 3-5 that HIV-1-infected ($HIV\text{-}1^{+}$) subjects have dysfunctional HDL.[331] However, no study has directly explored the relationships between dysfunctional HDL, HIV-1 infection and progression of atherosclerosis as well as predictors of dysfunctional HDL in HIV-1 infection.

Immune dysregulation is thought to contribute to increased CVD disease in HIV.[332,333] Even as HIV depletes CD4 T cell reservoirs, the infection precipitates activation of other innate and adaptive immune cells, with potential relevance to atherogenesis and atherothrombosis.[332,333] Among ART-treated HIV-infected patients, monocyte/macrophage activation relates to coronary atherosclerotic plaque inflammation[334] and vulnerability[335] and to increased carotid intima media thickness.[262] Since residual immune activation despite ART may contribute to the presence of dysfunctional HDL in HIV-1^+ persons[331] and progression of atherosclerosis,[262] we investigated possible associations between HRA and biomarkers of macrophage activation, such as soluble CD14 (sCD14), sCD163 and lipopolysaccharide (LPS)[41] and T cell activation such as CD38 expression. Herein, we hypothesized that the interplay between HDL function and immune activation may also contribute to increased CVD risk in HIV.

Using recently developed assays outlined in Chapters 4 and 5 that quantify the redox activity of HDL based on the oxidation rate of the fluorogenic probe dihydrorhodamine 123 (DOR)[75] and Amplex Red and serum specimens from established clinical studies, we examined the association of HRA with atherogenesis, as evaluated by measurement of carotid artery intima-media thickness (CIMT) and non-calcified coronary plaque, in HIV-1-infected (HIV-1^+) and -uninfected (controls) individuals. In view of the possible role of oxidized lipids and lipoproteins in immune activation and atherogenesis as summarized in Chapter 1, we investigated the association of oxidized HDL with biomarkers of macrophage and T cell activation.

A pathway that interacts with oxidized lipids and oxidized HDL and that is important for atherogenesis and regulation of immunity is the nuclear factor-kB (NF-kB) pathway. A soluble product of activation of the NF-kB pathway is the receptor activator of nuclear factor-kB ligand (RANKL) and its receptor (RANK), and its soluble (decoy) receptor, osteoprotegerin (OPG), mediate interactions (RANKL-OPG axis) that exert pleiotropic effects on bone metabolism, atherosclerosis and the immune system (outlined in Chapter 1).[336-338] Although reports of associations between circulating levels of OPG or RANKL with

cardiovascular disease have been conflicting, [336,337,339] the most recent studies have shown that increased RANKL concentration and decreased OPG level each are associated with vascular calcification, and increased RANKL/OPG ratio is significantly associated with coronary artery disease.[336,337,339] Moreover, atherosclerosis and osteoporosis share hyperlipidemia as an etiologic factor, [340,341] helper T cells can produce RANKL, [39,216] and oxidized lipids can directly increase the expression of RANKL from T cells through scavenger receptors.[39,216]

Human Immunodeficiency Virus type 1 (HIV-1) infection is characterized by a chronic state of systemic inflammation with increased levels of proinflammatory cytokines, hyperlipidemia, and helper T cell depletion,[155] and should therefore affect the RANKL-OPG axis, but these effects have not been defined. In view of reported positive associations of atherosclerosis, osteoporosis, and HIV-1 infection with pro-inflammatory activation of the RANKL-OPG axis and hyperlipidemia, and the stimulatory effects of oxidized lipids in production of RANKL from T cells, [216] we hypothesized that increased serum RANKL/OPG is independently associated with cholesterol levels and progression of atherosclerosis in HIV-1 infected subjects. Limited data exist regarding the relationship between oxidized HDL which has been shown to activate the NF-kB pathway *in vitro* and the RANKL-OPG axis in HIV infection. Using blood specimens from a previously described matched cohort study, we determined associations of baseline characteristics, HIV-1 infection, and the risk of development of subclinical atherosclerosis and its progression with the RANKL-OPG axis (serum soluble RANKL, OPG, and RANKL/OPG ratio) in HIV-1-infected and -uninfected subjects.[260] Finally, we determined the associations of oxidized (Low density lipoprotein [LDL], non-High density lipoprotein [non-HDL], HRA) and non-oxidized (HDL and total cholesterol) lipids with circulating levels of the RANKL-OPG axis.

6.3 Results

STUDY 1

HIV-1 infected subjects had more metabolic abnormalities than the control subjects. As reported previously the HIV-1 infected subjects in this cohort had significantly ($p<0.05$) higher incidence of metabolic syndrome, higher levels of total cholesterol, triglycerides and non-HDL cholesterol and insulin levels compared to the control subjects.[262] Due to matching the groups were similar with respect to age, sex, race, body mass index (BMI), LDL and HDL levels.[262]

HIV-1 infection was not significantly associated with baseline HDL function, but HDL function changed significantly over time within the HIV-1^+ subjects. In view of our previous findings that HIV-1 infected subjects have dysfunctional HDL, [75,331] we investigated possible associations between treated HIV-1 infection and changes in serum HRA. At baseline and week 96/144, serum HRA were not significantly different between both HIV-1^+ groups (with and without PI treatment) and in the combined HIV-1^+ group compared to the controls ($p>0.2$).[342] Although the HIV/non-PI group had a significant increase in relative median levels of HRA of 0.17 ($p=0.01$) by 96/144 weeks compared to baseline, there was a trend towards significant increases in HRA over time (yearly rate of change in nHRA or ΔnHRA within the combined HIV-1^+ group ($p=0.08$); this was not seen within the HIV/PI and not HIV groups ($p>0.3$).

Lower baseline HDL levels and anthropometric parameters of obesity were independently associated with HDL function. It is unknown which parameters may independently predict changes in HDL function in HIV-1 infected subjects. In univariate analysis of HIV-1 infected subjects, HDL cholesterol <35 mg/dl ($p=0.007$), increased baseline waist-to-hip ratio (WHR; $p=0.02$), and higher baseline BMI ($p=0.086$) were associated with higher baseline nHRA. There was a trend ($p=0.09$) for correlation of HRA with baseline CIMT **(Figure 6.1)**. In the control subjects, HDL cholesterol <35 mg/dl ($p=0.03$), baseline waist circumference ≥90 cm ($p=0.097$), and increased baseline WHR ($p=0.071$) were univariately associated with higher baseline nHRA. These data confirm

previous findings in HIV-1-uninfected subjects that obesity and lower HDL levels are associated with dysfunctional HDL.[72]

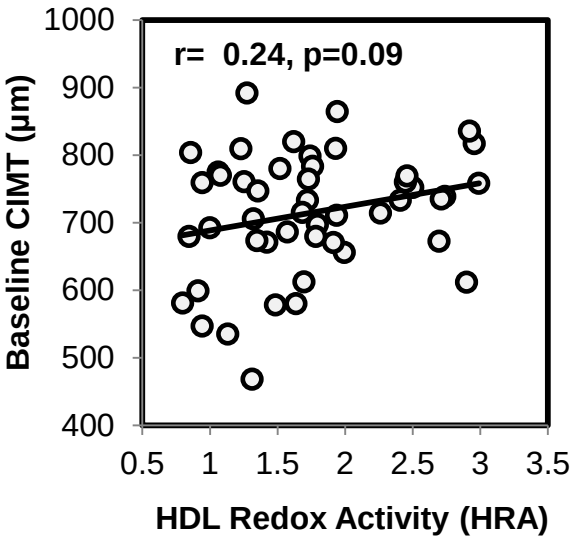

Figure 6.1. Association of HRA with baseline CIMT in HIV-1 infected subjects

Changes in HDL function over time were associated with baseline HDL, race, nadir CD4 count, and CIMT in HIV-1$^+$ subjects. Because there were significant changes in HRA over time within the HIV-1$^+$ group, we sought factors that were associated with yearly *rate* of change in HDL function (ΔnHRA) in HIV-1$^+$ subjects. Similar to the univariate analysis, multivariate analysis in HIV-1$^+$ subjects showed a positive ΔnHRA was associated with lower baseline CIMT (parameter estimate 0.45, 95% CI: 0.23-0.67; p<0.001), white race (estimate 0.12, 95% CI: 0.01-0.23; p=0.03), baseline HDL ≥35 mg/dl (estimate 0.07, 95% CI: 0.02-0.12; p=0.007), and nadir CD4$^+$ >200 cells/mm^3 (estimate 0.08, 95% CI: 0.04-0.12; p<0.001). In the control subjects, lower baseline LPS (p=0.03) and decreased baseline insulin (p=0.002) were associated with a positive ΔnHRA in multivariate analysis. Finally, increased *rate* of change in nHRA in HIV-1$^+$ subjects was associated with favorable metabolic and immunologic parameters with higher baseline HDL levels, in contrast to baseline nHRA, which was associated with lower HDL levels. Further larger prospective studies will be required to explore these findings and determine predictors of both baseline HDL function and changes in HDL function over time.

HRA as measured with the DHR assay was not associated with progression of subclinical atherosclerosis in HIV-1$^+$ subjects. Considering the previously described role of HDL function in progression of atherosclerosis in HIV-1-uninfected subjects, we investigated the possible relationship between HDL function and progression of atherosclerosis in HIV-1$^+$ subjects. In a prior multivariate analysis in the HIV-1$^+$ subjects reported in Chapter 2, baseline HDL <35 mg/dl (p=0.04) and higher levels of baseline sCD14 (p=0.04) and LPS (p=0.003) were associated with a positive ΔCIMT.[262] In univariate analysis in HIV-1$^+$ subjects, baseline nHRA was not associated with baseline CIMT (estimate: 53.2; 95% CI: (-16.6-123; p=0.13), ΔCIMT (estimate: -7.5; 95% CI: (-15.6- 0.7; p=0.07), and CIMT progression (estimate: 0.8; 95% CI: 0.2-3.6; p=0.81). It is possible that different factors influence the presence of plaque and CIMT, and thus the relationship of HDL function with different measures of atherosclerosis[262] requires further examination in larger studies.

The interaction of the HDL redox activity with levels of cholesterol may be important in HIV-1$^+$ subjects. The redox activity of HDL may depend on the interplay between HDL and LDL and the concentrations of both HDL and LDL and their ratio (LDL/HDL).[75] In HIV-1$^+$ subjects, the baseline interaction model of nHRA*(LDL/HDL), including HDL, LDL/HDL, and nHRA *HDL, nHRA*(LDL/HDL) was significantly associated with baseline CIMT (estimate: 120; 95% CI: 15.4-225; p=0.03), but not with CIMT progression (OR: 0.5; 95% CI: 0.06-4.5; p=0.55), and ΔCIMT (estimate: -3.5; 95% CI: -14.2-7.3; p=0.52). Thus, the interaction of the HDL redox activity with levels of cholesterol may be taken into consideration when studying the association of dysfunctional HDL with atherosclerosis in HIV-1$^+$ subjects who are known to have dyslipidemia and dysfunctional HDL. [75]

The HRA as measured with the novel Amplex Red assay can be used as a marker of cardiovascular disease in HIV-1 infected subjects. In view of the limitations of the DHR assay when cryopreserved samples are used as outlined in Chapter 5 and previously published[311] and the relative small size of this cohort, we used the novel Amplex Red assay to measure HRA that is based on immunoaffinity capture of HDL and specific enzymatic measurement of redox activity that may limit the biochemical interactions seen with DHR

(Chapter 5). In multivariate analysis of the HIV-infected subjects, higher baseline HRA was associated with the ΔCIMT increasing by 2.3 mm/yr (95% CI = (0.24, 5.6); p = 0.03) but no association between ΔCIMT and HRA was seen in the controls (not shown) **(Figures 6.2, 6.3)**. Thus dysfunctional HDL was independently associated with progression of subclinical atherosclerosis in HIV-infected subjects but not in uninfected controls.

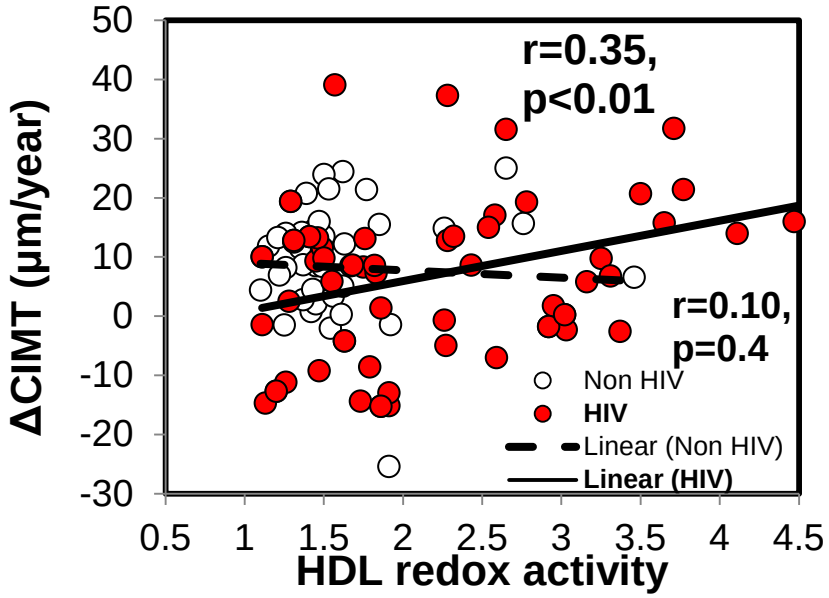

Figure 6.2. Increased HDL redox activity (HRA), as measured by the Amplex Red Method and the immunoaffinity capture, is independently associated with progression of atherosclerosis in HIV-1- infected subjects *in vivo*. Scatter plot of the Rate of Change in Carotid intima–media thickness (CIMT) (ΔCIMT) and HRA for 55 HIV-infected subjects (solid circles) and 36 uninfected controls (white circles). HDL ELISA kit was used to capture HDL in 96-well plates (kit B) as described in Methods. HRA was determined as described in Chapter 5. The values from HRA for each subject are plotted against ΔCIMT.

Higher baseline cholesterol levels were associated with higher RANKL/OPG serum ratio in HIV-infected subjects. To confirm our hypothesis that hyperlipidemia is independently associated with circulating levels of RANKL and/or OPG in HIV-1 infection and that the interplay of lipids with the RANKL-OPG axis may be important for progression of atherosclerosis in HIV-1 infection, we investigated factors significantly associated with baseline serum RANKL, OPG, and RANKL/OPG, as identified by univariate and multivariate models. In the HIV-1^+ subjects only, univariate analysis found that higher baseline total cholesterol was associated with higher baseline serum RANKL and RANKL/OPG and lower baseline serum OPG ($p \leq 0.07$). In multivariate analysis in the HIV-1^+ subjects, lower

baseline serum OPG was associated with higher baseline total cholesterol (parameter estimate -4.69; 95% CI: -8.47, -0.92; p = 0.02), lower baseline insulin (parameter estimate -4.35; 95% CI: -7.35, -1.35); p = 0.008), and lower baseline hs-CRP (parameter estimate -4.22; 95% CI: -7.59, -0.86); p = 0.02). In multivariate analysis in the HIV-1^{+} subjects, only higher baseline HDL (parameter estimate -7.51, 95% CI: -13.76, -1.26; p = 0.02) remained significantly associated with a negative yearly rate of change in RANKL. However, in the control subjects, there were no associations of lipids with serum levels of the RANKL-OPG axis **(Figure 6.4)**.[342]

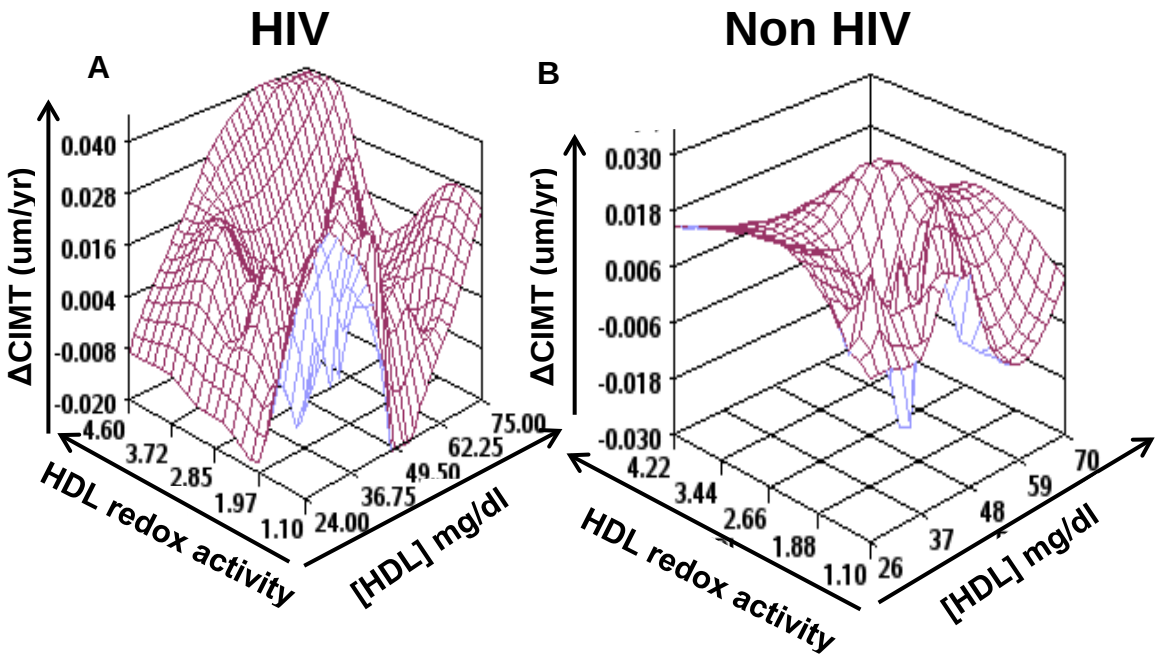

Figure 6.3. 3-D surface plot illustrating the relationship of the yearly rate of change in CIMT (ΔCIMT) (z axis) with baseline levels of HRA (normalized ratio) (x axis) and baseline levels of HDL cholesterol (mg/dl) (y axis) for the 55 HIV-1^{+} subjects (A) and uninfected control subjects in the study.

Higher HDL redox activity was associated with higher RANKL/OPG serum ratio in HIV-infected subjects. In view of previous *in vitro* studies that oxidized HDL may directly upregulate the NF-kB pathway[217] we hypothesized that increased HDL redox activity (HRA) is independently associated with circulating levels of RANKL and/or OPG in HIV-1 infection and that the interplay of oxidized HDL with the RANKL-OPG axis may be important for progression of atherosclerosis in HIV-1 infection. Thus, we investigated whether baseline HRA was significantly associated with baseline serum RANKL, OPG, and RANKL/OPG in

HIV-1 infection, as identified by univariate and multivariate models. In the HIV-1^{+} subjects only, univariate analysis found that higher baseline HRA was associated with higher baseline serum RANKL and RANKL/OPG ($p < 0.05$). In multivariate analysis in the HIV-1^{+} subjects,[343] higher baseline serum HRA was associated with higher baseline RANKL/OPG ($p=0.02$). However, in the control subjects, there were no associations of HRA with serum levels of the RANKL-OPG axis **(Table 6.1., Figure 6.5).**

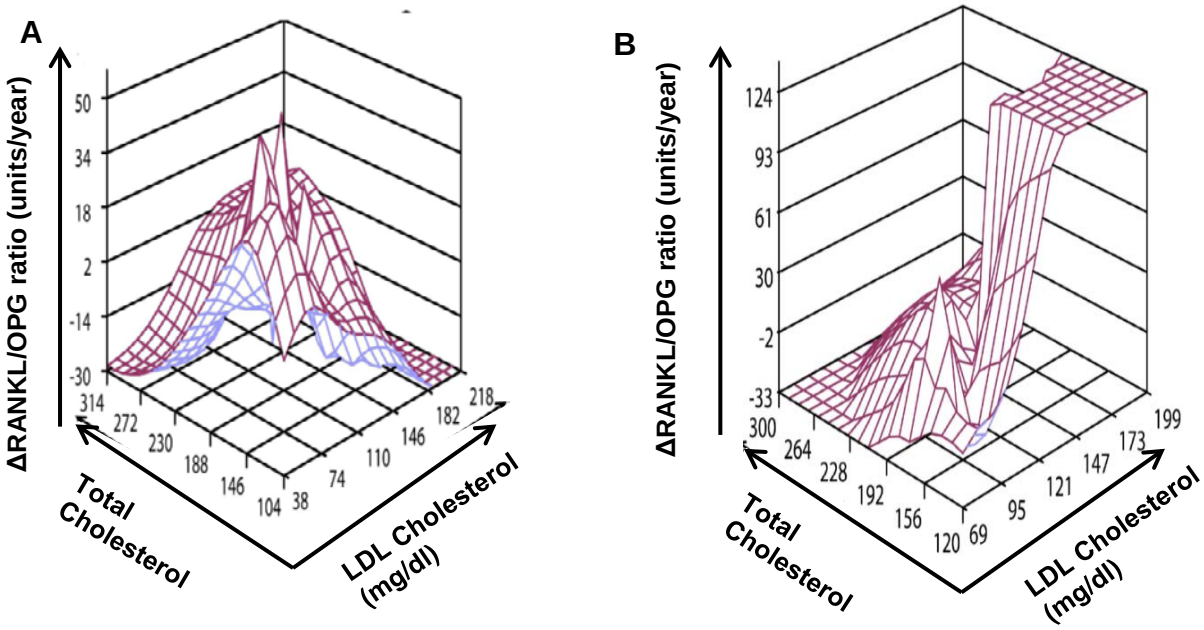

Figure 6.4. 3-D surface plot illustrating the relationship of the yearly rate of change in RANKL/OPG ratio (z axis) with baseline levels of total cholesterol (mg/dl) (x axis) and baseline levels of LDL cholesterol (pg/ml) (y axis) for the 55 HIV-1^{+} subjects **(A)** and the 36 controls **(B)** in the study.

	Univariate models		**Multivariate models**	
Covariate	Parameter Estimate (95% CI)	P value	Parameter Estimate (95% CI)	P value
Baseline DOR (per 10,000 FU/min)				
Waist-to-hip ratio (per 0.1 units)	-3.97(-6.94,-0.99)	0.01	-3.75(-6.57,-0.92)	0.01
Baseline RANKL (per 1000 pg/ml)	0.07 (0.01, 0.13)	0.02	(-)	(-)
Baseline OPG (per 100 pg/ml)	-0.06 (-0.33, 0.21)	0.64	(-)	(-)
Baseline RANKL/OPG (per 10 units)	0.87 (0.22, 1.53)	0.01	0.81 (0.16, 1.47)	0.02

Table 6.1: Baseline serum levels of RANKL and RANKL/OPG predict oxidative (functional) properties of HDL (reflected by the rate of oxidation of the fluorogenic probe dihydrorhodamine-DOR) in HIV-infected subjects

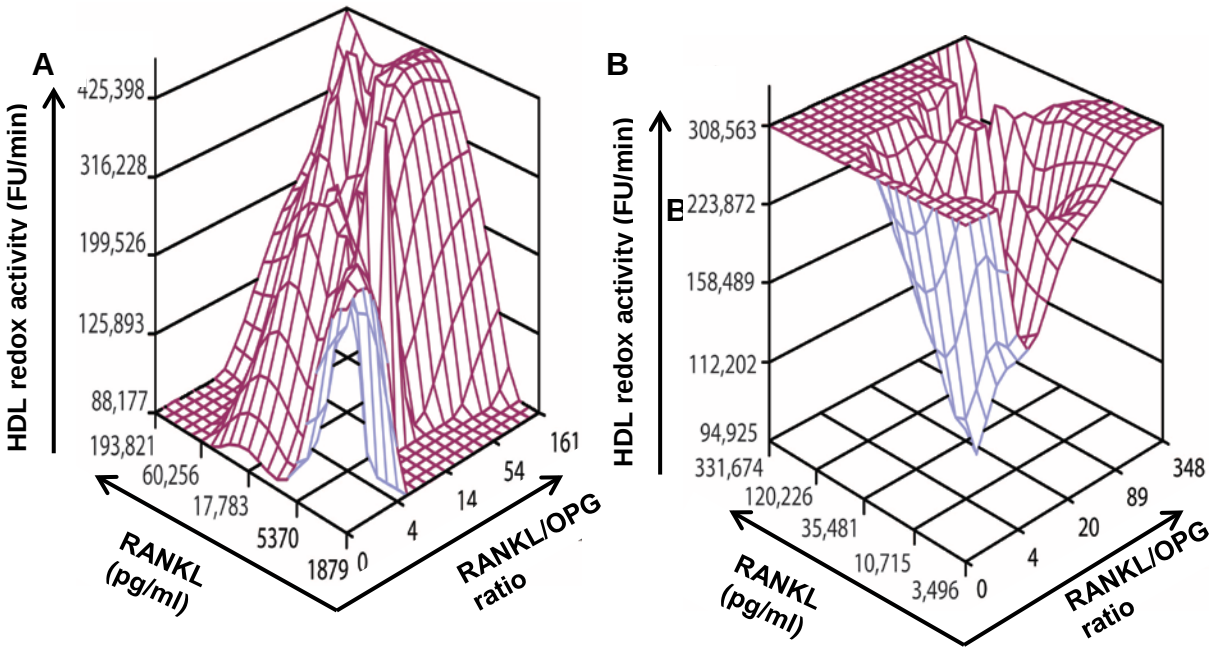

Figure 6.5. 3-D surface plot illustrating the relationship of the HRA (z axis) with baseline levels of serum RANKL (pg/ml) (x axis) and baseline levels of serum RANKL/OPG (pg/ml) (y axis) for the 55 HIV-1^{+} subjects **(A)** and the 36 controls **(B)** in the study.

STUDY 2

Characteristics of study participants Study participants included 102 HIV-infected men and 41 non-HIV infected men prospectively recruited to match HIV-subjects on traditional cardiovascular risk factors. HIV-infected subjects had been diagnosed, on average, 13.8 years prior. 95% of HIV-infected subjects were receiving stable ART, and the median duration of therapy was 7.9 years. 81% of HIV-infected subjects had achieved an undetectable viral load. The median current CD4 count in the HIV-infected group was 473 cells/mm^3, whereas the median historical nadir CD4 count was 175 cells/mm^3. Comparing the HIV-infected group and the non-HIV control group, there was no significant difference in age, family history of premature heart disease, smoking, diabetes prevalence, anthropometric parameters, or blood pressure. Levels of HDL cholesterol were not significantly different between groups (46 mg/dl (41, 52) versus 45 mg/dl (38, 55), non-HIV-infected versus HIV-infected, p=0.75.) Levels of total and LDL cholesterol were also comparable between groups, whereas triglyceride levels were higher in the HIV-infected group (109 mg/dl (81,182) versus 80 mg/dl (64, 123), p=0.001)**.** The differences in the

inflammatory and immune activation markers between HIV-1 infected and uninfected subjects are shown in **Table 6.2**.

Characteristic	Non-HIV-infected controls (n=41)	HIV-infected subjects (n=102)	p-value
IL-6, pg/mL	0.6 (0.5, 1.0)	0.9 (0.7, 1.5)	0.01
D-dimer, ng/mL	<220 (<220, 333)	<220 (<220, 322)	0.93
LPS, ng/mL	0.07 (0.06, 0.1)	0.1 (0.07, 0.1)	0.0004
MCP-1, pg/mL	235 (190, 299)	275 (179, 363)	0.13
sCD14, ng/mL	211 (121,374)	305 (157, 440)	0.08
sCD163, ng/mL	765 (572, 1054)	1063 (695, 1577)	0.0007

Table 6.2: Inflammatory and Immune Activation Markers. Abbreviations: IL-6: Interleukin-6; LPS: Lipopolysacharide; MCP-1: Monocyte chemoattractant protein-1; sCD14: Soluble CD14; sCD163: Soluble CD163

Comparison of HRA in HIV-infected group versus non-HIV group HRA was significantly higher in the HIV-infected group versus the non-HIV group (1.4 +/- 0.01 versus 1.3 +/- 0.01, p=0.03) **(Figure 6.6).** HRA was not significantly different between HIV-infected subjects with detectable viral load and HIV-infected subjects with undetectable viral load.

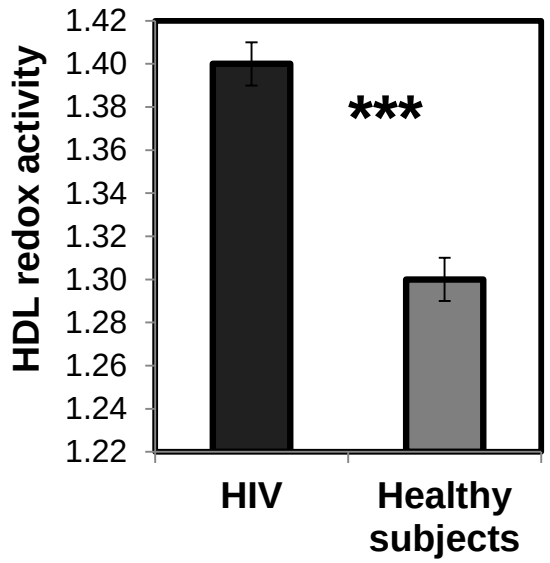

Figure 6.6: HDL Redox Activity (HRA) in HIV-infected Subjects versus matched non-HIV Subjects without clinically evident cardiovascular disease (HRA in HIV-infected group 1.4 +/- 0.01 versus HRA in non-HIV group 1.3 +/- 0.01, p=0.03).

Relationship between HRA and demographic parameters in the non-HIV group and HIV-infected group. Among non-HIV controls, there was a statistically significant relationship

between HRA and age (rho=0.31, p=0.05) **(Table 6.3).** There was no statistically significant relationship in non-HIV-infected subjects between HRA and family history of premature heart disease, history of diabetes, or history of hepatitis C (data not shown). Among HIV-infected subjects, there was no statistically significant relationship between HRA and any of the aforementioned demographic parameters, including age **(Table 6.3).**

	Non-HIV-infected controls (n = 41)		**HIV-infected subjects (n = 102)**	
	Correlation	**P-Value**	**Correlation**	**P-Value**
Demographic Parameters				
Age	rho=0.31	0.05*	rho=0.02	0.88
Pack years	rho=-0.29	0.08	rho=-0.08	0.48
Immunologic Parameters				
Log C-reactive protein (CRP) (mg/L)	r=-0.21	0.19	r=-0.07	0.50
Log IL-6 (pg/mL)	r=-0.24	0.20	r=-0.03	0.80
Log D-dimer (ng/mL)	r=-0.02	0.93	r=-0.004	0.97
Log lipopolysacharide (LPS) (ng/mL)	r=-0.03	0.85	r=-0.04	0.72
Log MCP-1 (pg/mL)	r=0.15	0.36	r=0.10	0.33
Log soluble CD14 (sCD14) (ng/mL)	r=0.17	0.30	r=0.04	0.70
Log soluble CD163 (sCD163) (ng/mL)	r=0.03	0.84	r=0.24	0.02*
Duration of HIV infection (years)	N/A	N/A	r=0.10	0.36
Duration of ART (years)	N/A	N/A	rho=0.19	0.14
Current CD4 count (cells/mm3)	N/A	N/A	rho=-0.006	0.96
Nadir CD4 count, (cells/mm3)	N/A	N/A	rho=-0.009	0.94
Log HIV RNA-level (viral load)	N/A	N/A	r=0.18	0.11
Cardiometabolic Parameters				
Body mass index (kg/m2)	rho=0.30	0.06	rho=0.11	0.30
Waist circumference (cm)	rho=0.28	0.11	rho=0.02	0.84
Visceral adipose tissue (cm2)	rho=0.28	0.08	rho=0.10	0.35
Systolic blood pressure (mm Hg)	rho=0.15	0.35	rho=-0.04	0.67
Diastolic blood pressure (mm Hg)	r=0.22	0.18	r=-0.10	0.34
Total cholesterol level (mg/dL)	rho=0.17	0.31	rho=-0.10	0.35
HDL cholesterol level (mg/dL)	rho=-0.17	0.29	rho=-0.32	0.002*
LDL cholesterol level (mg/dL)	r=0.27	0.09	r=0.03	0.74
Triglyceride level (mg/dL)	rho=0.20	0.23	rho=0.11	0.29
Log adiponectin (ng/mL)	r=-0.24	0.14	r=-0.28	0.006*

Percentage non-calcified coronary atherosclerotic plaque (%)	r=-0.43	0.11	r=0.29	0.03

Table 6.3: Relationship between HDL Redox Activity (HRA) and Demographic, Immunologic/Inflammatory, and Cardiometabolic Parameters in HIV-infected Subjects and Non-HIV Controls. Abbreviations: ART: antiretroviral therapy; IL-6: high sensitivity interleukin 6; LPS: lipopolysacharide; MCP-1: Monocyte chemoattractant protein-1

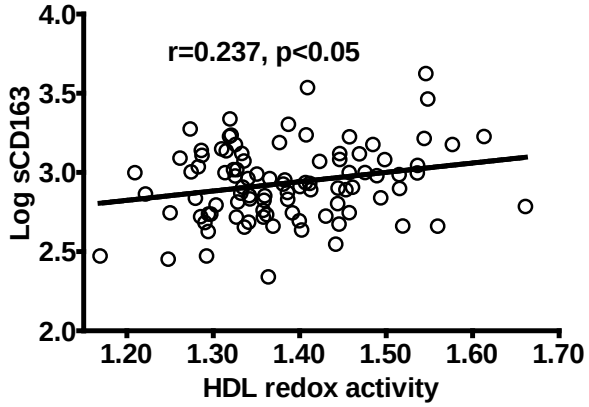

Figure 6.7: Relationship between HDL Redox Activity (HRA) and log levels of the monocyte/macrophage activation marker soluble CD163 among HIV-infected subjects (r=0.24, p=0.02).

Relationship between HRA and immunologic/inflammatory parameters in the non-HIV group and HIV-infected group. Among non-HIV controls, there was no statistically significant relationship between HRA and log levels of circulating markers of inflammation and immune activation including C-reactive protein (CRP), high-sensitivity interleukin-6 (IL-6), D-dimer, lipopolysaccharide (LPS), monocyte chemoattractant protein-1 (MCP-1), soluble CD14 (sCD14), and soluble CD163 (sCD163). In contrast, among HIV-infected subjects, there was a statistically significant relationship between HRA and log levels of the circulating levels of the monocyte/macrophage activation marker, sCD163 (r=0.24, p=0.02), but no significant relationship between other circulating markers of inflammation and immune activation **(Table 6.3,).** Among the HIV-infected group, there was no statistically significant relationship between HRA and duration of HIV, duration of ART, current CD4 count, nadir CD4 count, and log viral load **(Table 6.3)**. There was also no significant relationship between HRA and ART class used among the HIV-infected group.

Relationship between HRA and cardiometabolic parameters in the non-HIV group and HIV-infected group. Among non-HIV controls, there was no statistically significant

relationship between HRA and body mass index, waist circumference, visceral adipose tissue, systolic and diastolic blood pressure, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, log adiponectin, and the % of non-calcified coronary atherosclerotic plaque on coronary CT angiography. In contrast, among HIV-infected subjects, there was a significant inverse relationship between HRA and HDL levels (rho=-0.32, p=0.002) and between HRA and log adiponectin levels (r=-0.28, p=0.006). Moreover, there was a statistically significant positive relationship between HRA and the % of non-calcified coronary atherosclerotic plaque (r=0.29, p=0.03) **(Table 6.3).** HRA did not relate to other coronary CTA parameters including Agatson calcium score among the HIV-infected group.

Secondary analysis of relationship between HRA and HOMA-IR among non-diabetic subjects Among non-diabetic HIV-infected subjects, there was a statistically significant relationship between HRA and log HOMA-IR, a measure of insulin resistance (r=0.34, p=0.005). In this subgroup, the inverse relationship between HRA and log adiponectin remained statistically significant (r=-0.31, p=0.004). In multivariate modeling for HOMA-IR among non-diabetic HIV-infected subjects, HRA remained significantly associated with HOMA-IR, even after controlling for known diabetes risk factors including age, family history of diabetes, and visceral adipose tissue (R^2 for model 0.29, p=0.0005, ß-estimate for HRA 6.3 +/- 2.8, p=0.03) **(Table 6.4).**

	ß-estimate	**standard error**	**p-value**
Age	-0.03	0.04	0.53
Family history diabetes	-0.05	0.26	0.86
HDL Redox activity (HRA)	6.3	2.8	0.03*
Visceral adipose tissue	0.008	0.002	0.001*

Table 6.4: Multivariate Model for HOMA-IR among Non-Diabetic HIV-infected Subjects
R^2 for model=0.29, p = 0.0005*

STUDY 3

Limited data suggest that residual immune activation in HIV infection has an effect on HDL function. We investigated whether HDL function is associated with T cell activation in HIV-infected subjects.

HIV-1 infected subjects with suppressed viremia and low cardiovascular risk have dysfunctional HDL compared to uninfected controls. Using the DHR assay we determined redox properties of HDL isolated from fresh plasma from 150 HIV-1 infected patients with HIV-1 RNA <500 copies/mL on antiretroviral therapy (ART) and from 50 healthy volunteers. The characteristics of the HIV-1 infected subjects are shown in **Table 6.5**. The mean nHRA value for the HIV-1-infected group was 1.33 ±0.21 versus 0.87 ±0.15% for 50 healthy subjects ($p<0.0001$). Thus, we confirmed our previous findings shown in study 2 of this Chapter and also in Chapters 3-5 that HIV-1 infected subjects with suppressed viremia have dysfunctional HDL.

Higher HRA is associated with biomarkers of increased T cell activation. Higher nHRA was associated with increased immune activation markers such as higher frequency of activated T $CD8^+$ cells ($r=0.40$; $p<0.0001$) and CD38 expression on $CD8^+$ T cells ($r=0.36$; $p<0.0001$) **(Figure 6.8)**. Higher HRA was significantly associated with age, BMI, duration of HIV infection and ART, nadir CD4, baseline CD4, and lipids **(Table 6.5)**. After adjustment for significant covariates in multiple regression analysis, HRA remained significantly associated with markers of immune activation ($p=0.002$ for CD38 mol in $CD8^+$; $p<0.001$ for % activated $CD8^+$ T Cells.

Higher HRA is associated with a lower nadir $CD4^+$ T-cell count. Higher nHRA was associated with lower nadir $CD4^+$ T cell count ($r=-0.52$; $p<0.0001$) **(Figure 6.9)** which is a predictor of disease progression, immune activation and increased CVD among HIV-1 infected subjects.[344,345]

Characteristic	N(%)/Median (IQR)	r	P value
Male	138 (93.2%)	(-)	0.603
Age (years)	47 (21-79)	-0.170	0.039
White Non-Hispanic	95 (64.2%)	(-)	0.001
Hispanic	27(18.2%)	(-)	(-)
Body mass index (BMI) (kg/m^2)	25.3 (23.6, 27.4)	0.153	0.063
Duration of HIV infection (years)	12 (9, 17)	0.395	< 0.001
Duration of ART (years)	9 (5, 14)	0.322	< 0.001
Nadir $CD4^+$ T cells (cells/mm3)	250 (189, 350)	-0.439	< 0.001

Baseline $CD4^+$ T cells (cells/mm3)	528 (408, 704)	-0.146	0.080
Fasting glucose	91 (84, 104)	0.014	0.873
Total Cholesterol (mg/dl)	179 (153, 206)	0.162	0.058
LDL (mg/dl)	99 (81, 129)	0.210	0.013
HDL (mg/dl)	40 (33,48)	-0.174	0.041
Triglycerides (mg/dl)	134 (93, 92)	-0.229	0.007
Non HDL Cholesterol (mg/dl)	138 (112, 163)	0.182	0.032
Usage of lipid-lowering drugs	43 (29 %)	(-)	0.226
Metabolic syndrome	25 (17 %)	(-)	0.096

Table 6.5. Baseline characteristics of HIV-1 infected subjects and correlations (Spearman correlation r coefficient) with HRA.

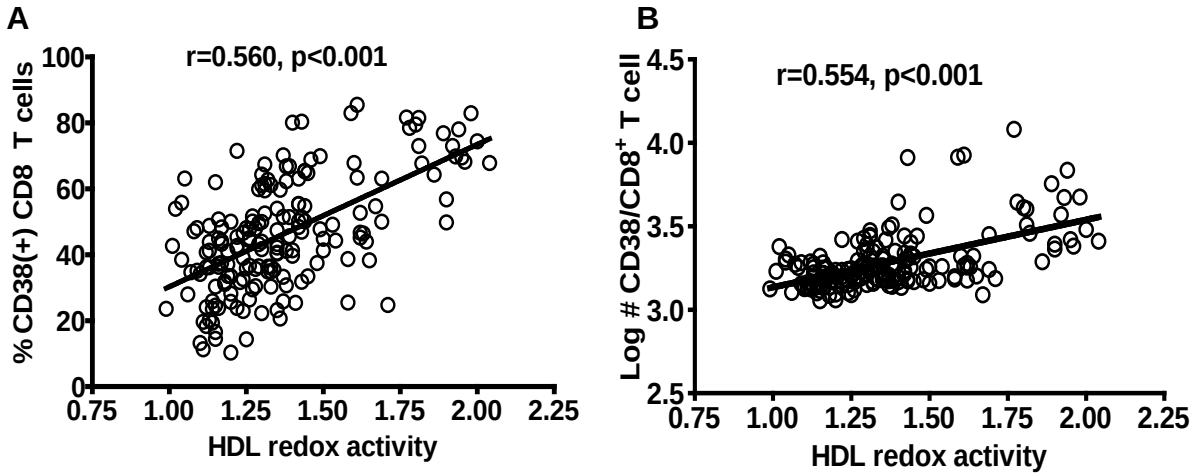

Figure 6.8: Relationship between HDL Redox Activity (HRA) and T cell activation markers among HIV-infected subjects. The number of CD38 molecules per $CD8^+$ T cell and the % $CD38^+DR^+CD8^+$ T cells were determined using flow cytometry as described in methods and the HRA was determined in HDL isolated from fresh plasma using the DHR assay.

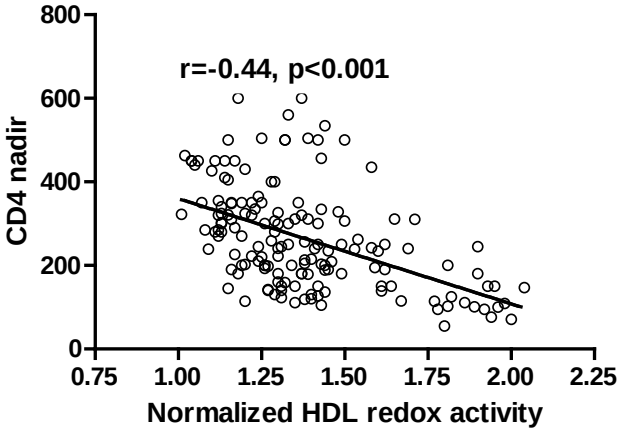

Figure 6.9: Relationship between HDL Redox Activity (HRA) and nadir $CD4^+$ T cell among HIV-infected subjects.

6.4 Discussion

Considering the previously described role of HDL function and immunity in progression of atherosclerosis in HIV-1-uninfected subjects, we investigated the possible relationship between HDL function, immune activation and atherosclerosis in HIV-1 infected subjects. Our data are among the first to show increased HDL redox activity (HRA) among chronically infected, ART-treated HIV-patients versus matched non-HIV subjects with comparable HDL levels. This observation suggests that HDL functional properties - specifically HRA - are not fully captured by simple measurement of HDL levels among HIV-infected patients. Although the biological significance of high HRA has yet to be fully determined, high HRA is felt to represent a measure of HDL dysfunction.[75] Using an *in vitro* monocyte chemotaxis assay, we have previously shown that oxidized HDL (which has higher HRA) directly increased monocyte chemotaxis, a key event in atherosclerosis.[75] In addition, higher HRA, as measured by the biochemical assay, correlated significantly with pro-inflammatory HDL, as measured by a cell based assay.[75] Indeed, dysfunctional HDL may have a reduced capacity to prevent oxidation of LDL particles, [50] which, when taken up by subendothelial macrophages, give rise to the core of an incipient coronary atheroma.[13,158] By this mechanism, HRA could have potential relevance as a biomarker of atherosclerotic disease risk in HIV. As discrepant lipid levels do not appear to account for the heightened CVD risk in HIV-infected patients versus non-HIV controls, [153] it is reasonable to further investigate the degree to which lipoprotein functional properties, such as HRA, contribute.

An important finding from studies 1 and 2 is the positive association between HRA and CIMT, one of the best measures of cardiovascular disease, [261] and percent non-calcified plaque among HIV-infected patients. Previous work in non-HIV subjects with known heart disease has shown that the amount of non-calcified plaque in nonobstructive coronary lesions prospectively predicts risk of clinical cardiac events, whereas the amount of calcified plaque does not.[346] It is possible that different factors influence the presence of plaque and CIMT, and thus the relationship of HDL function with different measures of atherosclerosis[262] requires further examination in larger studies. The above observation from our study

reinforces that HDL levels and HDL functional assessments may provide complementary information, and it suggests the need to further explore HRA as a biomarker of atherosclerotic disease risk in HIV. Supporting this line of investigation are data from non-HIV cohorts with autoimmune diseases demonstrating that acute-phase HDL is indeed a marker of atherosclerosis in these inflammatory disease states.[63,66,347] We did not observe an association between HRA and percent non-calcified coronary plaque in the non-HIV-infected group, and this may have been due to the smaller sample size of this group and the lower frequency of plaque or to differences in mechanisms of atherogenesis between HIV-infected and non-HIV infected patients.

In study 2, among HIV-infected subjects, HRA was significantly associated with circulating levels of the monocyte/macrophage activation marker soluble CD163 (sCD163). One possible explanation for this relationship is that systemic inflammation and immune activation in HIV alters HDL structural, and, in turn, functional properties. Previous studies have demonstrated structural/functional[50] changes to HDL circulating in experimental or naturally occurring infectious/inflammatory conditions (i.e. "acute-phase HDL"). The biology of CD163/sCD163 further reinforces the notion that systemic inflammation and immune activation in HIV may alter HDL functionality: CD163, found on the cell-surface of monocytes and macrophages, serves as a scavenger receptor for hemoglobin-haptoglobin complexes.[348-350] In inflammatory states, this receptor is cleaved from the cell surface, releasing soluble CD163 (sCD163).[351-354] There appears to be an inverse relationship between availability of cell-surface CD163 and circulating levels of sCD163.[355] In our study, high sCD163 levels among the HIV-infected subjects may not only reflect activation of circulating monocytes, but also imply a relative dearth of cell-surface CD163 receptors available to scavenge hemoglobin-haptoglobin molecules. Consequently, free hemoglobin/haptoglobin may be available in excess to complex HDL particles, altering their functionality, as has been described in inflammatory disease states such as rheumatoid arthritis.[64] The interplay of CD163 and hemoglobin-haptoglobin and the importance of the latter for HDL function[64] may explain the association of HRA only with sCD163 and not with

sCD14, another marker of macrophage activation. An alternate explanation for the relationship between HRA and sCD163 in our study could be that oxidative stress, immune activation, and inflammation in HIV-infected patients simultaneously and independently promotes increase in HRA and release of sCD163.[356]

Of note, consistent with linkage of oxidized HDL to immune activation in HIV-1 infection, we showed that increased HRA correlates to markers of T cell activation such as HLA-DR$^+$, CD38$^+$ that have been independently associated with a fall in CD4$^+$ T-cell counts and the development of AIDS.[357] HRA was significantly associated with CD38 level on CD8$^+$ T cells, a key indicator of immune activation that drives disease progression, [358] in HIV-1 infected subjects with suppressed viremia. In view of the immunomodulatory effects of oxidized lipoproteins on both adaptive and innate immunity as outlined in Chapter 1, and the data presented herein, we hypothesize that there is a vicious cycle of HIV-1-induced immune activation, inflammation, production of oxidized HDL, and further immune activation. Further studies are needed to elucidate the cross-talk between dysfunctional HDL and HIV-associated immune activation/inflammation.

One mechanism that may mediate the interplay of oxidized HDL with atherogenesis is activation of the NF-kB pathway. The soluble product of activation of the NF-kB pathway, RANKL and the RANKL-OPG axis have an important role in both regulation of immunity and atherogenesis as outlined in Chapter 1. In this analysis of stored serum samples from a prospective three-year study (study 1), we found that HIV-1-infected subjects on ART had significantly lower levels of RANKL and RANKL/OPG ratio, but similar OPG levels, compared to control subjects. In HIV-1-infected subjects, cholesterol and HRA was significantly associated with baseline serum levels and/or changes over time in the RANKL-OPG axis. These findings underscore the complicated interactions between HIV-1 infection, HDL and the RANKL-OPG axis.

Although the RANKL-OPG axis has not been studied extensively in HIV-1 infection, *in vitro*[225,241] and *in vivo* studies[359] suggest that HIV-1 infection has numerous effects on the RANKL-OPG axis. *In vitro* studies suggest that HIV-1 infection *per se,*[225] immune

dysregulation,[224] and changes in blood levels of TNF-related apoptosis-inducing ligand (TRAIL), a basic member of the TNF superfamily,[236] during HIV-1 infection as well as ART[360] can affect the RANKL-OPG axis. Limited data from *in vivo* studies on levels of circulating RANKL[361-363] and OPG [362,363] in untreated and treated HIV-1 infection are conflicting. A recent study found that serum RANKL was lower in HIV-infected individuals than controls and was negatively associated with the number of coronary segments with plaque and Agatston coronary artery calcium score in HIV-1-infected individuals even after adjusting for traditional cardiovascular risk factors.[364] Similar to these findings and contrary to the increased RANKL/OPG ratio found in other systemic inflammatory conditions,[336] we found that HIV-1-infected subjects on either NNRTI- or PI-based ART had significantly lower levels of total serum RANKL and RANKL/OPG ratio compared to control subjects at both baseline and after 2-3 years of follow-up. However, few studies have previously determined the RANKL/OPG ratio in HIV-1 infection and suggested that HIV-1 infection[365] and ART[361] increased[362] or did not affect the RANKL/OPG ratio. Without longitudinal data on patients initiating different ART regimens, it is not possible to define the role of ART on RANKL levels. Differences in the assays used,[224] the potency of OPG to neutralize RANKL,[366] the metabolic activity of different tissues that express the majority of RANKL and OPG,[366] the state of immune activation,[362] and the use of different antiretrovirals[367] between different groups of HIV-1-infected subjects could all explain discrepancies between different studies.

The interplay between T lymphocytes, lipids and bone may also explain the lower circulating levels of RANKL in HIV-1 infected compared to uninfected subjects. Increased RANKL expression in T cells and reduced OPG expression in T cells have previously been shown to be associated with reduced bone density and hyperlipidemia.[216] HIV-1 infection is associated with T cell lymphopenia and reduced bone density.[368] Thus, it is possible that reduced systemic levels of RANKL in HIV-1-infected subjects may reflect reduced T cell-derived RANKL. Moreover, the nadir $CD4^+$ T-cell count, a marker of HIV-1 disease severity, was associated with the yearly rate of change in RANKL and RANKL/OPG, and this is consistent with previous *in vitro* studies that have shown that HIV-1 can induce changes in

production of RANKL and RANKL/OPG in $CD4^+$ T-cells[224] and recent *in vivo* studies that serum RANKL was positively associated with $CD4^+$ counts in HIV-1-infection and that patients with lower $CD4^+$ T lymphocyte counts had lower serum RANKL levels.[364] Further studies are needed to investigate the contribution of T-cell dysfunction to RANKL production in HIV-1-infected patients.

Another explanation for our unexpected findings that HIV-1-infected patients had lower serum levels compared to the controls may be that increased RANKL tissue expression in HIV-1 infection may lead to a negative feedback loop and lower circulating levels of RANKL. Indeed, serum levels of RANKL may be very different from local tissue expression and activity.[364] RANKL mRNA levels have been found to be higher in human atherosclerotic plaques than in normal vessels, [224] and an inverse relationship between serum RANKL levels and measures of coronary artery disease has been reported.[369,370] It may be possible that with increased local RANKL activity there is less release of cell surface RANKL to soluble RANKL.[364] Consistent with this hypothesis, circulating RANKL levels were inversely associated with local tissue RANKL mRNA levels.[371] In HIV-1 infection, *in vitro* data suggests that RANKL activity is increased at the tissue level whether via HIV-1 infection itself [224] or secondary to medication effects.[224] This raises an intriguing possibility that these factors increase local RANKL activity in HIV-1-infected patients that may be associated with lower circulating RANKL levels. Our findings highlight the need for further studies to evaluate local RANKL activity within the tissues of HIV-1-infected individuals.

We also found that higher serum RANKL/OPG was significantly associated with higher baseline total cholesterol and HRA. Our findings are consistent with previous studies that indicate that the molecular mechanisms of osteoclastogenesis and RANKL signaling are highly dependent on cholesterol[372,373] and that cholesterol levels can be a significant predictor for serum RANKL/OPG level.[374] Moreover, we found that lower HDL cholesterol was independently associated with positive changes over time in serum levels of RANKL in HIV-1-infected subjects. Lower HDL levels are inversely associated with higher levels of oxidized LDL and increased HDL redox activity in HIV-1-uninfected subjects.[375] HIV-1

infected patients have lower HDL levels[155] and HDL with higher redox activity[75] compared to -uninfected patients. Previous *in vitro* studies have shown that oxidized lipoproteins can directly upregulate production of RANKL from T cells through scavenger receptors[39,216] and that oxidized HDL can also upregulate the NF-kB pathway. [217] Consistent with these findings increased HDL redox activity was independently associated with higher serum levels of RANKL/OPG in HIV-1-infected subjects (but not in -uninfected subjects). Higher levels of oxidized LDL may increase serum levels of RANKL.[216] However, in our study we did not find significant associations of the circulating levels of oxidized fractions of cholesterol (LDL and non-HDL) with serum levels of RANKL and OPG. The RANKL-OPG-LDL cholesterol interplay may be more evident in those patients with a higher underlying cardiovascular risk [336,337] and our small study may be underpowered to demonstrate these associations in subjects who were otherwise at low risk for atherosclerosis. Thus, both total levels of cholesterol as well as oxidized lipoproteins/cholesterol may be associated with regulation of the RANKL-OPG pathway in HIV-1-infected patients and further mechanistic studies need to elucidate the interplay between lipids and the RANKL-OPG pathway in HIV-1 infection.

Finally, HRA was significantly associated with parameters of cardiovascular disease, metabolic parameters and anthropometric parameters of obesity (all 3 studies). Notably, among non-diabetic HIV-infected patients in our study, HRA was inversely associated with adiponectin (an anti-inflammatory, anti-oxidant adipokine which protects against insulin resistance), [376,377] and positively associated with HOMA-IR (a measure of insulin resistance). The latter relationship held even when controlling for known diabetes risk factors such as age, family history of diabetes, and visceral adiposity. These observations are especially notable in light of the fact that "healthy" HDL is thought to promote adiponectin production and insulin sensitivity.[378] The relationships between HIV-specific immune activation, HDL dysfunction, adiponectin production, and insulin resistance remain to be determined. Further exploration of HRA as a biomarker of metabolic risk in a larger cohort of HIV-infected subjects with a wider range of HOMA-IR is needed.

Limitations of this study include the cross-sectional design, which precludes conclusions about causality. In addition, the limitations of the biochemical assays of HDL function have previously been described in detail.[379] [311] Moreover, despite preliminary data from *in vitro* studies that support a role of oxidized HDL in atherosclerosis,[75] the significance of the fluorescent readout as a measure of HRA, which is a biochemical and not a physiological parameter, to study atherosclerosis *in vivo,* needs to be validated in large-scale human studies.

Despite the above limitations, our study provides some of the first information on the interplay between lipids, oxidized HDL, immune activation, RANKL-OPG axis and atherogenesis in HIV-1-infected subjects versus matched non-HIV subjects with comparable HDL levels. Moreover, the data shows that HRA among HIV-infected subjects is significantly associated with measures of macrophage activation and cardiometabolic risk, including levels of sCD163, insulin resistance, CIMT and percent non-calcified coronary plaque. The strong association of HRA to relevant cardiometabolic and immunological indices among HIV-infected subjects lends credence to the potential clinical relevance of the difference in HRA observed between HIV and non-HIV patients. In light of the reproducibility of our cell-free assay to assess HRA, this study paves the way for future studies exploring both the biologic significance of HRA and the utility of HRA as a biomarker for cardiometabolic risk among HIV-infected patients. Thus, this study may set the basis for further studies on the role of HDL function in HIV-1 infection given that standard clinical lipid profile testing may not be an adequate measurement of the risk for atherosclerosis.

6.5 Methods

Study 1

The current study is a subset analysis of samples obtained from a prospective, matched cohort study[262] in which subjects were enrolled as risk factor-matched triads of HIV-1^+ individuals with viremia < 500 RNA copies/ml with (n=29) or without (n=26) use of PI therapy, and HIV-1-uninfected (control) individuals (n=36) from 41 triads.

Presence of metabolic syndrome, CIMT and baseline variables (fasting glucose, lipids, insulin, cardiovascular disease-related measurements, $CD4^+$ T cell counts and HIV RNA levels, high-sensitivity C-reactive protein, serum sCD14, serum LPS) have previously been determined.[262] Stored samples from baseline and week 144 (or 96 if week 144 was missing) were assayed. HDL was isolated using ultracentrifugation and the DOR of each sample was determined as previously[75] and was normalized (nDOR) as ratio to the DOR of a control HDL isolated from pooled serum from healthy subjects.

Matched analyses comparing the combined HIV-1^+ group to the controls (N=32) assessed the effect of HIV-1 infection on nDOR. Progression of atherosclerosis was evaluated as yearly rate of change in CIMT (ΔCIMT). [262] Mixed models regression with triad as a random effect and repeated measures regression evaluated associations of baseline measurements of nDOR and yearly rates of change in the outcome, respectively, with baseline covariates. SAS 9.2 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Study 2

Study participants

102 HIV-Infected men and 41 non-HIV-infected men (ages 18-55) without known or clinically evident cardiovascular disease were recruited from the Boston area to participate in the original study. The majority of HIV-infected subjects were on ART, but no subject had newly initiated ART within 3 months. To achieve between-group matching on traditional

cardiovascular risk factors, non-HIV subjects were prospectively recruited with consideration of age, blood pressure, lipids, and cigarette smoking. The clinical study was approved by Institutional Review Boards from the Massachusetts General Hospital (Partners Healthcare) and Massachusetts Institute of Technology, and all subjects gave informed consent. Data on the relationship between coronary atherosclerosis and circulating inflammatory biomarkers were previously published, [205,380] but data on HRA in this cohort have not been reported.

Assessment of demographic, immunologic, and cardiometabolic parameters

History was used to ascertain demographic data on all subjects including age, race, family history of premature heart disease, smoking, diabetes, use of anti-hypertensive medications, use of lipid-lowering medications, and hepatitis C. For HIV-infected subjects, history was also used to determine date of diagnosis, current and past antiretroviral therapy, and nadir $CD4^+$ count. Blood was drawn and tested for immunologic and inflammatory parameters: $CD4^+$ T cell counts were assessed by flow cytometry, and HIV viral load was determined by ultrasensitive reverse-transcription polymerase chain reaction (PCR) (Roche Amplicor Monitor; lower limit of detection - 50 copies/ml). High sensitivity CRP was measured by the Cobas Integra C-Reactive Protein (Latex) Test. The Enzyme-linked immunosorbent assay (ELISA) was used to test levels of interleukin-6 (IL-6) (R&D), monocyte chemoattractant protein 1 (MCP-1) (R&D), soluble CD14 (sCD14) (R&D) and soluble CD163 (sCD163) (Trillium Diagnostics). Levels of lipopolysaccharide (LPS) were tested via the endpoint LAL assay (Associates of Cape Cod). D-dimer levels were measured quantitatively using an immuno-turbidometric method. With respect to cardiometabolic parameters, standard techniques were used for weight and body measurements, as well as calculation of body mass index (BMI). Ileac crest was the site for waist circumference measurement. Visceral adipose tissue area was calculated using images from single-slice abdominal CT scans at the level of L4. Fasting lipid, glucose, and insulin levels were determined using standard techniques. Adiponectin levels were measured by ELISA (R&D).

Multidetector row computed tomography coronary angiography (coronary CT angiography)

Coronary CT angiography data were used to characterize coronary plaque features. CT angiography images were obtained using a 64-slide CT scanner (Sensation 64; Siemens Medical Solutions, Forchheim, Germany), as per our previously published protocols.[205,380]

HDL Redox Activity (HRA)

HDL purification by ultracentrifugation

Cryopreserved serum samples were thawed and adjusted to a density of 1.085 g/ml by the addition of 0.118 g potassium bromide (KBr, Sigma) per ml serum. The adjusted serum was overlaid with 1.085 g/ml density solution (KBr in water) and mixed, and the samples were centrifuged for 20 hours at 50k rpms and 4 ºC (Beckman Coulter Rotor type 70.1 Ti). The bottom layer containing the HDL was then added to a new tube, and its density was increased to 1.21 g/ml by the addition of 0.198 g KBr per ml serum. The resulting solution was then overlaid with 1.21 g/ml KBr, mixed, and centrifuged as above. The purified HDL was removed by needle aspiration and dialyzed for eight hours against 100mM NaCl, 10mM Tris, and 1mM EDTA (American Bioanalytical). The KBr was dried overnight prior to each usage to maintain accurate density measurements.

Determination of HDL cholesterol concentration:

HDL cholesterol was quantified using a standard colorimetric assay (Thermo DMA Co., San Jose, CA), as previously described.[75]

Fluorometric biochemical cell-free assay for assessment of HRA

HDL redox activity was assessed using a validated fluorometric biochemical cell-free assay described in Chapter 4.[75] This assay measures the effect of purified HDL from cryopreserved serum on the rate of oxidation of the fluorogenic probe dihydrorhodamine 123 (DHR).[75] The DHR oxidation rate (DOR) of each sample was normalized to the DOR of HDL from pooled serum of healthy blood bank donors. The ratio DOR subject/ DOR pooled serum was used as a measure of HRA, with higher HRA suggesting dysfunctional HDL. Of note, individuals conducting assessment of HRA were blinded as to whether HDL samples were derived from HIV-infected or non-HIV infected subjects. HRA values for samples determined

to be hemolyzed (samples from 7 HIV-infected subjects and 1 non-HIV subject) were excluded *a priori* from analysis.

Statistical Analysis

Demographic, immunologic/inflammatory, and cardiometabolic parameters were assessed for normality by histogram and the Wilk-shapiro test. Normally distributed data are presented as mean +/- standard deviation; non-normally distributed data as median (interquartile range). Between-group comparisons were performed using the Student's t-test for normally distributed variables and using the Wilcoxon rank sum test for non-normally distributed variables. Dichotomous parameters were compared between groups using chi-square test likelihood ratios. Univariate regression analyses were performed to assess the relationship of demographic, immunologic/inflammatory, and cardiometabolic parameters to HRA. Non-normally distributed parameters were related to HRA using Spearman's rho or were log-transformed and related to HRA via the Pearson's correlation coefficient. Dichotomous variables were related to HRA via Student's t-test. These univariate associations were performed separately in the HIV-infected and non-HIV groups. A secondary analysis was performed to compare HRA with log adiponectin and log HOMA-IR among HIV-infected subjects after excluding the limited number of subjects (n=7) with known diabetes. Moreover, in this group of non-diabetic HIV-infected subjects, multivariate modeling for HOMA-IR was performed in which HRA was entered as an independent variable, along with known risk factors for insulin resistance, including age, family history of diabetes, and visceral adipose tissue. SAS (JMP 9.0, SAS Institute) was used to perform statistical analyses. Two-tailed probability values were assessed, and statistical significance was designated at a p value <0.05.

Study 3

Study subjects: 150 HIV-1 infected patients with HIV-1 RNA <500 copies/mL on antiretroviral therapy (ART) and 50 healthy volunteers without known or clinically evident

cardiovascular disease were recruited in the Los Angeles Areas. The characteristics of the HIV-1 infected subjects are shown in **Table 6.5**.

Immunophenotypic analysis: Measurement of T cell activation. In brief, aliquots of 1 × 10^6 PBMCs were incubated with appropriate fluorochrome-conjugated antibodies, including anti-CD3 (APC-H7), anti-CD4 (PE-Cy7), anti-CD8 (APC), anti-CD38 (PE), and anti-HLA-DR (FITC), for 30 min at 4°C in the dark. Cells were then washed once and were ready for flow cytometry analysis. T cell activation was determined by the percentage of T cells expressing both HLA-DR^+ and $CD38^+$. PBMCs were also stained with 7-aminoactinomycin D (Calbiochem, La Jolla, CA) for dead cell discrimination. In addition, we measured CD38 expression as median fluorescence intensities (MFI) set and the QuantiBRITE beads (BD Biosciences) were used to define the equivalent number of PE molecules and calculate the number of CD38 molecules per cell as previously described.[381] Stained PBMCs were analyzed on a BD LSRII.

Chapter 7

Dysfunctional HDL directly upregulates T cell activation in HIV-1 infection: *in vitro* studies

7.1 Abstract

HIV-1 infection is characterized by a chronic state of immune activation that is an independent predictor of disease progression but the mechanisms that drive immune activation remain unclear. Oxidized lipids play key roles in both viral replication and T cell biology. High density lipoprotein (HDL) has many functions including the disposal of oxidized lipids and is generally anti-inflammatory. However, in systemic inflammatory states, it can become oxidized (HDLox) and dysfunctional. In Chapters 3-6 I have shown that HIV-1-infected subjects have dysfunctional HDL that is associated with biomarkers of immune activation and atherosclerosis. Based on these in vivo observations that support our hypothesis that dysfunctional HDL is major play in the interplay between immune activation and atherogenesis, we sought out to determine whether dysfunctional (oxidized) HDL can directly affect cell activation and immune responses of T cells that are important for HIV-1 immunopathogenesis, immune activation and atherogenesis. Herein, we found that both *in vivo* modified dysfunctional HDL from HIV-1 infected subjects and *in vitro* oxidized HDL directly upregulated markers of T cell activation *in vitro*. In addition HDLox directly increased T cell proliferation and production of cytokines, specific killing of HIV-1 specific cytotoxic $CD8^+$ T cells (CTLs) and expression of chemokine receptors (CXCR4, CCR5) in $CD4^+$ T cells important for HIV-1 infectivity, whereas it reduced antiviral immune responses of HIV-1 specific CTLs. These data identify dysfunctional/oxidized HDL as a novel mechanism of immune activation associated with HIV-1 infection. Understanding the specific mechanisms causing chronic T-cell immune activation is crucial to target immune activation with new therapeutic agents that may reduce the morbidity and mortality of HIV-1 infection.

7.2 Introduction

Human Immunodeficiency Virus (HIV-1) infection is characterized by a chronic state of systemic inflammation with increased levels of proinflammatory cytokines, hyperlipidemia, and helper T cell depletion.[198] Chronic immune activation, and inflammation predict and likely contribute to the excess risk of morbidity and mortality in HIV-1 infected subjects despite effective antiretroviral therapy (ART).[198] Thus, defining the mechanisms for persistent inflammation and immune activation despite ART is a key research question. However, the causal link between HIV-1 and immune activation remains unclear.[198]

Lipids play key roles in both viral replication[106,107] and T cell biology[382,383] and oxidized lipids may directly affect HIV infectivity.[107,114,116] Inflammation increases oxidized lipids that mediate atherogenesis[207] and also regulate both innate[30,144] and adaptive immunity.[39,145,147] Modified lipoproteins such as oxidized low density lipoprotein (oxLDL) carry oxidized lipids and play an important role in the regulation of immunity.[207,384] While High density lipoprotein (HDL) has major immunoregulatory function,[385,386] and has protective effects against oxidized lipids and cardiovascular disease, during systemic inflammation it can be modified, oxidized and become dysfunctional[61,75] and may contribute to atherogenesis.[48,61] However, the precise impact of HIV-1 infection on these processes is unclear.

We have recently shown that HIV-1-infected subjects have dysfunctional HDL[75,331] that is associated with biomarkers of T cell activation.[387] Thus it is likely that there are complex interactions of oxidized HDL with HIV-1 immunopathogenesis. We hypothesize that there is a vicious cycle of HIV-1-induced immune activation, inflammation, production of oxidized HDL, and further immune activation. To elucidate this hypothesis we investigated direct *in vitro* effects of oxidized lipoproteins (HDLox, LDLox) on T cells and markers of T cell activation such as HLA-DR$^+$, CD38$^+$ that have been independently associated with a fall in CD4$^+$ T-cell counts and the development of AIDS.[357]

7.3 Results

The immune cells and lipoproteins that were used in this study were isolated from subjects with no metabolic abnormalities. All the PBMC and lipoproteins used for our *in vitro* studies were isolated from male HIV-1 infected subjects with a median age of 41 years (IQR: 33-47), with suppressed viremia (< 50 copies/ml), on ART with normal $CD4^+$ T-cell count (median: 534 IQR: 427-639 cells/mm^3), normal lipid profile and no metabolic abnormalities. Similarly, all the uninfected subjects in the control group were matched for age, sex and race and had no metabolic abnormalities.

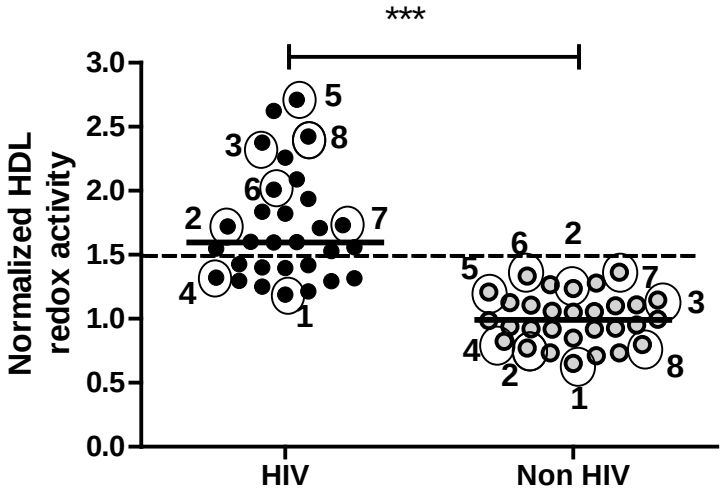

Figure 7.1: A biochemical cell free assay can be used to determine HDL function in human subjects. ApoB depleted serum was isolated by PEG precipitation from 30 healthy subjects and 30 patients with HIV infection that have previously been shown to have acute phase HDL.[311] HDL redox activity (HRA) was determined with the dihydrorhodamine (DHR) assay as described in Methods. Non-cryopreserved apoB depleted serum was used for the DHR assay and the readout was normalized by the readout of a pooled control from healthy subjects as previously described.[311] The HIV-infected subjects had significantly higher HRA (median 1.60, IQR: 1.38, 1.96) compared to the uninfected subjects (0.99±1.36) ($p<0.001$). Each symbol represents the HDL function score for an individual subject. Short horizontal lines indicate median scores for each group. The long horizontal dotted line at 1.5 indicates the dividing line for abnormal HDL function scores (scores ≥ 1.5 indicate the presence of dysfunctional HDL). Circles around symbols in each group (HIV vs non HIV) indicate the 8 subjects from which lipoproteins were isolated and tested in each experiment. For HIV-1 infected subjects mostly HDL samples with a score > 1.5 were used to better study the *in vitro* effects of dysfunctional HDL but samples 1 and 4 were also used to also determine effects of HDL from HIV-1 infected subjects with more favorable HDL function.

In vivo modified HDL isolated from patients known to have dysfunctional HDL can be used *in vitro* to study the effects of dysfunctional HDL on T cell immune activation. Although *in vitro* oxidation is the most widely used method to study effects of oxidized lipids on immune cells, this may not resemble the complex modifications of dysfunctional HDL that occur *in vivo.*[54] Thus, we have included HDL of defined functional status from HIV-1-infected persons, as a more physiologic source of dysfunctional HDL, based on our previous findings that HIV-1 infected subjects have dysfunctional HDL. A previously validated biochemical cell free assay that measures HDL redox activity, a biochemical property that correlates well to function of HDL[75] can be used to determine HDL function in human subjects. Using this assay we defined functional status of HDL from HIV-1 infected (HIV-HDL) and uninfected subjects as described in **Figure 7.1** which was then used for *in vitro* experiments to determine the possible *in vitro* effects of HIV-HDL on T cell activation.

In vivo modified native HDL from HIV-1 infected subjects (HIV-HDL) directly upregulated T cell activation. HIV-HDL directly upregulated the number of $CD8^+$ T-cells positive for CD38 and DR antigens, an established measure of T cell activation, when added to PBMC from healthy subjects (**Figure 7.2A**). In addition, compared to normal HDL, HIV-HDL directly upregulated CD38 antigen expression on $CD8^+$ T-cells (**Figure 7.2B-D**). These effects were observed in both activated and unstimulated (not stimulated *in vitro* with anti-CD3 antibody) healthy PBMC (**Figures 7.2D, E**), suggesting a costimulatory effect of dysfunctional HDL on T cells. This direct relative upregulation in $CD8^+$ T cell activation was more prominent with CD38 antigen expression rather than combinations of HLA-DR and CD38 expression (**Figures 7.2 D, E**). Similarly, HIV-HDL directly increased both CD38 antigen expression and combinations of HLA-DR and CD38 expression in $CD4^+$ T cells (data not shown).

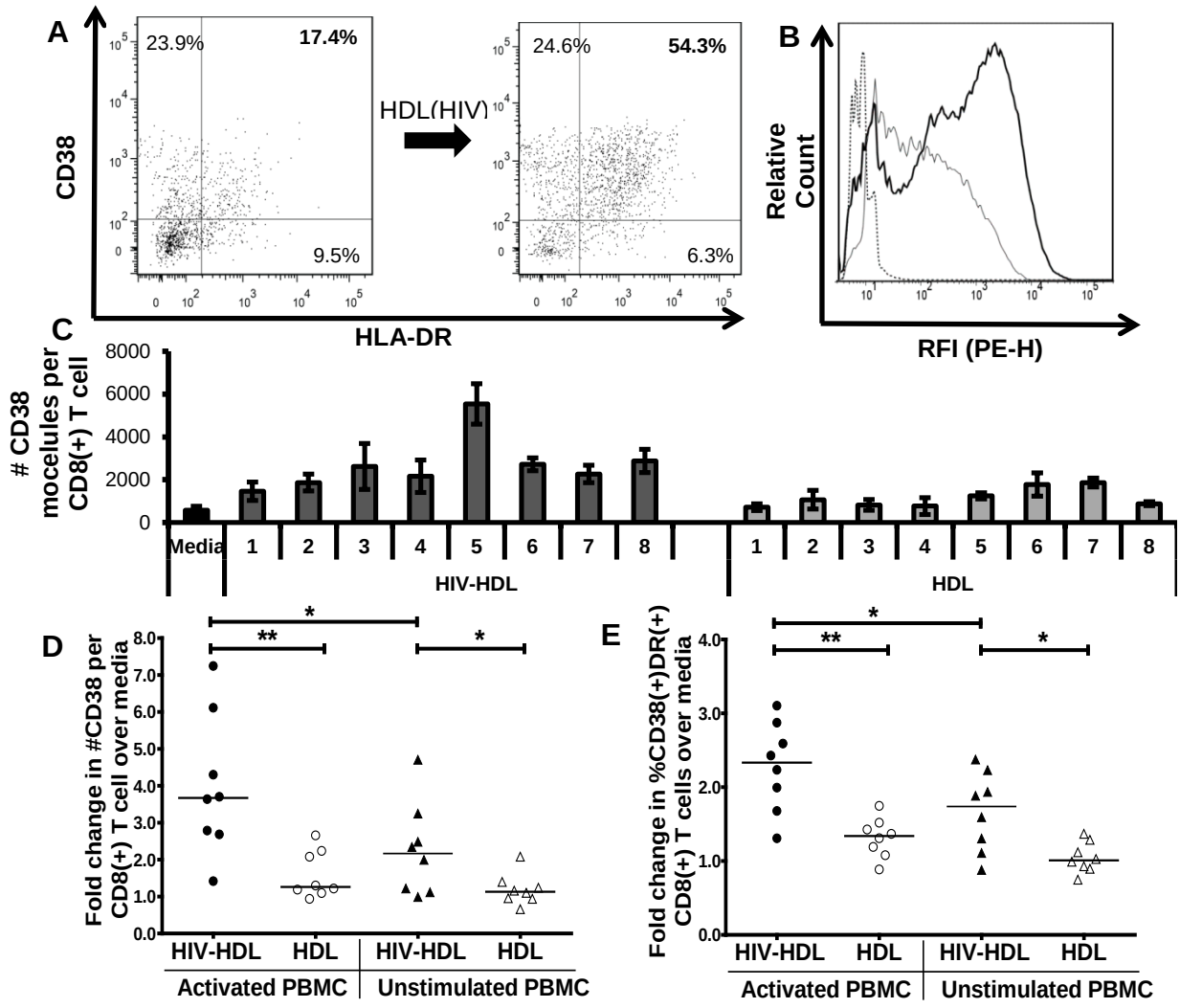

Figure 7.2. ***In vivo*** **modified native HDL directly upregulates T cell activation in HIV-1 infected subjects. A.** Native HDL was isolated from 8 HIV-1 infected subjects with suppressed viremia and 8 uninfected subjects. The patients were selected based on the HDL functional phenotype (HDL redox activity) that was determined using a biochemical assay as described in Methods and in Figure 7.1. PBMCs from healthy subjects (n=3) were incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without all the above native HDLs (20 ug/ml) and T cell activation was determined by measuring the expression of CD38 in $CD8^+$ T cells (number of CD38 molecules per cell) as described in Methods. A. Dysfunctional HDL from HIV-1 infected subjects directly upregulated % $CD38^+$ DR^+ $CD8^+$ T cells when added to PBMC from healthy subjects. A representative sample is shown. **B.** Dysfunctional HDL directly upregulates expression of CD38 in $CD8^+$ from HIV-1 infected subjects. A representative sample is shown (isotype: dotted line, media alone: thin solid line, Dysfunctional HDL: thick solid line). Dysfunctional HDL directly increased relative fluorescence intensity (RFI) for PE compared to media alone. Using the Quantibrite Method as described in Methods this increase in relative MFI corresponds to an increase in the median number of CD38 molecules per $CD8^+$T cell from 378 (media) to 1036. **C.** Results from PBMCs from one healthy subject that were treated with HDL from 8 HIV-1 infected subjects and from 8 uninfected subjects are shown. The error bars represent standard deviations of duplicate experiments. Similar results

were obtained for 2 other subjects (not shown). **D, E.** The fold change in expression of CD38 (# CD38 molecules per $CD8^+$ T cell)**(D)** and % $CD38^+DR^+$ $CD8^+$ T cells **(E)** in cells treated with HDL vs media was determined for all different PBMC samples(n=3) and for all different HDLs (8 HIV-HDL and 8 normal HDL). Identical HDLs were used for all 3 different PBMCs and the averages from the 3 different PBMCs are plotted. Both activated PBMC (n=3) and unstimulated PBMCs (n=3) were used. The relative change in T cell activation for 8 HIV-HDL and 8 normal HDLs (median) is shown. Similar results were obtained for $CD4^+$ T cells (not shown). * p<0.05, ** p<0.01.

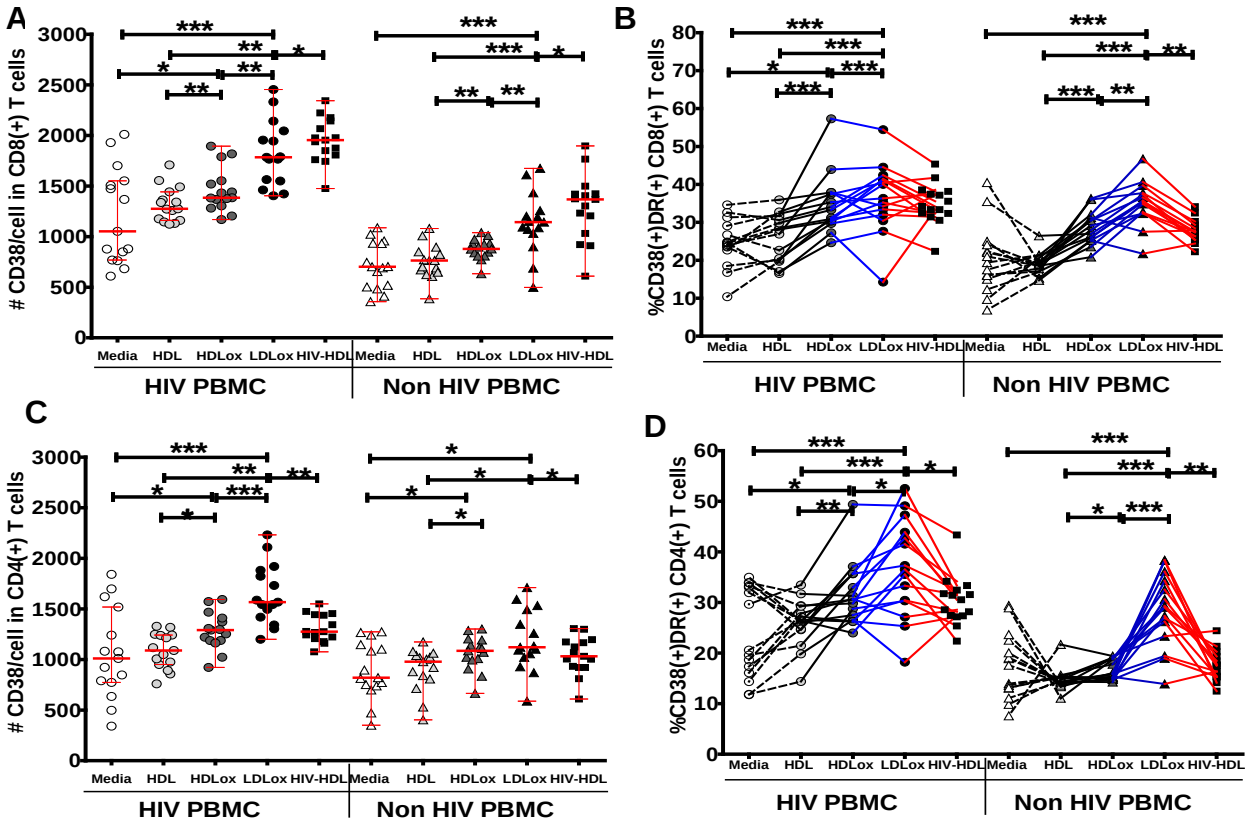

Figure 7.3. *In vitro* oxidized HDL directly upregulates T cell activation. *In vitro* oxidized HDL directly upregulates expression of CD38 **(A, C)** and $CD38^+DR^+$ in $CD8^+$ **(B, D)** in $CD8^+$ **(A, B)** and $CD4^+$ **(C, D)** cells from HIV-1 infected and uninfected subjects. Commercially available HDL from healthy subjects was oxidized *in vitro* using CuSO4 as described in Methods. PBMC from HIV-1 infected (n=15) and uninfected (n=15) subjects with suppressed viremia were incubated with serum free media, anti-CD3 antibody and IL-2 for 72 hours with or without HDLox (25 ug/ml). Oxidized LDL that was also oxidized *in vitro* with CuSO4 was commercially available and *in vivo* modified HDL from an HIV infected subject known to have dysfunctional HDL was also added to compare the effects on T cell activation of *in vitro* versus *in vivo* modified HDL. The medians and IQR are shown in **A, C** and the paired samples in **B, D**. Paired T-test was used for comparison of values between pairs * p<0.05, ** p<0.01, *** p<0.001

In vitro oxidized HDL directly upregulates T cell activation. Given the limitations of biochemical assays of HDL function and the inherent potential variability of dysfunction of native HDL among different subjects, we then explored the possible costimulatory effects of *in vitro*

oxidized HDL on T cells to confirm the costimulatory effects of native HIV-HDL. Indeed, *in vitro* oxidation of lipoproteins using CuSO4 is the most widely used method to study effects of oxidized lipids on immune cells and may allow direct modification of normal HDL into oxidized HDL (HDLox) and dissection of the specific effects of lipoprotein oxidation on immunity *in vitro*.[39,145] Preliminary titration experiments were performed to identify the optimum concentration of oxidized HDL that did not compromise cell viability and oxidized HDL at concentrations ≤ 25 ug/ml had minimal effect on cell viability, whereas the higher

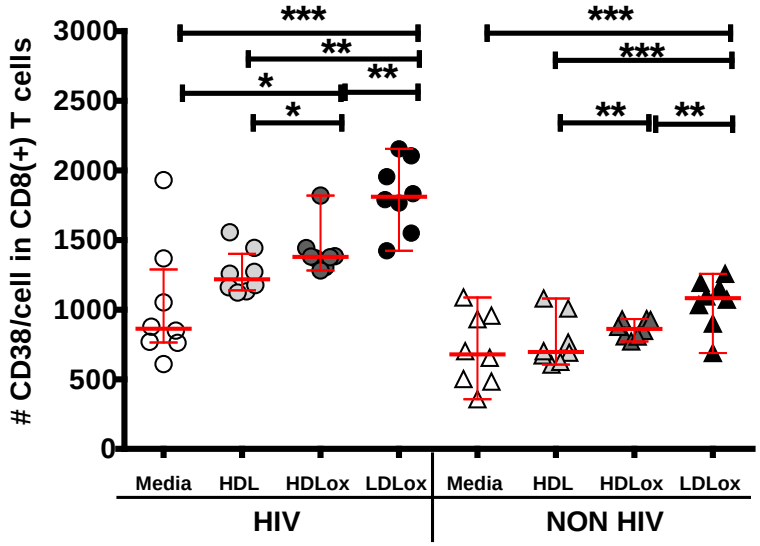

Figure 7.4. *In vitro* oxidized HDL directly upregulates cell activation of purified T cells. T cells from HIV-1 infected and uninfected subjects (n=8) were isolated using an immunomagnetic negative selection kit as described in methods and were treated as in Figure 7.3. The medians and IQR are shown. Similar results were obtained for $CD4^+$ T cells (data not shown). Paired T-test was used for comparison of values between pairs * $p<0.05$, ** $p<0.01$, *** $p<0.001$

concentrations were toxic (data not shown). HDLox directly upregulated both the CD38 antigen expression on $CD8^+$ T-cells (**Figure 7.3A**) and combinations of HLA-DR and CD38 expression (**Figure 7.3B**) in PBMC and isolated T cells **(Figure 7.4)** from both healthy and HIV-1 infected subjects. These immunostimulatory effects of HDLox were more prominent in $CD38^+HLA\text{-}DR^+$ T cells, an immunophenotype that has been associated with more rapid disease progression in HIV-1 infected subjects (**Figure 7.5**).[201] HDLox also directly downregulated the $CD38^-HLA\text{-}DR^+$

T cell immunophenotype that has been associated with more favorable disease progression in HIV-1 infected subjects (**Figure 7.6**).[201,388]

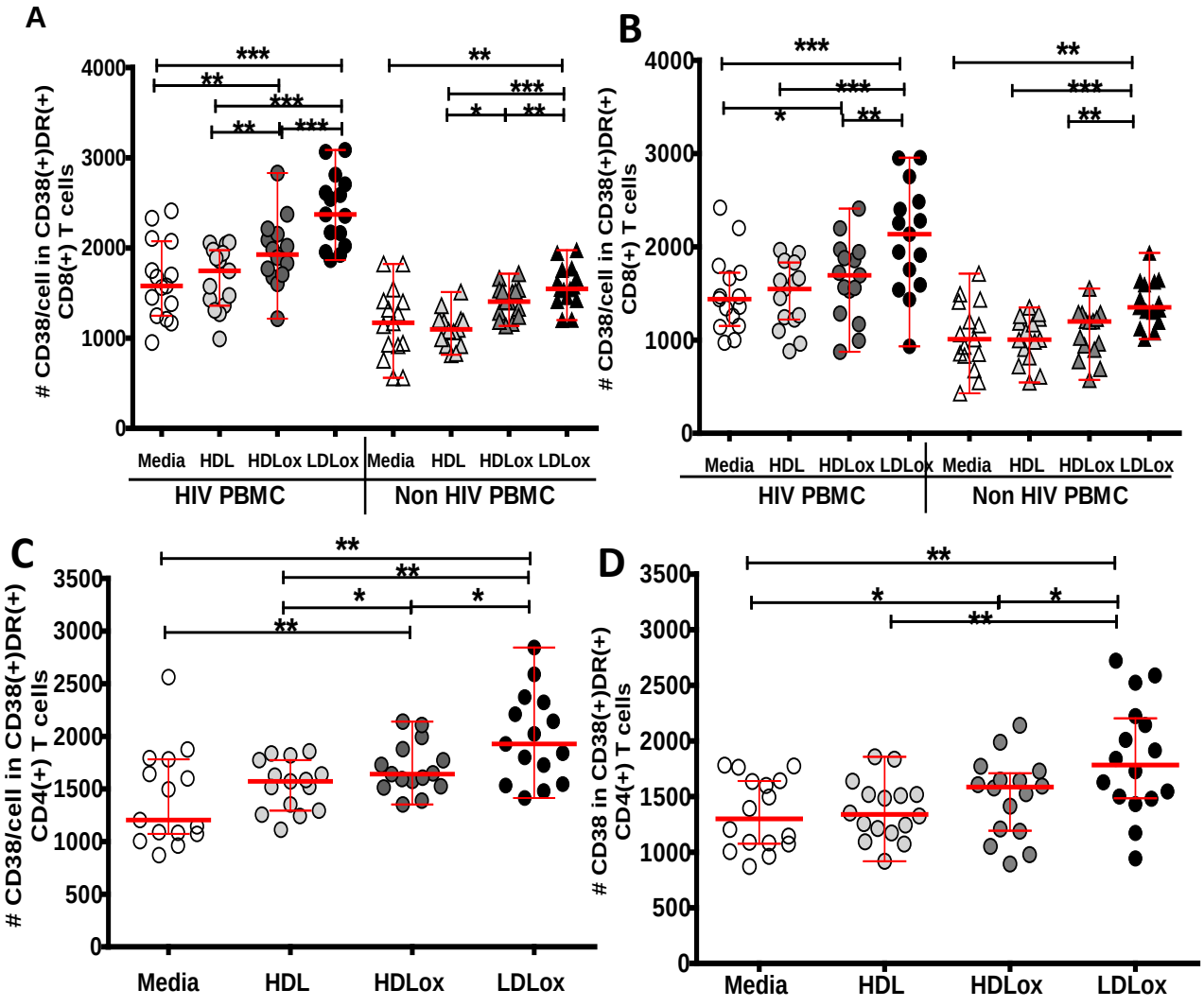

Figure 7.5. *In vitro* modified lipoproteins upregulate CD38 expression in $CD38^+DR^+$ T cells, an immunophenotype that has been associated with more rapid disease progression in HIV-1 infected subjects. A. Commercially available HDL from healthy subjects was oxidized *in vitro* using CuSO4 as described in Methods. PBMC from HIV-1 infected subjects with suppressed viremia (n=15) and uninfected subjects (n=15) were incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without lipoproteins (HDL, HDLox, LDLox all at 25 ug/ml). The median number of CD38 molecules per cell was determined using flow cytometric analysis gated in $CD38^+$ DR^+CD4^+ T cells as described in Methods. The medians and IQR are shown. **B.** Modified HDL oxidized *in vitro* using a more physiological method directly downregulates the $CD38^-DR^+$ T cell immunophenotype. Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an

in vivo generated potent lipid oxidant, as described in Methods. PBMC from HIV-1 infected subjects with suppressed viremia (n=15) or uninfected subjects (n=15) were incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without lipoproteins (HDL, HDLox, LDLox all at 6.25 ug/ml. The Medians and IQR are shown. **C, D.** Commercially available HDL from healthy subjects was oxidized *in vitro* using CuSO4 **(C)** or 13(S) HPODE **(D)** and PBMC from HIV-1 infected subjects with suppressed viremia (n=15) and uninfected subjects (not shown) were treated as above. The median number of CD38 molecules per cell was determined using flow cytometric analysis gated in $CD38^+$ DR^+ $CD4^+$ T cells as described in Methods. The medians and IQR are shown. Similar results to (A, B) were obtained for the $CD4^+$ T cells in uninfected subjects (data not shown). Paired T-test was used for comparison of values between pairs * $p<0.05$, ** $p<0.01$, *** $p<0.001$

The HDLox-induced increase in T cell activation was more prominent in PBMC from HIV-1 infected subjects compared to healthy controls matched for age, sex and race when identical lipoproteins were used (**Figures 7.2-7.6**) suggesting that although the costimulatory effects of oxidized HDL on T cells are not specific for HIV-1 infection, they may be more prominent in immune cells from HIV-1 infected subjects. The costimulatory effects of HDLox were less prominent compared to oxidized LDL (LDLox), a lipoprotein previously shown to increase T cell activation in other inflammatory states.[145,146,389] Overall, similar results with HDLox were observed with $CD4^+$ T cells (**Figures 7.3-7.6 C, D**).

We then compared the effects on T cell activation of *in vitro* (HDLox) versus *in vivo* modified HDL (HIV-HDL) to determine whether the *in vitro* effects observed with HDLox may be physiologically meaningful. Overall, HDLox had similar effects on T cell activation compared to HIV-HDL (**Figure 7.3**) except for a significantly more prominent upregulation in CD38 antigen expression on $CD8^+$ T-cells with HIV-HDL compared to HDLox (**Figure 7.3A**).

Although, *in vitro* oxidation of lipoproteins with CuSO4 is the most widely used method to oxidize lipids *in vitro*, it is not physiological since this type of oxidation does not occur *in vivo*. In addition we found that addition of CuSO4 can cause concentration dependent cell toxicity in PBMCs (data not shown). We have previously shown that HDL can be oxidized and become dysfunctional *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant important for oxidation of lipoproteins and atherogenesis.[390] Using this more physiological method of *in vitro* oxidation of HDL we confirmed the direct upregulation of T cell activation with HDLox (**Figure**

7.7) which was comparable to the effects of addition of HIV-HDL (**Figure 7.7**) without the cellular toxicity of CuSO4 (data not shown). Thus, this method was subsequently used for all our *in vitro* experiments to investigate the possible immunomodulatory effects of HDLox.

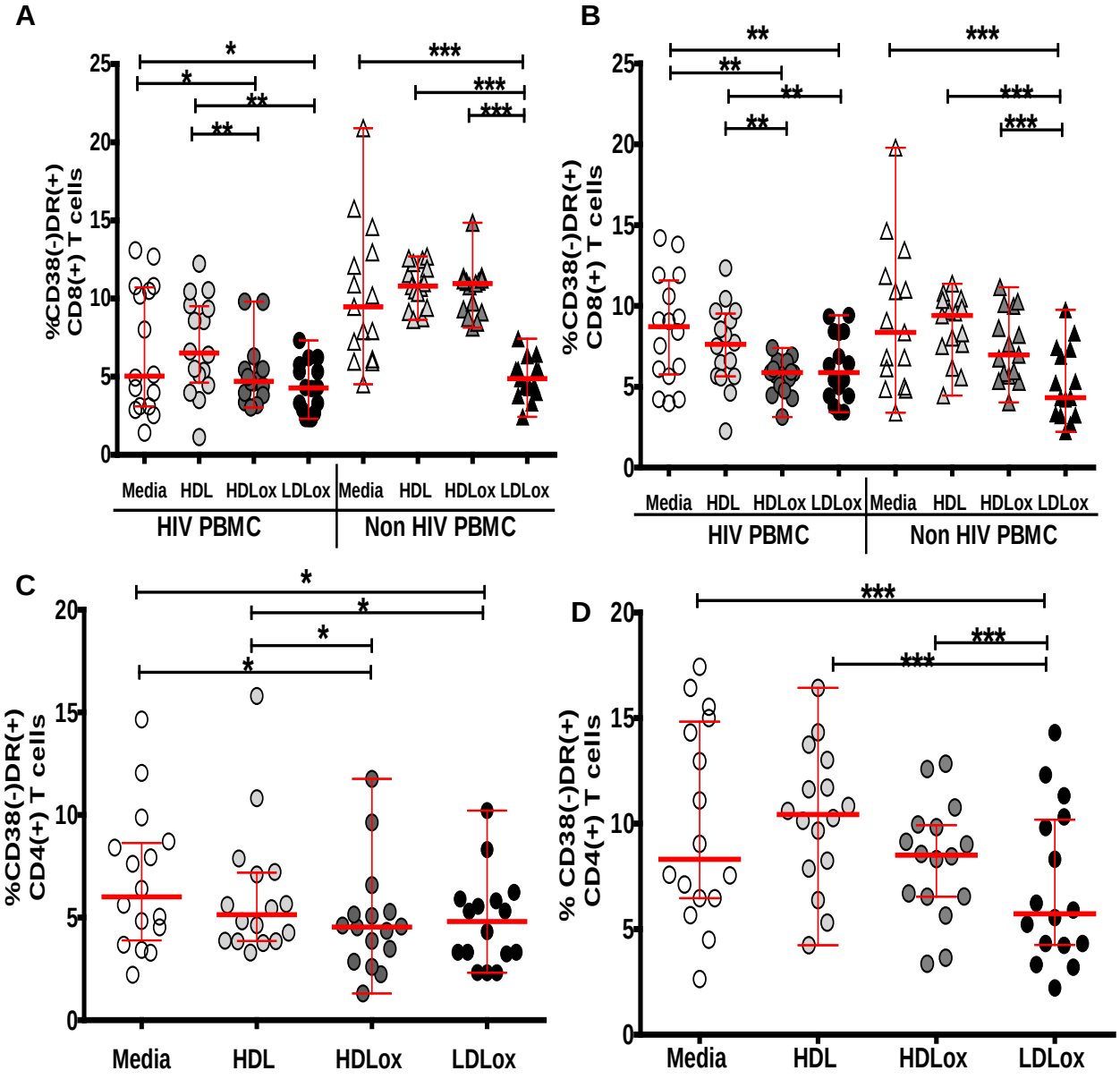

Figure 7.6. ***In vitro*** **modified lipoproteins directly downregulate the CD38-DR$^+$ T cell immunophenotype that has been associated with more favorable disease progression in HIV-1 infected subjects. A.** Commercially available HDL from healthy subjects was oxidized *in vitro* using CuSO4 as described in Methods. PBMC from HIV-1 infected subjects with suppressed viremia (n=15) and uninfected subjects (n=15) were incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without lipoproteins (HDL, HDLox,

LDLox all at 25 ug/ml). The immunophenotype $CD38^{-}DR^{+}$ was determined using flow cytometric analysis gated in $CD8^{+}$ T cells. The medians and IQR are shown. **B.** Modified HDL oxidized *in vitro* using a more physiological method directly downregulates the $CD38^{-}DR^{+}$ T cell immunophenotype. Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. PBMC from HIV-1 infected subjects with suppressed viremia (n=15) or uninfected subjects (n=15) were incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without lipoproteins (HDL, HDLox, LDLox all at 6.25 ug/ml. The Medians and IQR are shown. **C, D.** Commercially available HDL from healthy subjects was oxidized *in vitro* using CuSO4 **(C)** or 13(S) HPODE **(D)** and PBMC from HIV-1 infected subjects with suppressed viremia (n=15) and uninfected subjects (not shown) were treated as above. The immunophenotype CD38-DR^{+} was determined using flow cytometric analysis gated in $CD4^{+}$ T cells. The medians and IQR are shown. Similar results to (A, B) were obtained for the $CD4^{+}$ T cells in uninfected subjects (data not shown). Paired T-test was used for comparison of values between pairs * p<0.05, ** p<0.01, *** p<0.001

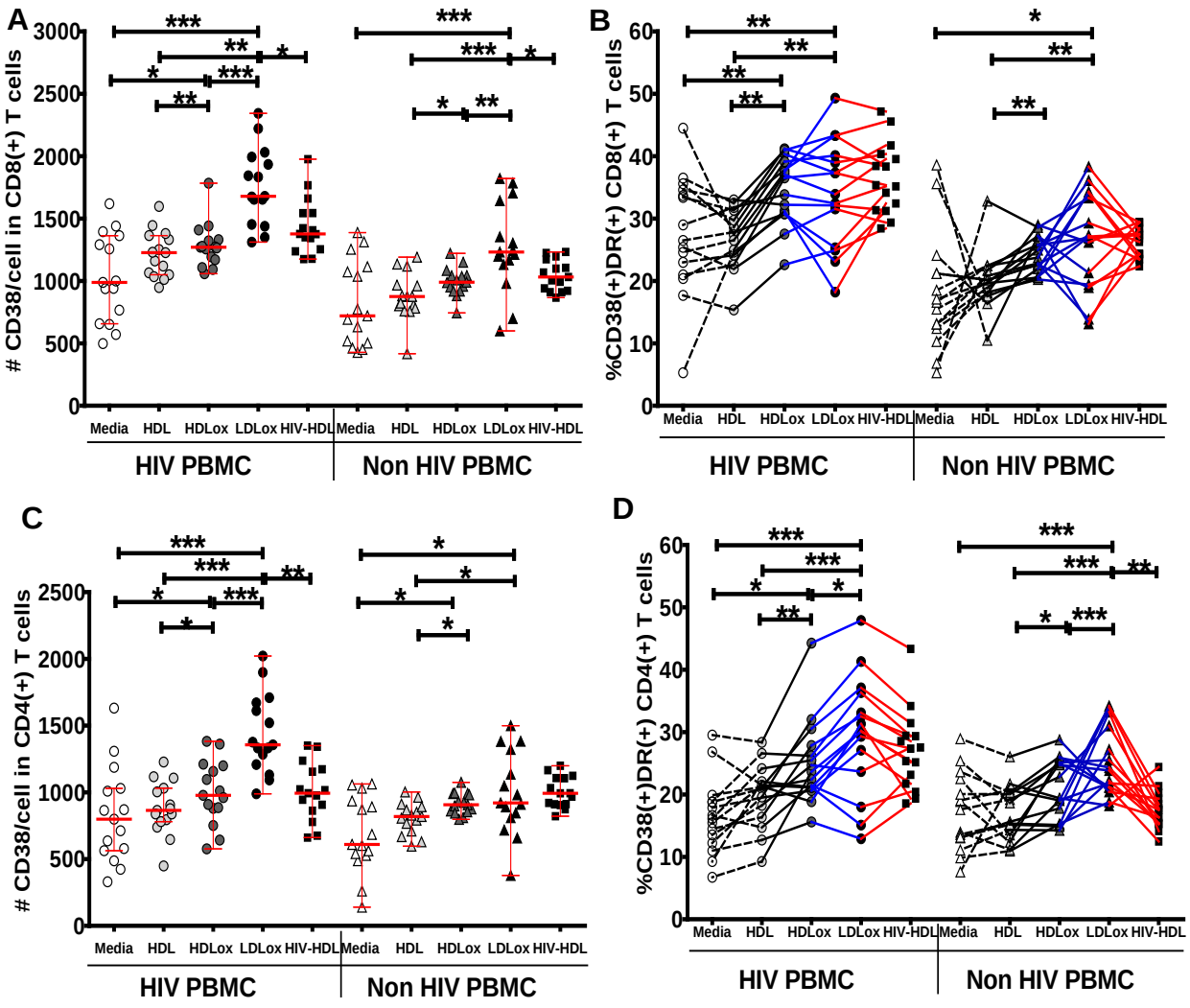

Figure 7.7. Modified HDL oxidized *in vitro* using a more physiological method directly upregulates T cell activation. *In vitro* oxidized HDL directly upregulates expression of CD38 **(A, C)** and $CD38^{+}DR^{+}$ in $CD8^{+}$ **(B, D)** in $CD8^{+}$ **(A, B)** and $CD4^{+}$ **(C, D)** cells from HIV-1 infected

and uninfected subjects. Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. PBMC from HIV-1 infected (n=15) and uninfected (n=15) subjects with suppressed viremia were incubated with serum free media, anti-CD3 antibody and IL-2 for 72 hours with or without HDLox (25 ug/ml). Oxidized LDL that was also oxidized *in vitro* with CuSO4 was commercially available and *in vivo* modified HDL from an HIV infected subject known to have dysfunctional HDL was also added to compare the effects on T cell activation of *in vitro* versus *in vivo* modified HDL. The medians and IQR are shown in A, C and the paired samples in B, D. Paired T-test was used for comparison of values between pairs * p<0.05, ** p<0.01, *** p<0.001

In vitro oxidized HDL directly promoted proliferation of T cells. Consistent with its immunostimulatory effects on T cell activation as outlined above, HDLox directly increased proliferation of T cells from HIV-1 infected but not from uninfected subjects (**Figure 7.8**).

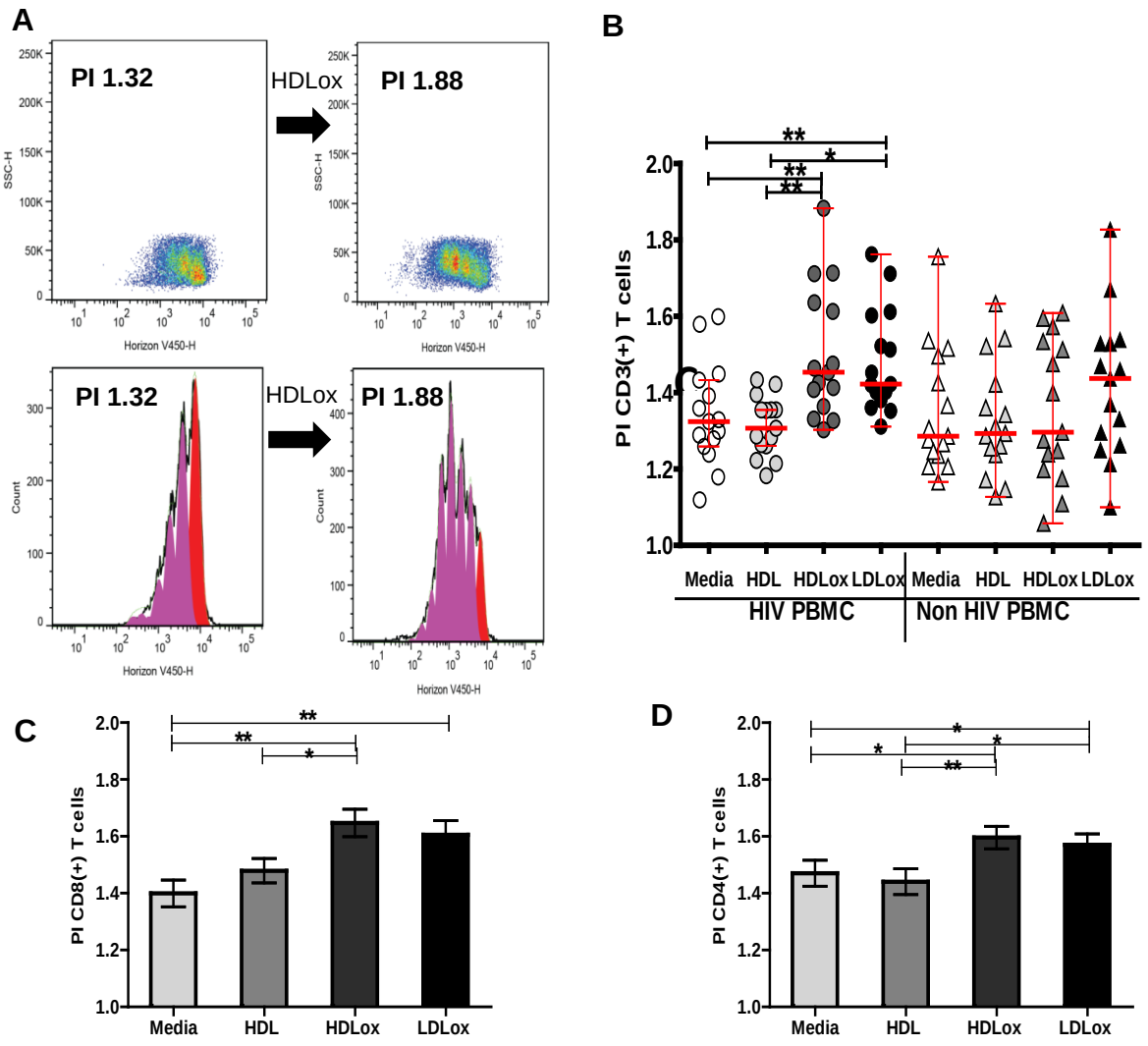

Figure 7.8. Modified HDL directly upregulates T cell proliferation. *In vitro* oxidized HDL directly upregulates proliferation of T cells from HIV-1 infected subjects. Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. PBMC from HIV-1 infected subjects with suppressed viremia (n=15) and uninfected subjects (n=15) were incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without lipoproteins (HDL 6.25 ug/ml; HDLox 6.25 ug/ml). Oxidized LDL that was also oxidized *in vitro* with CuSO4 was commercially available. The proliferation analysis (bottom) was performed in gated live, single $CD3^+$ T cells and the proliferation index (PI) was calculated as described in Methods. A representative sample is shown in **(A)**. The Median and IQR per group are shown in **(B)**. The proliferation analysis was also performed in gated live, single $CD3^+$, $CD8^+$ T cells **(C)** and gated live, single $CD3^+$, $CD4^+$ **(D)** from HIV-1 infected subjects (n=15). The Mean and SEM are shown. Paired T-test was used for comparison of values between pairs * p<0.05, ** p<0.01, *** p<0.001

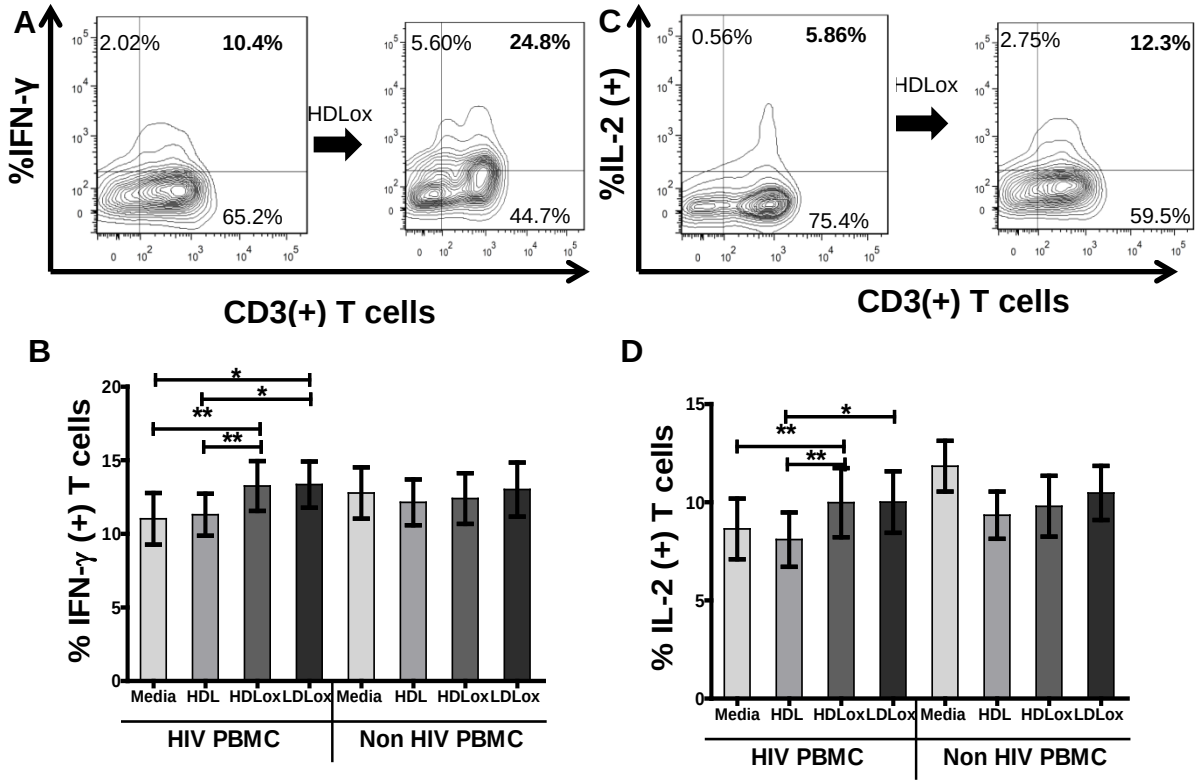

Figure 7.9. Modified HDL directly upregulates T cell cytokine production in HIV-1 infection. A. *In vitro* oxidized HDL directly upregulates production of INF-γ **(A, B)** and IL-2 **(C, D)** from T cells of HIV-1 infected subjects. Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. Oxidized LDL that was also oxidized *in vitro* with CuSO4 was commercially available. PBMC from HIV-1 infected subjects with suppressed viremia (n=15) and uninfected subjects (n=15) were incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without lipoproteins (HDL 6.25 ug/ml; HDLox 6.25 ug/ml). Intracellular cytokine staining for INF-γ **(A, B)** and IL-2 **(C, D)** were performed as described in Methods.

Representative samples are shown in **(A, C)**. The mean and SEM are shown. Paired T-test was used for pairs * p<0.05, ** p<0.01, *** p<0.001

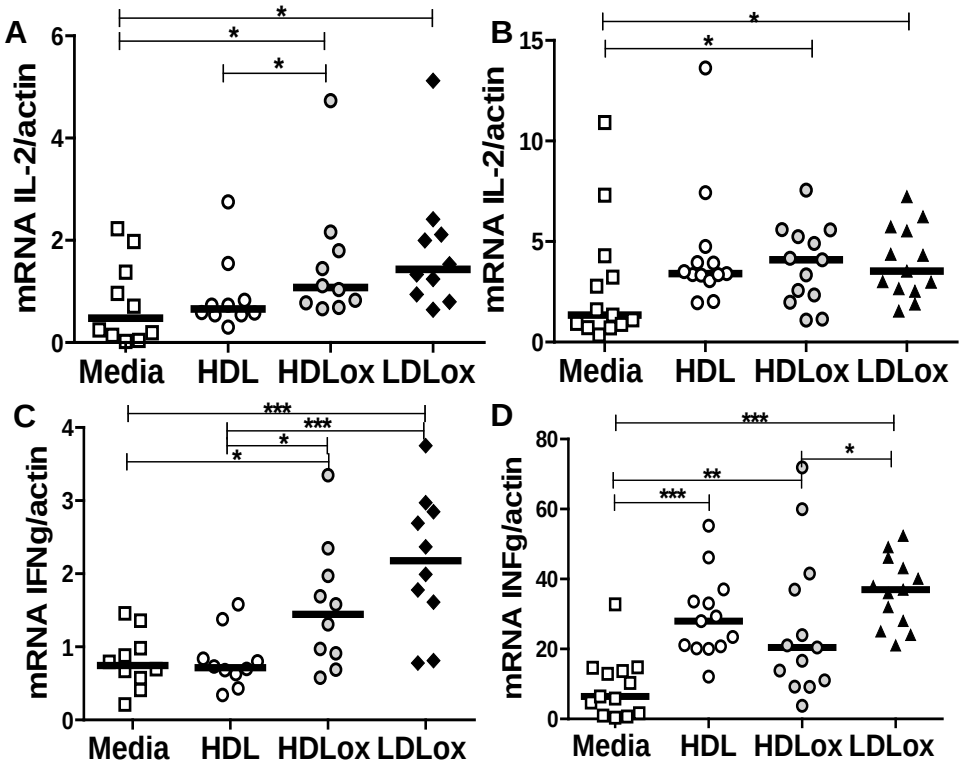

Figure 7.10. ***In vitro*** **modified lipoproteins directly upregulate mRNA expression of cytokines that are mainly secreted by T cells.** Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. PBMC from HIV-1 infected subjects with suppressed viremia (n=10) and from uninfected subjects (n=13) was incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without lipoproteins (HDL, HDLox, LDLox all at 6.25 ug/ml and on day 3 mRNA expression of INF-γ and IL-2 was determined as described in Methods. The Medians and IQR are shown for IL-2 **(A,B)** and INF-γ **(C,D)** for HIV-1 infected **(A,C)** and uninfected subjects **(B,D)**. Paired T-test was used for comparison of values between pairs * p<0.05, ** p<0.01, *** p<0.001

<u>*In vitro* oxidized HDL directly increased T cell cytokine production in HIV-1 infection.</u>

Similarly, HDLox directly increased production of both INF-γ (**Figures 7.9 A, B**) and IL-2 (**Figures 7.9 C, D**) from T cells of HIV-1 infected subjects but not from uninfected controls. HDLox also directly upregulated mRNA expression of cytokines that are mainly secreted by T cells (INF-γ, IL-2) in PBMC (**Figure 7.10**).

In vitro oxidized HDL directly upregulates specific killing of HIV-1 specific cytotoxic $CD8^+$ T-cells (CTLs). In view of the increased T cell activation induced by HDLox, we then determined whether this increased activation may also be associated with increased killing of HIV-1 specific CTLs. HDLox directly increased killing of HIV-1 specific CTLs as determined using a standard chromium release assay (**Figure 7.11**) and also increased expression of granzyme B and perforin (**Figure 7.12**) in HIV-1 specific CTLs.

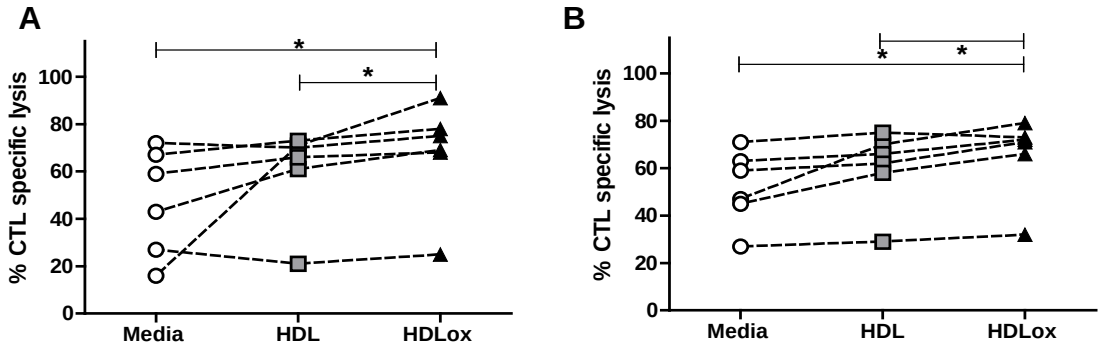

Figure 7.11. Modified HDL directly upregulates specific killing of HIV-1 specific cytotoxic CD8 $^+$ T cells (CTLs). Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. HIV-1 specific CTLs as outlined in **Table 7.1** were incubated with serum free media alone, for 72 hours with or without lipoproteins [HDL, HDLox, at 100 ug/ml **(A)** or at 50 ug/ml **(B)**]. On day 3 these HIV-1-specific clones were utilized in standard chromium release assays (effector to target ratio of 3:1) using the HLA A*0201-expressing cell line T1 pulsed with the appropriate synthetic peptide. The % specific CTL lysis was calculated as described in Methods. Paired T-test was used for comparison of values between pairs * $p<0.05$, ** $p<0.01$, *** $p<0.001$

In vitro oxidized HDL directly potentiates expression of chemokine receptors important for HIV-1 infectivity. To confirm our hypothesis that oxidized HDL may directly increase T cell activation but also HIV-1 infectivity that may also drive immune activation and given the previous findings that oxidized lipids may increase expression of CXCR4 on T lymphocytes[114,391] and modify expression of CCR5,[107] we investigated the direct effect of HDLox on expression of CXCR4 and CCR5 on $CD4^+$ T cells, the main target cell population of HIV-1. HDLox directly upregulated expression of both CXCR4 and CCR5 on $CD4^+$ T cells in HIV-1 infected subjects but not in uninfected controls (**Figure 7.13**).

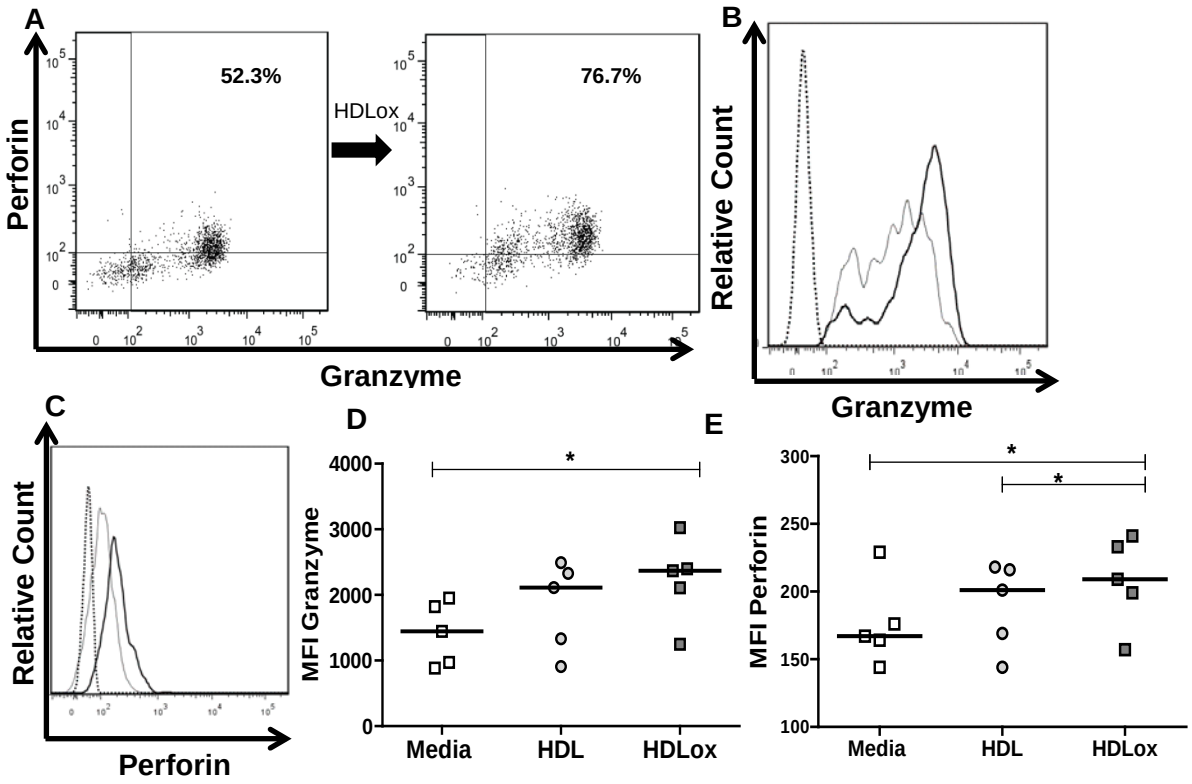

Figure 7.12 Modified HDL directly upregulates granzyme and perforin in HIV-1 specific cytotoxic CD8$^+$ T cells (CTLs). Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. HIV-1 specific CTLs as outlined in **Table 7.1** were incubated with serum free media alone for 72 hours with or without lipoproteins [HDL, HDLox, at 100 ug/ml or at 50 ug/ml (not shown)]. Flow cytometric analysis for granzyme and perforin was performed in gated live, single CD8$^+$ T cells as in methods. **A.** Representative dot plots are shown. **B.** A representative histogram that shows an increase in the mean fluorescence intensity (MFI) of granzyme expression in gated live single CD8$^+$ T cells in the presence of HDLox 100 ug/ml (thick line) vs media alone (thin line) is shown. An isotype control (dotted line) is also shown. **C.** A representative histogram that shows an increase in the MFI of perforin expression in gated live single CD8$^+$ T cells in the presence of HDLox 100 ug/ml (thick line) vs media alone (thin line) is shown. An isotype control (dotted line) is also shown. HDLox upregulated MFI expression of granzyme **(D)** and perforin **(E)** compared to media alone. Paired T-test was used for pairs * p<0.05, ** p<0.01, *** p<0.001

In vitro oxidized HDL directly reduces antiviral immune responses. To examine effects of oxidized HDL that may potentiate HIV-1 replication and further drive immune activation we evaluated the *in vitro* effects of HDLox on the antiviral activity of HIV-1-specific CD8$^+$ T-cells (CTLs). HDLox but not HDL reduced the ability of HIV-1-specific

CTLs to suppress viral replication (**Figure 7.14**). Thus, oxidized HDL may contribute to viral persistence *in vivo* through dysfunction of cellular antiviral immunity.

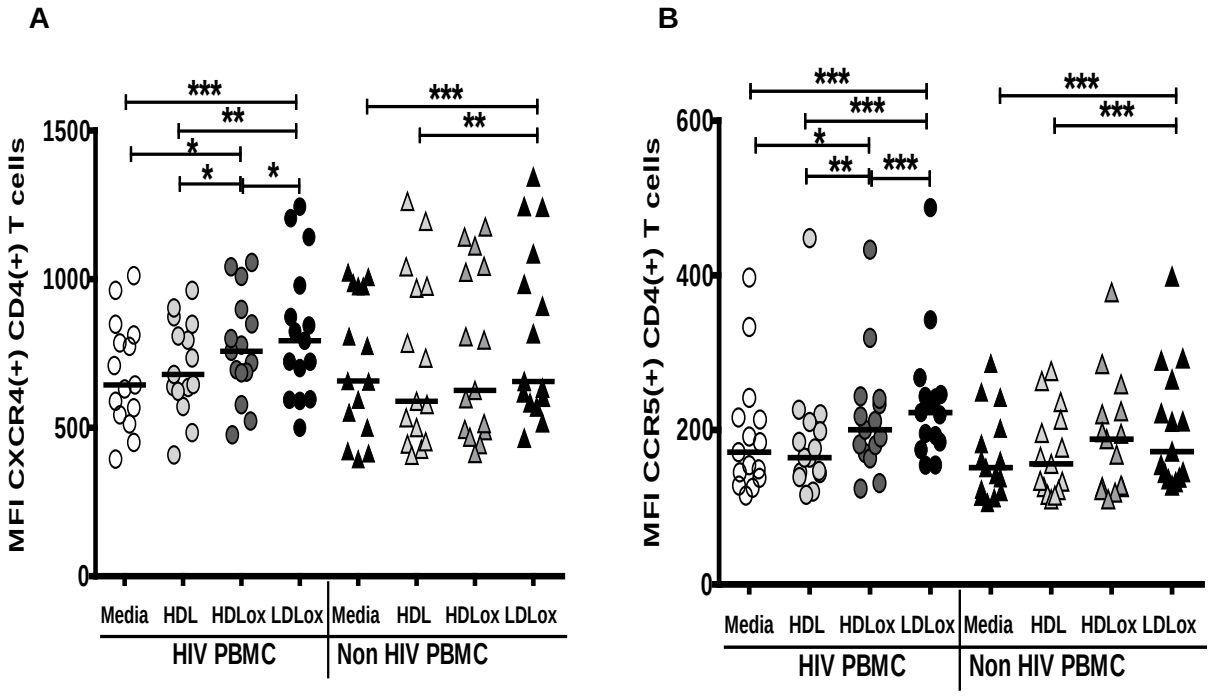

Figure 7.13. Modified HDL directly upregulates expression of chemokine receptors important for HIV-1 infectivity. A. *In vitro* oxidized HDL directly upregulates expression of CXCR4 in $CD4^+$ T cells of HIV-1 infected subjects. Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. Oxidized LDL that was also oxidized *in vitro* with CuSO4 was commercially available. PBMC from HIV-1 infected subjects with suppressed viremia (n=15) and uninfected subjects (n=15) were incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without lipoproteins (HDL, HDLox, LDLox all at 6.25 ug/ml). Flow cytometry analysis for CXCR4 **(A)** and CCR5 **(B)** expression was performed in gated $CD4^+$ T cells. The mean fluorescence intensity (MFI) was calculated as described in Methods. The medians are shown. Paired T-test was used for comparison of values between pairs * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 7.1: HIV-1 specific CTL clones used in this study

Epitope	Sequence	HLA restriction	Location	CTL clone
SL9	SLYNTVATL	A*02	Gag (75–85) (p17)	S1/SL9 3.23 T p11
IV9	ILKEPVHGV	A*02	RT (309-318)	S68A62t p12
KW10	KIATESIVIW	B*57	RT (374-383)	S34 KW10 3.4p3
AW9	AVRHFPRIW	B*57	vpr (30-38)	S34/AW9 3.8 p2
HQ10	HTQGYFPDWQ	B*57	nef (116-125)	S11/HQ10 nef 10.10 p1
KF11	KAFSPEVIPMF	B*57	Gag (162-172)(p24)	S14/10.6KF11 p6

The dysfunction of cellular antiviral immunity induced by oxidized HDL is not secondary to changes in functional avidity. The function of HIV-1-specific CTLs is markedly abnormal in most infected persons showing inappropriate activation and abnormal differentiation and

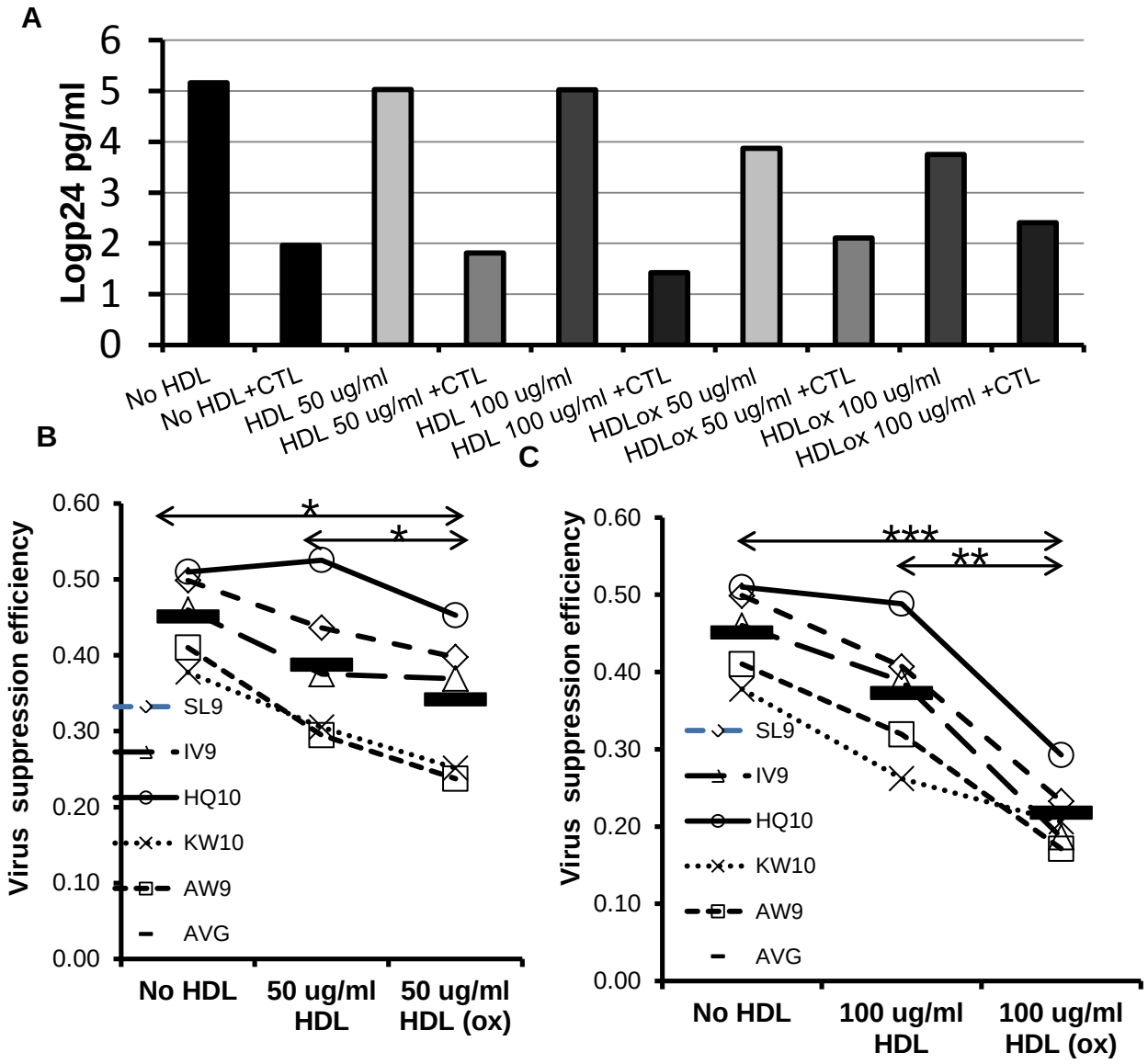

Figure 7.14. Modified HDL directly downregulates the virus suppression efficiency of HIV-1 specific cytotoxic CD8$^+$ T cells (CTLs). T1 cells were infected with HIV-1 NL4-3.1 at low multiplicity and cultured with or without HIV-1 specific CTLs as outlined in **Table 7.1** in the presence of normal or 13(S) HPODE oxidized HDLox [HDL, HDLox, at 50 ug/ml **(A)** or at 100 ug/ml **(B)**]. Viral replication was assessed by p24 ELISA. A representative experiment from a Gag-specific CTL clone is shown in **(A)** and raw data from Day 6 are plotted. The same lipoproteins were used for all CTL clones and virus inhibition assay was performed and the virus

suppression efficiency for each CTL clone **(B)** was calculated as described in Methods. The medians are shown **(B, C)**. Note that T1 cells are immortalized and maximally activated at baseline, and therefore the effect of HDL on cellular activation and HIV-1 replication cannot be assessed with these cells. The reduction in HIV-1 with HDLox may be due to over-activation and cell death. Paired T-test was used for comparison of values between pairs * p<0.05, ** p<0.01, *** p<0.001

exhaustion.[142] While chronic antigenic stimulation most likely plays a major role in these changes, suppressive antiretroviral therapy is not fully restorative and dysfunctional HDL also persists.[331] It therefore is conceivable that abnormalities of CTLs could be driven by oxidized HDL. We investigated whether oxidized HDL may directly affect functional avidity of HIV-1 specific CTL clones that may be translated to reduced antiviral activity against HIV-1.[392] HDLox did not significantly affect the functional avidity of HIV-1 specific CTL clones (**Figure 7.15**). Thus, further studies are needed to elucidate the mechanisms of reduced cellular antiviral immunity induced by oxidized HDL despite increases in $CD8^+$ T cell activation and killing.

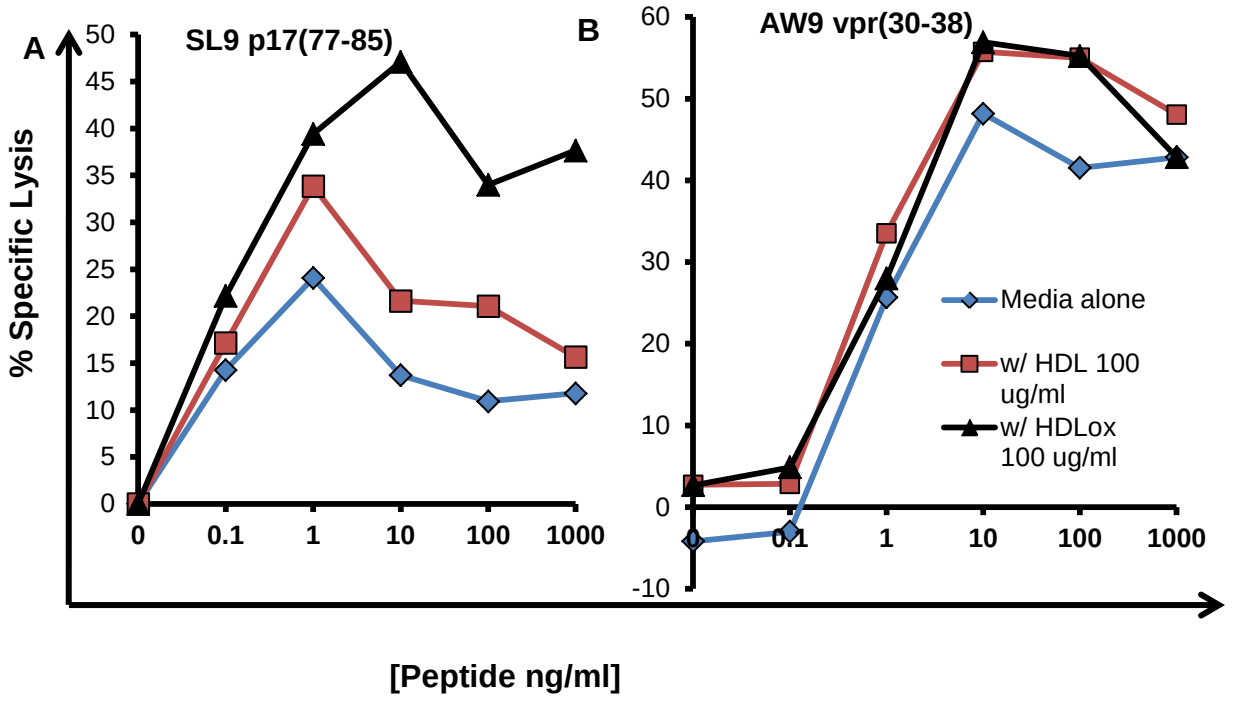

Figure 7.15. HDLox does not significantly affect the functional avidity of HIV specific CTL clones. Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, as described in Methods. HIV-1 specific CTLs as outlined in **Table 7.2** were incubated with serum free media alone for 72 hours with or without lipoproteins [HDL, HDLox, at 100 ug/ml]. The same lipoproteins were used for all CTL clones and the functional avidity and SD50

for each CTL clone were calculated on day 3 as described in Methods. The data from 2 CTL clones SL9 p17 (77-85) **(A)** and AW9 vpr (30-38) **(B)** are shown. The SD50 for the SL9 p17 (77-85) was 84.4, 98.7, 172.8 pg/ml in the presence of serum free media, plus HDL or HDLox, respectively. The SD50 for the AW9 vpr (30-38) was 987.6, 870.2, 1143.7 pg/ml in the presence of serum free media, plus HDL or HDLox, respectively.

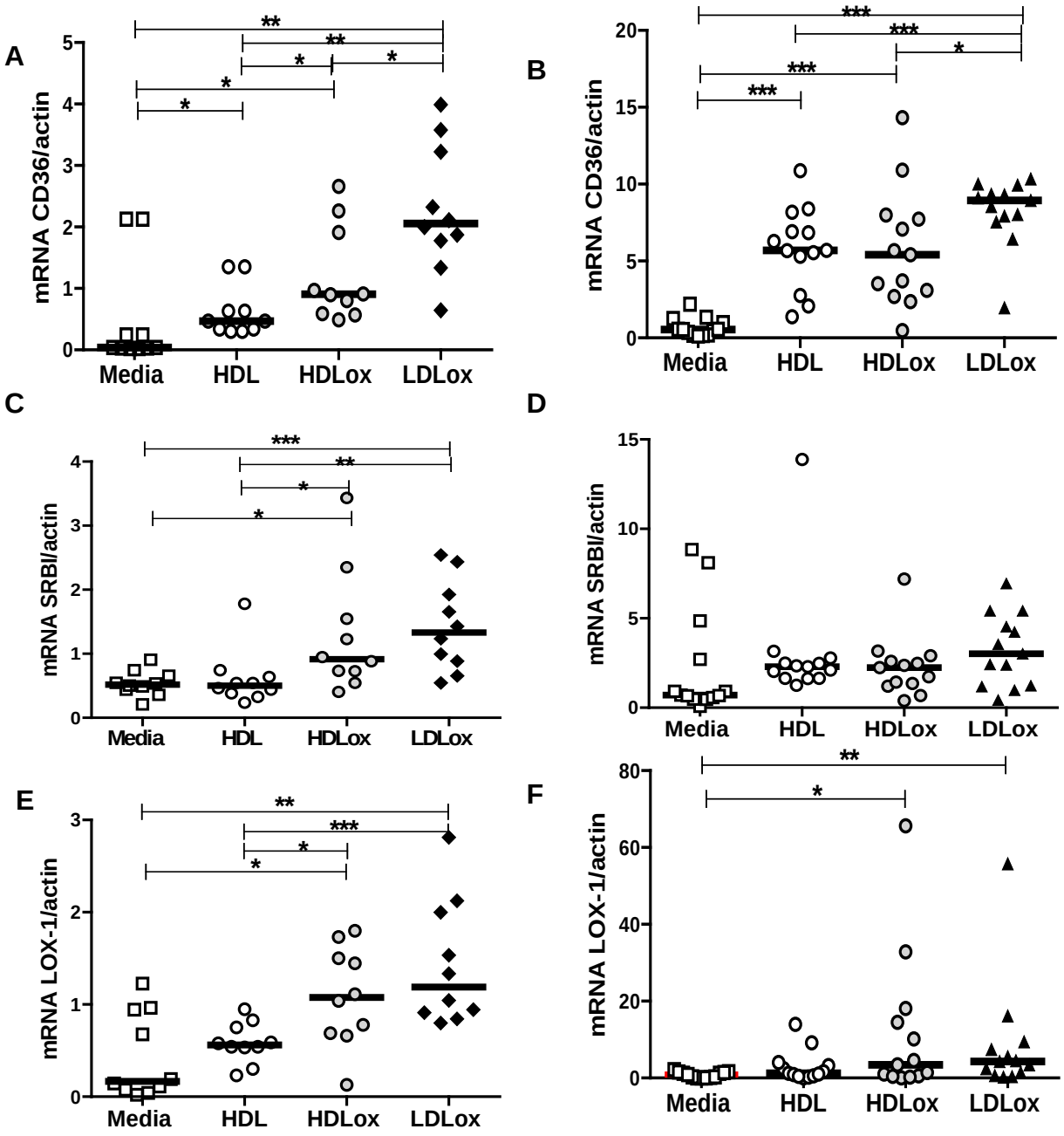

Figure 7.16. ***In vitro*** **modified lipoproteins directly upregulate mRNA expression of scavenger receptors.** Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. PBMC from HIV-1 infected subjects with suppressed viremia (n=10) and from uninfected subjects (n=13) was incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours

with or without lipoproteins (HDL, HDLox, LDLox all at 6.25 ug/ml and on day 3 mRNA expression of CD36 **(A, B)**, SRBI **(C, D)** and LOX-1 **(E, F)** was determined as described in Methods. The Medians and IQR are shown for HIV-1 infected **(A, C, E)** and uninfected subjects **(B, D, F)**. Modified lipoproteins directly upregulated mRNA expression of all three scavenger receptors in both HIV-1 infected and uninfected subjects. Paired T-test was used for comparison of values between pairs * p<0.05, ** p<0.01, *** p<0.001

HDLox may directly upregulate expression of scavenger receptors in immune cells of HIV-1 infected subjects. As outlined in Chapter 1, scavenger receptors may be one of the mechanisms that mediate effects of oxidized lipoproteins such as oxidized LDL on immune cells.[21-23] CD36,[23] scavenger receptor class B1 (SR-B1),[24] and lectin-like oxidized LDL receptor-1 (LOX-1)[24,25] also bind HDLox and thus we determined the effects of HDLox on the expression of scavenger receptors in immune cells (PBMCs) from HIV-1 infected subjects. HDLox directly upregulated expression of CD36, SR-B1 and LDLox in immune cells of HIV-1 infected and uninfected subjects **(Figure 7.16)**. However, these observations need to be confirmed with further mechanistic studies using purified cell populations (T cells, macrophages).

HDLox likely activates immune cells through the NFκB pathway. Although scavenger receptors are mainly expressed on macrophages and endothelial cells, [24,25] oxidized lipids can activate T cells *in vitro*, increasing receptor activator of NFκB ligand (RANK-L) production and LOX-1 expression [39,40,216] and thereby activating the NFκB pathway.[393] *In vivo*, we found that cholesterol [342] and HDLox[343] correlate with serum RANK-L in HIV-1 infected (but not in uninfected) persons, suggesting the importance of the NF-kB pathway triggering by HDLox. Herein, we show that HDLox directly upregulated expression of RANKL and OPG, important mediators of the NF-kB pathway, in immune cells of HIV-1 infected subjects **(Figure 7.17)**. These results confirm previous *in vitro* studies that oxidized HDL may directly upregulate the NF-kB pathway[217] which may provide a mechanistic link for HDLox and immune activation in HIV-1 infection. However, these preliminary observations need to be confirmed with further mechanistic studies using purified cell populations (T cells, macrophages).

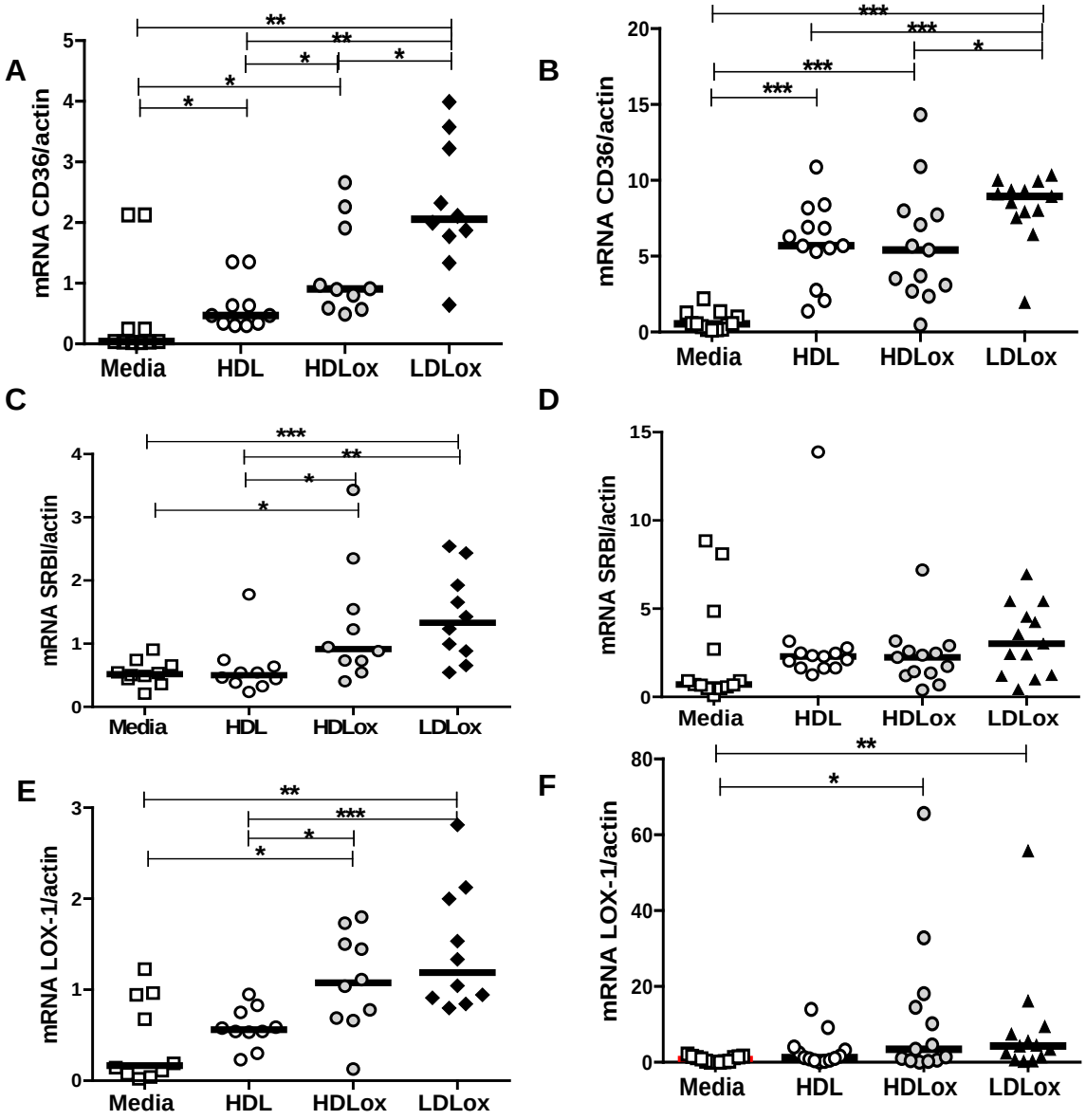

Figure 7.17. *In vitro* modified lipoproteins directly upregulate mRNA expression of RANKL and OPG, important mediators of the NF-kB pathway. Commercially available HDL from healthy subjects was oxidized *in vitro* using 13(S) HPODE, an *in vivo* generated potent lipid oxidant, as described in Methods. PBMC from HIV-1 infected subjects with suppressed viremia (n=10) and from uninfected subjects (n=13) was incubated with serum free media alone, anti-CD3 antibody and IL-2 for 72 hours with or without lipoproteins (HDL, HDLox, LDLox all at 6.25 ug/ml and on day 3 mRNA expression of RANKL **(A, B)**, OPG **(C, D)** and their ratio **(E, F)** was determined as described in Methods. The Medians and IQR are shown for HIV-1 infected **(A, C, E)** and uninfected subjects **(B, D, F)**. In HIV-1 infected subjects but not in uninfected subjects oxidized HDL directly upregulated (compared to media alone) mRNA expression of RANKL and OPG. Oxidized LDL directly upregulated mRNA expression of RANKL and OPG in both HIV-1 infected and uninfected subjects. Paired T-test was used for comparison of values between pairs * $p<0.05$, ** $p<0.01$, *** $p<0.001$

7.4 Discussion

The current research suggests that dysfunctional/oxidized HDL is a novel mechanism of immune activation associated with HIV-1 infection. We found that both *in vivo* modified dysfunctional HDL from HIV-1 infected subjects and *in vitro* oxidized HDL directly upregulated markers of T cell activation *in vitro*. In addition HDLox directly increased T cell proliferation and production of cytokines, specific killing of HIV-1 specific cytotoxic $CD8^+$ T-cells (CTLs) and expression of chemokine receptors important for HIV-1 infectivity, whereas it reduced overall antiviral immune responses. Thus, we hypothesize that HDLox has a central role in HIV-1 pathogenesis through a combination of: increasing immune activation, enhancing expression of chemokine receptors important for HIV-1 replication, and dampening antiviral activity of CTLs.

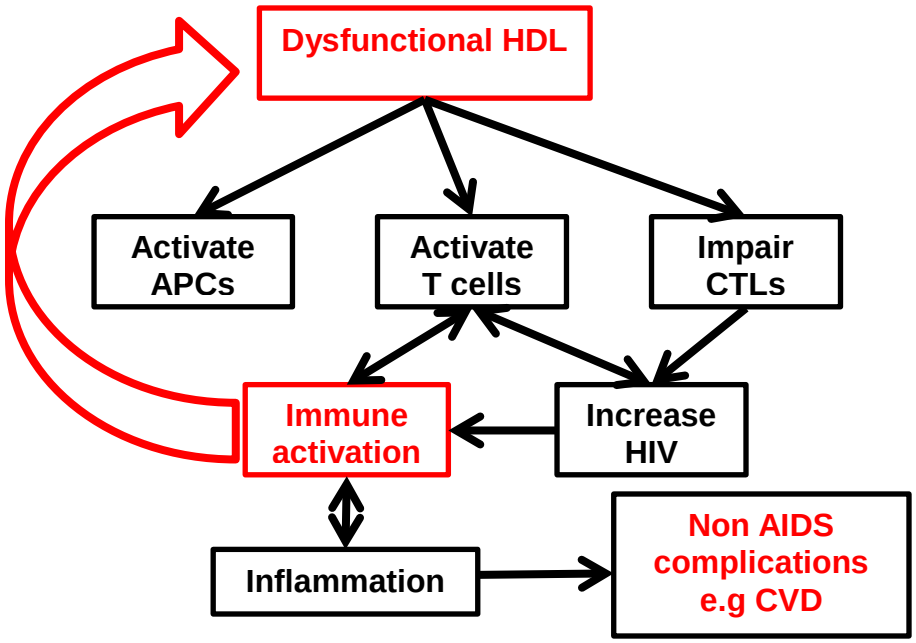

Figure 7.18. Overall hypothesis. Abbreviations: AIDS: acquired immunodeficiency syndrome, APCs: Antigen Presenting Cells, CTLs: Cytotoxic $CD8^+$ T cells, CVD: Cardiovascular disease, HIV: Human Immunodeficiency virus

This hypothesis (shown in **Figure 7.18**) identifies dysfunctional HDL as a key factor that both

results from and contributes to the immune activation of HIV-1 infection.

HIV-1 infection is characterized by a chronic state of immune activation that is an independent predictor of disease and probably the central process causing immune damage and immunosuppression.[198] Although viremia is apparently suppressed with ART, this immune activation does not fully normalize.[171] There are multiple mechanisms by which HIV-1 infection could induce systemic immune activation including HIV-1 replication, increased production of pro-inflammatory molecules, microbial translocation and increased homeostatic proliferation in response to $CD4^+$ T-cell depletion.[198] Each mechanism may feed to the others, thus creating an uncontrolled positive feedback.[198] This residual immune activation may be largely responsible for the increased morbidity and mortality observed in HIV-infected subjects on ART.[394] In this context, we have also shown that in HIV-1 infected subjects with suppressed viremia,[262] markers of microbial translocation and macrophage activation were associated with atherosclerosis progression, an important cause of morbidity and mortality among people with treated HIV-1 infection.[155]

Inflammation is a hallmark of immune activation in HIV-1 pathogenesis[155] and may increase oxidized lipids that mediate atherogenesis[207] and may regulate both innate[144] and adaptive immunity.[39,147] HDL has several key effects including regulation of innate immunity,[386] the disposal of oxidized lipids and is generally anti-inflammatory. However, in systemic inflammatory states, it can become oxidized/dysfunctional, [49,54,61] which is associated with atherogenesis in patients with inflammatory disorders.[49,54,61] We have recently shown that HIV-1-infected subjects have dysfunctional HDL[331] that is associated with biomarkers of T cell activation[387] and percent non-calcified coronary plaque, a measure of subclinical atherosclerosis.[395] HDL has been reported to suppress HIV-1 replication *in vitro*, [121] but the impact of HDL oxidation on this process is unknown. Thus it is likely that there are complex interactions of dysfunctional HDL with HIV-1 replication and immune activation given the key roles that lipids play in both viral replication[106,107,114] and T cell biology.

Although increased numbers of HLA-DR$^+$, CD38$^+$ CD8$^+$ T-cells were associated with disease progression in HIV-1 infection[199] and this marker is widely used to study T cell activation, it is less stronger marker for the risk of chronic HIV disease progression to AIDS and death compared to elevated CD38 antigen expression on CD8$^+$ T-cells.[201] Consistent with dysfunctional HDL linkage to immune activation in HIV-1 infection, we showed that HDLox correlates to CD38 level on CD8$^+$ T-cells, a key indicator of immune activation that drives disease progression.[201] In addition, HDLox had a more prominent relative effect in upregulation of CD38 expression in T cells compared to its effect in relative increase in numbers of HLA-DR$^+$, CD38$^+$ CD8$^+$ T-cells.

The mechanisms that may mediate these direct effects of HDLox on T cells remain to be determined. It is well established that oxidized lipoproteins such as LDLox contribute to immune activation and atherogenesis [30,145] through previously described mechanisms such as immune cell increases in scavenger receptors for oxidized lipids, and chemokine receptors.[23,114,396] These processes are less explored for HDLox, and have been understudied in HIV-1 infection. For example scavenger receptors also bind oxidized HDL[24,25] and oxidized lipids can activate T cells *in vitro*, increasing receptor activator of NFκB ligand (RANK-L) production and lectin-like oxidized LDL receptor-1 (LOX-1) expression [39,216] and thereby activating the NFκB pathway. [217] *In vivo*, we found that cholesterol[342] and HDL redox activity[343] correlate with serum RANK-L in HIV-1 infected (but not in uninfected) persons, suggesting the importance of the NF-kB pathway triggering by HDLox[217] which may provide a mechanistic link for HDLox and immune activation in HIV-1 infection. *In vitro*, HDLox directly upregulated expression of scavenger receptors and RANKL, OPG in immune cells of HIV-1 infected subjects. In addition, except for the NF-kB pathway other activation pathways in T cells may also be triggered by oxidized lipoproteins such as activation of platelet activating factor (PAF).[145,397] These proposed mechanisms may mediate the observed effects of oxidized HDL on T cell activation and are the focus of current research within our group.

Dysfunctional HDL may also have indirect effects on adaptive immunity through effects on innate immunity. In a cohort of 102 HIV-1 infected subjects and 41 non-HIV controls without clinical cardiovascular disease presented in Chapter 6, increased HDL redox activity, a measure of HDL function, correlated positively with the macrophage activation marker soluble CD163 ($r=0.24$, $p=0.02$).[395] Dysfunctional HDL has previously been shown *in vitro* to directly influence monocyte[214] and dendritic cell[67] function in systemic inflammatory states such as autoimmune diseases[214] and leprosy.[67] We have also studied the direct effects of oxidized HDL on monocytes and macrophages from HIV-1 infected subjects and found that HDLox can directly upregulate cellular proliferation, activation and expression of proinflammatory cytokines (unpublished data). Further studies are needed to define the direct effects of HDLox on innate immunity and their downstream effects on T lymphocytes.

Interestingly, oxidized HDL inhibited CTL antiviral activity despite inducing increases in activation of T cells and killing of HIV-1 specific $CD8^+$ T-cells. This observation could not be explained by changes in functional avidity of HIV-1 specific CTLs. Lipid rafts in the host cell membrane have key roles in viral replication and contain CD4 and chemokine receptors utilized by HIV-1 to enter target cells, and are key for the assembly of virions.[110] We found that HDLox may directly upregulate expression of CXCR4 and CCR5 in $CD4^+$ T cells and thus these effects may increase HIV-1 infectivity. Normal HDL may have antiviral role; amphipathic domains of apoA-I reduce gp41-mediated membrane fusion through direct interactions with virions.[121] The effect of HDL oxidation on this process is unknown, but lipid oxidation decreases the stability, alters the orientation of apoA-I on HDL particle[131] and thus could interfere with the normal antiviral activity of HDL. Oxidized lipoproteins may also directly modify lipid rafts,[130] a major determinant of HIV-1 antiviral responses.[123,124,128] Viral cholesterol-binding proteins such as Nef may mobilize lipid rafts in natural target cells of HIV-1 (macrophages, $CD4^+$ T cells) [114,119] through a pathway that competes with cholesterol efflux, a major function of HDL.[122] Thus,

further studies are needed to determine the effects of HDLox on lipid rafts in target cells of HIV-1 that may overall increase HIV-1 infectivity and lead to reduced antiviral responses.

Our study has several limitations. Although *in vitro* oxidation is the most widely used method to study effects of oxidized lipids on immune cells, this may not resemble the complex modifications of dysfunctional HDL that occur *in vivo*.[54] However, this limitation is shared by most experimental studies of this topic and our *in vitro* data described herein are consistent with our previous *in vivo* observational data that increased HDL redox activity is associated with T cell activation in HIV-1 infected subjects.[387] Finally, although we have included HDL of defined functional status from HIV-1-infected persons, as a more physiologic source of dysfunctional HDL, the biochemical assay that was used to define HDL function has limitations that have previously been described in detail.[75,379]

Despite the above limitations, our study provides some of the first information that support a novel hypothesis that there is a vicious cycle of HIV-1-induced immune activation, inflammation, production of dysfunctional HDL, that contributes to further immune activation. The immediate clinical implication would be that novel therapeutics to address qualitatively abnormal HDL, such as HDL mimetics, [247,248] could reduce the morbidity and mortality of HIV-1 by reducing the immune activation that drives both progressive $CD4^+$ T lymphocyte loss and CVD. A deeper understanding of the specific mechanisms causing chronic T-cell immune activation is crucial to target immune activation in HIV-infected individuals.

7.5 Materials and Methods

Human subjects: Fifteen HIV-1-infected men on combination ART with suppressed viremia (below 50 copies of RNA/ml) (median age 40, range 18-53 years) were recruited at the University of California, Los Angeles (UCLA). These patients had no documented coronary atherosclerosis, normal lipid profile, were not receiving hypolipidemic medications and were not diabetic (Table 1). Fifteen normal men (median age 42, range 20-57 years) matched by age and race were recruited under a protocol approved by an institutional Human Research Subject Protection Committee (UCLA IRB).

Cell isolation and culture: Human peripheral blood mononuclear cells (PBMCs) were isolated from heparin-anticoagulated blood by Ficoll-Hypaque gradient centrifugation from donors with normal lipid levels, as documented by the UCLA Clinical Labs. T lymphocytes were purified using an immunomagnetic negative selection kit using tetrameric antibody complexes recognizing CD4, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ, glycophorin A and dextran-coated magnetic particles (StemCell Technologies). Purity of T lymphocytes (>98%) was confirmed by flow cytometry (Becton Dickson) using anti-CD3 antibody (BD Pharmingen). Cell viability following purification was consistently >90%, as assessed by trypan blue exclusion. Cells were plated at a concentration of 1 × 106/ml in AIM-V serum-free medium (Gibco) for cell culture experiments. For experiments testing activated cells, cells were stimulated using anti-CD3 antibody (200 ng/ml) supplemented with recombinant human interleukin-2 (NIH AIDS Reference and Reagent Repository, 50 IU/ml) and lipoproteins. For HDL treatment, cells were counted, washed in RPMI, and resuspended in serum free media to minimize effects of serum factors. PBMC (1×106/ml) were also treated in duplicate at 37°C in 5% CO2 for 72 hours with lipoproteins: a) HDL (6.25 ug/ml) b) *in vitro* oxidized HDL (HDLox, 6.25 ug/ml) c) *in vitro* oxidized LDL (LDLox, 6.25 ug/ml). Cells were also treated with HDL

isolated from HIV-1 infected subjects with known dysfunctional HDL or from healthy subjects with normal HDL at 20 μg/ml for 72 hours at 37°C in 5% CO2.

Chemicals and Materials: Human HDL was purchased from EMD Millipore Corporation, (Billerica, MA). Human oxidized LDL was purchased from Biomedical Technologies, Inc. (Stoughton, MA). 13(S)-hydroperoxy-9Z, 11E-octadecadienoic acid (13(S)-HPODE) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Dihydrorhodamine 123 (DHR) was obtained from Molecular Probes (Eugene, OR). DHR was prepared as concentrated stock of 50 mM in dimethyl sulfoxide as previously described.[75]

Lipoprotein preparation and oxidation: A cell-free assay that measures HDL redox activity, a measurement of HDL function,[75] was used to determine which HIV-1 infected patients had the most dysfunctional HDL. Native HDL was then isolated from plasma of these HIV-1-infected patients and from uninfected subjects using ultracentrifugation as previously.[75] HDL was isolated from plasma of healthy subjects and the lipoproteins were oxidized by use of copper ions as previously.[39,75,398] The lipoproteins were tested pre- and post-oxidation for lipopolysaccharide levels and found to have <30 pg of lipopolysaccharide/ml of medium. HDL was also oxidized *in vitro* with 13(S)-HPODE as described[390]: human HDL (5 μg HDL-cholesterol/ml) was incubated with 13(S)-HPODE (0.5 μg/ml), for 60 min (co-incubation). HDL-cholesterol concentration was quantified using a standard colorimetric assay (Thermo DMA Co., San Jose, CA) as previously described.[75]

Cell-free assay of HDL redox activity: HDL function was determined based on a previously established biochemical cell free assay that measures HDL redox activity based on the oxidation of the fluorogenic probe DHR (presented in Chapter 3).[75]

Immunophenotypic analysis: Measurement of T cell activation. In brief, aliquots of 1 × 10^6 PBMCs were incubated with appropriate fluorochrome-conjugated antibodies, including anti-CD3 (APC-H7), anti-CD4 (PE-Cy7), anti-CD8 (APC), anti-CD38 (PE), and anti-HLA-DR (FITC),

for 30 min at 4°C in the dark. Cells were then washed once and were ready for flow cytometry analysis. T cell activation was determined by the percentage of T cells expressing both HLA-DR^+ and $CD38^+$. PBMCs were also stained with 7-aminoactinomycin D (Calbiochem, La Jolla, CA) for dead cell discrimination. In addition, we measured CD38 expression as median fluorescence intensities (MFI) set and the QuantiBRITE beads (BD Biosciences) were used to define the equivalent number of PE molecules and calculate the number of CD38 molecules per cell as previously described.[381] Stained PBMCs were analyzed on a BD LSRII.

Intracellular cytokine staining. Intracellular expression of INF-γ and IL-2 in T cells was performed as described.[399] Briefly, 1 × 106 PBMCs were stimulated by antiCD3 antibody and treated with lipoproteins for 66 hours as described above. Cells were then incubated with brefeldin A (10 μg/ml) for 6 hours, followed by surface staining with anti-CD3 (APC-H7) and intracellular cytokine staining with anti-INF-γ (FITC) and anti-TNF-α (PE).

Intracellular staining of cytotoxic $CD8^+$ T-cells (CTLs) for granzyme and perforin. CTLs were incubated with lipoproteins for 66 hours and were then stimulated by peptide-loaded HLA-$A02^+$ B-LCLs. After 2 hours, brefeldin A (10 μg/ml; BD) was added, with incubation for 4 hours more. Cells were then harvested and fixed in Fixation/Permeabilization solution (BD Biosciences). Intracellular staining was performed as per the manufacturer's protocol using Perm/Wash buffer (BD Biosciences) FITC-conjugated granzyme B and PE-conjugated perforin antibodies (BD Biosciences). All antibodies were purchased from Becton Dickinson Biosciences.

Multiparameter proliferation analysis of human lymphocytes. PBMC were labeled with 10 μM CellTrace™ Violet (Life Technologies), and stimulated with anti–human CD3 and interleukin-2 for 72 hours in culture. After stimulation for 3 days, the Violet Cell Trace profiles of T cells were evaluated by flow cytometry. A gating strategy was employed to limit analysis to live, single cells T-cells. Cell divisions and proliferation index (PI) were determined using the Proliferation Wizard algorithm in FlowJo version 9.2 (TreeStar).

Analysis of flow cytometry data. Analysis was done using FlowJo version 9.2 (TreeStar). Single-color staining of cell surface antigens with isotype control antibodies were performed as controls.

Quantitative RT-PCR Gene Expression Analyses: PBMC cells that were incubated for 72 hours with or without lipoproteins (HDL, HDLox) were harvested and total RNA was then prepared using the RNAeasy Mini Kit (Qiagen). Quantitative PCR was performed using TaqMan gene expression primers (Applied Biosystems) specific for human INF-γ, IL-2. Reactions were carried out using the TaqMan 7730 system (Applied Biosystems) and standard reagents from Applied Biosystems. The PCR was then carried out for 40 cycles at 50°C for 2 min, 95°C for 10 min, 92°C for 15 sec, and 60°C for 1 min. Cycle Thresholds for each gene were determined and normalized with those for reactions performed with actin specific TaqMan primers.

Target cell lines: The HIV-1-permissive T1 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, l-glutamine, HEPES, and penicillin-streptomycin (R10) as previously described.[400] These were utilized as MHC-matched target cells for CTL clones.

HIV-1-specific CTL clones: CTL clones (Table 2) were derived from the peripheral blood mononuclear cells of HIV-1-infected individuals by limiting-dilution cloning and were maintained with periodic restimulations (anti-CD3 antibody and irradiated allogeneic PBMCs) in R10 supplemented with 50 U/ml interleukin-2 (R10-50).[400,401]

Chromium release assays: Target cell killing by CTL clones in the presence of lipoproteins was assessed by standard chromium release assays.[400,401] Briefly, CTLs were incubated with lipoproteins (HDL, HDLox) for 72 hours in serum free media. Target cells were ^{51}Cr labeled (in the presence or absence of 10 μg/ml synthetic epitope (Sigma) and incubated with or without CTLs for 4 hours at an excess effector-to-target cell ratio of 5:1. Specific lysis was calculated by subtracting the control spontaneous release from the test release and dividing that sum by the difference of the control maximum release minus the spontaneous

release: specific lysis = (observed chromium release − spontaneous chromium release) ÷ (maximal chromium release − spontaneous chromium release).

Testing of HIV-1 susceptibility to suppression by HIV-1-specific CTL clones in the presence of lipoproteins: The susceptibility of each virus to suppression by the CTL clones was quantified by using a virus suppression assay that has been described in detail.[400,401] In brief, the target cells were infected with SL-9 virus at a multiplicity of approximately 0.01 tissue culture infectious doses per cell, and co-cultured in 96-well cell culture plates at a CTL effector to target cell ratio of 1.25×10^4 with 5×10^4 cells, in triplicate for each condition with or without lipoproteins (HDL, HDLox). A control with no effector cells was included. Supernatant was assessed for p24 antigen concentration (p24 ELISA kit, Perkin Elmer, MA, USA).

Virus Suppression at six days after infection was calculated as:

"Suppression" = (Log10 pg/ml p24 antigen without CTL) - (Log10 pg/ml p24 antigen with CTL)

Virus Suppression Efficiency at six days after infection was calculated as:

"Suppression Efficiency" = "Suppression" ÷ (Log10 pg/ml p24 antigen without CTL)

Functional avidity measurements: Functional avidity of CTL clones was determined by standard peptide titration chromium release assays.[400,401] Briefly, the chromium-labeled target cells were preincubated with serial dilutions of the cognate peptide (Sigma) before the chromium release assay. Functional avidity was measured as the concentration of peptide yielding 50% of maximal CTL killing (SD_{50}).

Statistical methods: Medians were compared using the Wilcoxon rank-sum test or Wilcoxon signed-rank test. Group means were compared using the Student's t-test and analysis of variance for unpaired continuous variables and the paired Student's t-test for paired variates. *p* values of 0.05 or less were considered significant. The analysis was performed using the GraphPad Prism (version 5.04) program.

Chapter 8

Discussion

8.1 Abstract

This chapter is divided into 3 major sections **(8.2-8.4)**. The first section **(8.2)** summarizes the major findings and discussions of this dissertation. The next section **(8.3)** describes potential future work. Concluding remarks are provided in the final section.

8.2 Summary of Results

The overall goal of this research is to define the role of oxidized High Density Lipoprotein (HDL) in the intersection of Human Immunodeficiency virus (HIV-1), immune activation, and atherogenesis. I present *in vivo* and *in vitro* data that support the hypothesis that there is a vicious cycle of HIV-1-induced immune activation, inflammation, production of oxidized HDL, and further immune activation that may explain the increased rate of atherosclerotic disease in HIV-1 infection.

Residual immune activation in treated HIV-1 may be contributing to atherosclerosis. HIV-associated cardiovascular disease (CVD) is a growing clinical problem but the relative contributions of viremia, immune activation, ART and inflammation to the increased CVD risk are unclear (see **Chapter 1** and **section 1.4** for a detailed description on this topic). HIV-1 infection is characterized by a chronic state of immune activation that is an independent predictor of disease and probably the central process causing immune damage and immunosuppression. Residual immune activation in treated HIV-1 may be contributing to atherosclerosis but there is limited evidence supporting this hypothesis. To the best of my knowledge, the research presented in **Chapter 2** is among the first *in vivo* evidence that immune activation may be a contributing factor to progression of atherosclerosis in HIV-1 infection **(Figure 2.1)**. Given the complexity of the interaction between HIV and the host immune system, there are multiple molecular and cellular mechanisms by which HIV infection may induce immune activation but it is unclear what mechanisms drive immune activation in treated HIV-1 infection (see **Chapter 1** and **section 1.5** for a detailed discussion of this topic).

Dysfunctional HDL may mediate the interplay between HIV-1, immune activation and atherosclerosis. Lipids can regulate atherogenesis (**Chapter 1, section 1.2**), immunity (**Chapter 1, sections 1.2-1.5**) and HIV-1 immunopathogenesis (**Chapter 1, sections 1.3, 1.5**). Oxidized lipids may be a significant mediator in the interplay between inflammation, immune activation and atherosclerosis (**Chapter 1, section 1.5**). While normal HDL counteracts oxidized lipids and

protects against CVD, HDL can become oxidized and dysfunctional in the setting of chronic inflammatory diseases (**Chapter 1, section 1.2**). Thus oxidized (dysfunctional) HDL may have multiple roles in the pathogenesis of HIV-1 infection but it is not known whether HIV-1 infected subjects have dysfunctional HDL.

HIV-1 infected subjects have dysfunctional HDL. To the best of my knowledge, the research presented in **Chapter 3** is the first demonstration that HIV-1 infected subjects have dysfunctional HDL and that use of HDL mimetic peptides *in vitro* can improve HDL dysfunction **(Figure 3.1)**. However, this was a small pilot study and a cell based assay was used to determine HDL function which has significant experimental variability and cannot be used in large human studies (**Chapter 1, section 1.2**). Thus, in **Chapter 4** we developed a novel cell free biochemical assay that measures redox properties of HDL as a tool to study HDL function *in vivo* in the setting of large scale studies in humans **(Figures 4.1-4.6)**. We used this assay to demonstrate that HIV-1 infected subjects have dysfunctional HDL **(Figures 4.7-4.8, 6.7, 7.1)**. While using this tool to measure HDL function in cryopreserved samples, I found that this assay had certain biochemical limitations **(Chapter 5, Figures 5.1)** and I went on to develop another novel cell free assay based on immunoaffinity capture of HDL and enzymatic determination of lipid peroxidation of HDL using a well-established fluorochrome **(Chapter 5, Figures 5.2, 5.3)**. To validate this method, I used this assay to determine HDL function *in vivo* in animal models of atherosclerosis **(Figure 5.4)**, in HIV-1 infected subjects **(Figures 5.5-5.7)** and in uninfected humans **(Figure 5.7-5.9)**. Overall, using 3 different assays of HDL function (one cell based and two biochemical assays) I showed that HIV-1 infected subjects have dysfunctional HDL.

Dysfunctional HDL is associated with immune activation, indices of cardiometabolic risk and atherosclerosis in HIV-1 infected subjects. Dysfunctional HDL has reduced antioxidant properties and high HDL redox activity (HRA), but it is unknown whether abnormal HDL function is associated with immune activation and progression of atherosclerosis in HIV-1-infected subjects. Using the novel biochemical assays of HDL function, in **Chapter 6** we showed that

dysfunctional HDL was independently associated with two different measures of subclinical atherosclerosis, carotid artery intima-media thickness (CIMT) **(Figures 6.2-6.4)** and percent non-calcified coronary plaque **(Table 6.2)**, in HIV-1 infected but not in uninfected controls. We also found that increased HDL redox activity correlated positively to increased levels of biomarkers of macrophage activation **(Figure 6.8)** and predictors of disease progression such as higher T cell activation **(Figure 6.9)** and lower nadir $CD4^+$ T cell count **(Figure 6.10)**. HDL redox activity was also associated with indices of cardiometabolic risk **(Tables 6.1, 6.3-6.5)** such as obesity and insulin resistance.

Dysfunctional HDL can have direct effects on T cells that mediate the interplay between HIV-1, immunity and atherogenesis. In view of our *in vivo* observations that support a role of oxidized HDL in immune activation and CVD associated with HIV-1 infection, I investigated the direct *in vitro* effects of dysfunctional (oxidized) HDL on T cells, which are important for immune activation **(Chapter 1, section 1.6)**, HIV-1 immunopathogenesis **(Chapter 1, section 1.4)** and atherogenesis **(Chapter 1, section 1.3).** We found that both *in vivo* modified dysfunctional HDL from HIV-1 infected subjects **(Figures 7.1, 7.2)** and *in vitro* oxidized HDL **(Figures 7.3-7.7)** directly upregulated markers of T cell activation *in vitro*. In addition oxidized HDL directly increased T cell proliferation **(Figure 7.8)** and production of cytokines **(Figures 7.9, 7.10)**, specific killing of HIV-1 specific cytotoxic $CD8^+$ T cells (CTLs) **(Figures 7.11, 7.12)** and expression of chemokine receptors (CXCR4, CCR5) in $CD4^+$ T cells important for HIV-1 infectivity **(Figure 7.13)**, whereas it reduced antiviral immune responses of HIV-1 specific CTLs **(Figure 7.14)**. Together these results indicate that dysfunctional/oxidized HDL is a novel mechanism of immune activation associated with HIV-1 infection.

Oxidized HDL likely activates T cells through the NFκB pathway. Based on the importance of the NF-kB pathway **(section 1.6)** and scavenger receptors **(section 1.3)** for the effects of cholesterol on immunity and atherogenesis as outlined in **Chapter 1**, I then set out to investigate *in vivo* in HIV-1 infected subjects whether non-oxidized and oxidized lipoproteins

may be independently associated with changes in the receptor activator of NFκB ligand (RANK-L), the soluble product of activation of the NF-kB pathway that is produced by T cells. Our *in vivo* observations supported a role of lipids **(Figure 6.5)** and oxidized HDL **(Figure 6.6)** in the regulation of the NF-kB pathway in HIV-1 infected subjects but not in uninfected controls **(Chapter 6)**. Next, I showed that oxidized HDL can directly upregulate the mRNA expression of RANKL/OPG **(Figure 7.17)** and the mRNA expression of scavenger receptors **(Figure 7.16)** in immune cells of HIV-1 infected subjects **(Chapter 7)**. Further mechanistic studies are needed to elucidate the mechanisms that mediate the direct effects of oxidized HDL on T cells.

Apolipoprotein A-I (apoA-I) peptide mimetics potentially can reverse HDL dysfunction. ApoA-I mimetic peptides have been shown to restore function of dysfunctional HDL, and are promising therapeutic agents for reducing CVD risk **(Chapter 1, section 1.7)**. In Chapter 3 we demonstrated that an ApoA-I mimetic peptide improved HDL function *in vitro* in plasma from HIV-1-infected persons **(Figure 2.1).** In view of those data and the pro-inflammatory effects of oxidized lipids **(Chapter 1)**, we hypothesize that apoA-I mimetics can reverse the effects of oxidized HDL on the leukocytes important for both HIV-1-induced immune activation and atherogenesis.

These findings have great implications for improving morbidity and mortality among treated HIV-1 infected subjects with suppressed viremia. Given that HIV-1-infected persons on suppressive antiretroviral therapy may continue to have significantly elevated immune activation and oxidized HDL, such an approach could reduce the excess morbidity and mortality remaining despite antiretroviral therapy (ART), and directly address the mechanism of immune decline in HIV-1 infection. This work is innovative and may have a potential impact on public health since these findings may initiate further studies to explore the efficacy of novel therapeutic interventions (such as HDL mimetics) to reduce cardiovascular disease (CVD), a leading cause of death in treated HIV-1 infected subjects.

This research sets the basis for further studies that may establish dysfunctional HDL as a mechanistic link between HIV-1, immune activation and atherosclerosis. Although this dissertation does not include experiments that directly investigate effects of dysfunctional HDL on endothelial cells and atherogenesis, the current research has established whether dysfunctional HDL is a key player in atherogenesis in HIV-1 infection by demonstrating that dysfunctional HDL can directly upregulate a) immune activation, that may be independently associated with CVD in HIV-1 infected subjects b) phenomena and mechanisms that have been well established to contribute to the pathogenesis of atherosclerosis in HIV-1 uninfected subjects (such as activation of the NF-kB, upregulation of scavenger receptors and monocyte chemotaxis) **(Chapter 1)**. Future studies that are outlined in **section 8.3** are needed to further elucidate the interplay between oxidized HDL, HIV-1, immunity and atherogenesis.

8.3 Future work

OVERVIEW: The overall goal is to examine possible interactions that link oxidized HDL (HDLox) to immune activation and atherogenesis associated with HIV-1. The research presented in this dissertation supports vicious cycles of HIV-1-induced immune activation, inflammation, production of HDLox, and further immune activation. We will perform a broad screen of possible roles for HDLox. **Aim 1** examines potential effects of HDLox activating cells, while **Aim 2** examines how HDLox could contribute to HIV-1 persistence. **Tables 1 and 2** summarize the phenomena being investigated.

AIM 1: To delineate pathways by which HDLox directly increases systemic immune activation.

Hypotheses: HDLox facilitates immune (T cells, monocytes/macrophages) activation through mechanisms such as upregulation of scavenger receptors, the NF-κB pathway, and/or PAF. HDL mimetics can counteract these processes.

Hypothesized Oxidized HDL Effect	
T Lymphocytes	↑ Activation
	↑ NFKB
	↑ Scavenger Receptors
Monocytes/ Macrophages	↑ Activation
	↑ Stimulation of T Cells
	↑ NFKB
	↑ Scavenger Receptors

Table 1. Hypothesized effects to be tested in Aim 1.

Rationale: The rationale for the future work to try to elucidate the mechanisms that mediate the effects of oxidized HDL on immunity in HIV-1 infected subjects and why apolipoprotein A-I (apoA-I) peptide mimetics potentially can reverse HDL dysfunction is outlined in the current dissertation. In addition, another proposed mechanism that may mediate these effects is the Platelet Activating Factor (PAF) and the lipoprotein-associated phospholipase A2 (Lp-PLA2) pathway.

PAF and lipoprotein-associated phospholipase A2 (Lp-PLA2) may link HIV-1, HDLox, and atherogenesis. PAF is a mediator of inflammation implicated in CVD[397] and in HIV-1

infection.[402,403] It acts via the PAF receptor (PAFR) on T cells and monocytes/macrophages [404] through the same activation pathway as oxidized lipoproteins.[145] The major enzyme that inactivates PAF (Lp-PLA2) is found in both LDL and HDL, is produced by leukocytes[208] and stimulates lymphocyte proliferation.[405] However, it is unknown whether in HIV-1 infection, HDLox (versus normal HDL) upregulates PAF and PAFR, driving inflammation due to oxidized lipids, thus directly contributing to immune activation, inflammation, and CVD.

Approach

⇒ Exposure of T cells and M/M to HDL (ox). Freshly isolated $CD4^+$ and $CD8^+$T cells and monocytes will be isolated from healthy individuals using a negative selection kit. Each cell type will be cultured overnight in serum-free medium with: normal HDL, matched HDLox, and dysfunctional HDL from HIV-1-infected persons at different concentrations (0-50 mg/dL).

⇒ Evaluation of cellular activation in response to HDLox. Using flow cytometry the T cells will be analyzed for viability (7-AAD), CD3, CD4, CD8, HLA DR, CD38 and the monocytes will be analyzed for: viability (7-AAD), CD14, CD16, CD40, HLA DR, CD64, CD163.

⇒ Measurement of cytokines produced by T cells and M/M in response to HDLox. Supernatant from the cell cultures stimulated with different HDLs will be assessed by Luminex cytokine bead arrays using the Human Cytokine Array Kit Panel A (R&D) which provides a broad screen for inflammatory cytokines from T cells (e.g. IFN-γ,TNF,IL-2,IL-17) and M/M (e.g. IL-1, TNF,IL-12).

⇒ Examination of scavenger receptors on T cells and M/M exposed to HDLox. Flow cytometry will be performed on T cells with staining for viability (7-AAD), CD3, CD4, CD8, SR-B1, CD36, and LOX-1, and M/M staining for viability (7-AAD), CD14, CD16, SR-B1, CD36, and LOX-1 as previously.[39]

⇒ Evaluation of the NF-kB axis in T cells and M/M exposed to HDLox. The NF-kB axis will be evaluated through measurement of the key mediator RANKL and protein levels and secretion of RANKL will be assessed via Western Blot and ELISA as previously.[39] If changes are seen as

anticipated, the effects of NF-kB inhibitors will be assessed for impact of this pathway on cellular activation, cytokine profiles, and expression of scavenger receptors.

⇒ Evaluation of the PAF pathway in T cells and M/M exposed to HDLox. The mRNA and protein levels of PAF and its receptor PAFR, as well as enzymatic Lp-PLA2 activity will be assessed in T cells and M/M. If the expected upregulation of this pathway is observed, then the experiments on cellular activation, cytokine secretion, and scavenger receptor expression will be repeated in the presence of PAFR antagonists[406] to explore the role of this pathway for those effects.

⇒ Assessment of proliferative effects of T cells in response to M/M exposed to HDLox. M/M exposed to HDL overnight will be washed and added to autologous HDL-*unexposed* CFSE-labeled T cells to examine their effect on inducing T cell proliferation over the next 3-6 days (CFSE dilution) using flow cytometry and whether HDL causes increased nonspecific stimulation of lymphocyte proliferation by M/M.

⇒ Testing of reversibility of these processes by HDL mimetic peptides. For positive effects noted in the above experiments, the reversibility of those effects will be tested by using HDL mimetic peptides versus sham scrambled peptide at concentrations ranging from 1-10 µg/ml.

AIM 2: To examine effects of HDLox that potentiate HIV-1 replication.

Hypotheses: HDLox directly increases replication of HIV-1 through its effect(s) on $CD4^+$ T lymphocytes and contributes to viral persistence *in vivo* through dysfunction of cellular antiviral immunity.

Hypothesized Oxidized HDL Effect	
HIV-1/Target Cells	↑ CCR5
	↑ PAF Pathway
	↑ HIV-1 Production
CTLs	↓ MIP/RANTES
	↓ Killing
	↓ Antiviral Activity

Table 2. Hypothesized effects tested in Aim 2.

Rationale:

⇒ HIV-1 replication in $CD4^+$ T cells depends on activation, and HDLox is associated with inappropriate T cell activation. As outlined in this dissertation, oxidized lipids are believed to drive T cell activation. Cellular infection generally requires CCR5 expression, which depends on activation. Furthermore, oxidized lipids can increase CXCR4 on T cells, [107,114,391] although it is unclear whether CCR5 is also upregulated.[107] Viral replication requires activation-induced transcription factors and thus increased activation would favor enhanced HIV-1 replication.

⇒ PAF and Lp-PLA2 can mediate activation of T cells. As discussed in Aim 1, PAF acts through PAFR on T cells and M/M to drive activation, while Lp-PLA2 in LDL or HDL can augment the inflammatory effects of oxidized lipids and drive lymphocyte proliferation, again expected to augment viral replication.

⇒ The life cycle of HIV-1 heavily depends on lipid interactions **(Chapter 1)**[109,110] Normal HDL may have an antiviral role.[120,121] Oxidized lipoproteins like ox-LDL may directly modify lipid rafts.[130] Viral cholesterol-binding proteins such as HIV-1 Nef mobilize lipid rafts in macrophages through a pathway that competes with cholesterol efflux, a major function of HDL,[122] and lipid rafts are a major determinant of HIV-1 antiviral responses.[123-129]The effect of HDL oxidation on this process is unknown, but lipid oxidation decreases the stability, alters the orientation of apoA-I on HDL particle[131] and thus could interfere with the normal antiviral activity of HDL.

Experimental Plans:

⇒ Primary $CD4^+$ T cells, M/M, HIV-1-specific CTLs. Primary $CD4^+$ T cells, monocytes, HDL (ox) will be purified as in Aim 1. The Yang laboratory has over 300 cryopreserved HIV-1-specific CTL clones accumulated over 15 years of studying HIV-1 escape from CTLs.

⇒ HIV-1 strains. Initial experiments will be performed using JR-CSF (R5) and NL4-3.1 (X4) clones. Positive results will be generalized using primary HIV-1 swarms.[407]

⇒ Effect of HDLox on viral replication in $CD4^+$ T cells and M/M. These target cells will be cultured with HDL as in Aim 1 overnight, and then infected at low multiplicity with HIV-1. If viral replication is not observed, the $CD4^+$ T cells will be stimulated additionally with anti-CD3 antibody and the monocytes will be plate adhered and stimulated with M-CSF[408] during exposure to HDL. Viral replication will be monitored over 7 days.

⇒ Evaluation of effects of HDLox on HIV-1 target cells and viral replication. If upregulation of HIV-1 replication is seen (R5 JR-CSF and/or X4 NL4-3.1), CCR5 and CXCR4 will be assessed on the target cells by flow cytometry. If Aim 1 demonstrates upregulation of the PAF axis, the effect of PAFR antagonists on viral replication in the presence and absence of HDLox will be tested to examine the role of this pathway. If our studies demonstrate that HDLox may upregulate expression of the chemokines CXCR4 and CCR5 in macrophages and T cells, susceptibility of HIV-1 replication to maraviroc and AMD3100 serial titrations (to determine the 90% inhibitory concentration, IC_{90}) will be determined to further evaluate/confirm infection enhancement by HDLox via increase of chemokine receptors, which would cause increased IC_{90}. This approach may lead to further studies whether HIV-1 entry inhibitors in combination with HDL mimetic peptides can be used as therapies for immune activation associated with HIV-1 infection.

⇒ Examination of the effect of HDLox on virions. High titer HIV-1 stocks will be exposed to HDL for an hour, and then diluted to concentrations that do not affect target cells, and these stocks will be used to infect primary $CD4^+$ T cells or immortalized cells (T1, H9) to evaluate for an effect of HDL on virions rather than target cells. The effects of normal versus HDLox on virus replication and on the life cycle of HIV-1 will be determined using a p24 ELISA and a reporter virus, respectively, as described **(Figure 8.1)**.[400,409,410] This will evaluate whether normal HDL has a direct antiviral effect, and whether HDLox loses efficacy and can increase HIV-1 infectivity through changes in stages of the viral life cycle that are dependent on lipids (such as entry).

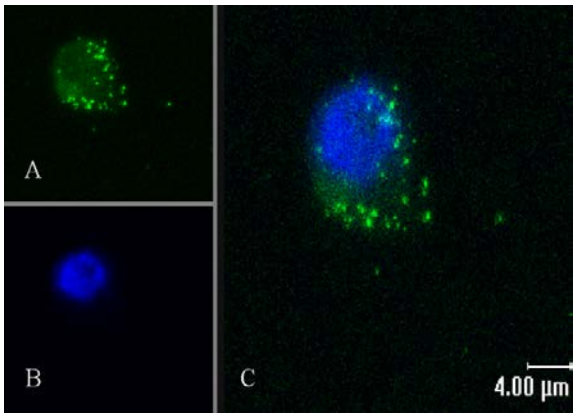

Figure 8.1: (A) To track entry of HIV-1 into the cytoplasm of CD4+ T cells using confocal microscopy, we have previously tagged virions by incorporation of HIV Vpr fused to the GFP (green) (B) Counterstain with DAPI stain (C) Merged picture.

⇒ Evaluation of CTL suppression of HIV-1 replication in the presence of HDLox. CTL virus suppression assays will be performed as described in Chapter 7. Immortalized target cells (which are maximally activated) will be utilized, to reduce the potentially confounding effects of HDL on HIV-1 replication via the target cell activation effects being explored above. In addition, specific killing of HIV-1 specific CTLs in the presence of HDL (ox) will be determined with standard chromium release assays as described in Chapter 7. The killing activity of both primary $CD8^+$ T cells from HIV-1 infected subjects and HIV-1 specific CTL clones will be determined by measurement of granzyme and perforin expression as in Chapter 7.

⇒ Evaluation of production of HIV-suppressive factors by $CD8^+$ T cells in the presence of HDLox. Primary $CD8^+$ T cells from HIV-1 infected subjects and HIV-1 specific CTL clones will be incubated with HDL(ox) as above and Luminex bead arrays using the Cytokine Array Kit Panel A (R&D), will be used to measure HIV-suppressive factors (MIP 1a, MIP 1b, RANTES) in cell cultures supernatants. This will determine whether HDLox reduces production of these factors by $CD8^+$ T cells and thus antiviral responses.

⇒ Evaluation of effects of HDLox on lipid rafts of macrophages and T cells. We will focus our experiments on determining the effects of HDLox on lipid raft-promoting capacities of viral cholesterol-binding proteins such as Nef in natural target cells of HIV-1 (macrophages, $CD4^+$ T cells) that may increase HIV-1 infectivity.[106,114,116,118,119]

⇒ Test of reversibility of the impact of HDLox using HDL mimetic peptides. This will be performed as in Aim 1.

Interpretation of Results/Statistical Analysis: The data will test our hypotheses regarding pathways of immune activation due to HDLox and directly address the hypotheses regarding HDLox effects on increasing HIV-1 replication through effects on target cells, as well as reducing efficacy of antiviral CTLs. These effects will be correlated (using Spearman's correlation and linear regression models) to the other parameters such as markers of immune activation in Aim 1. Student's t test or the Wilcoxon rank sum test will be used to compare the effects of normal versus HDLox.

8.4 Concluding Remarks

Since the start of the epidemic, more than 60 million people have been infected with HIV and nearly 25 million adults and children have died of AIDS, making it one of the deadliest epidemics in history. As such, the need to reduce morbidity and mortality associated with HIV infection has never been more urgent. Cardiovascular disease is becoming a major cause of death in persons with HIV infection since the advent of treatment regimens that halt and reverse the immunodeficiency associated with HIV. Chronic activation of the immune system in HIV infection, particularly due to viral persistence that provokes ineffective cellular immunity may drive both disease progression and accelerated cardiovascular disease (CVD) in persons infected by HIV. The mechanisms that connect inflammation, CVD and activation of the immune system in HIV infection remain to be elucidated. A deeper understanding of the specific mechanisms causing chronic T-cell immune activation is crucial to targeting immune activation in HIV-infected individuals in a specific and effective way. Even if we will be unsuccessful in eradicating or substantially reducing HIV reservoirs, the design of novel therapeutic interventions that might improve the recovery of a competent immune system and reduce activation/inflammation-induced cardiovascular disease would be of major benefit to people living with HIV.

The *in vivo* and *in vitro* studies presented in this dissertation provide evidence that oxidized HDL may be a link between HIV, activation of the immune system and CVD. Our preliminary *in vitro* data also demonstrate that the effects of abnormal (oxidized) HDL can be reversed experimentally with administration of a drug that can mimic the function of normal HDL (HDL mimetic). Demonstrating mechanisms that are associated with CVD in patients with HIV infection may initiate further studies to explore the efficacy of novel therapeutic interventions such as HDL mimetic peptides that may reduce the CVD and immune activation associated with HIV-1 infection and might improve the prognosis of HIV infected patients.

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