TESIS DOCTORAL

IL-12/IL-18 and M-CSF/GM-CSF trigger two new pathways of pro-oxidants enzymes up-regulation on macrophages. An increase in viral load during treatment interruptions induces a burst of factors implicated in cardiovascular diseases.

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Memoria de la tesis presentada para obtener el grado de Doctor en Inmunología por la Universidad Autónoma de Barcelona

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Certifica:

Que el trabajo experimental y la redacción de la memoria de la Tesis Doctoral titulada **"IL-12/IL-18 and M-CSF/GM-CSF trigger two new pathways of pro-oxidants enzymes up-regulation on macrophages. An increase in viral load during treatment interruptions induces a burst of factors implicated in cardiovascular diseases"** han sido realizados por Ferdinand Noukwe Noukwe debajo de su dirección y considera que es apto para ser presentado para optar al grado de Doctor en Inmunología por la Universidad Autónoma de Barcelona.

Y para que quede constancia, firmo este documento a Badalona, 30 de Mayo de 2011.

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Certifica:

Que el trabajo experimental y la redacción de la memoria de la Tesis Doctoral titulada **"IL-12/IL-18 and M-CSF/GM-CSF trigger two new pathways of pro-oxidants enzymes up-regulation on macrophages. An increase in viral load during treatment interruptions induces a burst of factors implicated in cardiovascular diseases"** han sido realizados por Ferdinand Noukwe Noukwe debajo de su dirección y considera que es apto para ser presentado para optar al grado de Doctor en Inmunología por la Universidad Autónoma de Barcelona.

Y para que quede constancia, firmo este documento a Bellaterra, 30 de Mayo de 2011.

Dra. Dolores Jaraquemada Pérez de Guzmán

A mes parents

A ma bien aimé Laure

A Alicia, Francesca et mes autres enfants

Abbreviations

LDL-c	= Low density lipoprotein cholesterol
HDL-c	= High density lipoprotein cholesterol
oxLDL	= Oxidized cholesterol
TG	= Triglycerides
CVD	= Cardiovascular disease
CAD	= Coronary artery disease
ROS	= Reactive oxygen species
H2O2	= Hydrogen peroxide
HOCl	= Hypochlorous acid
O_2^-	= anion superoxide / superoxide radical,
PKC	= Protein kinase C
MPO	= Myeloperoxidase
NADPH	= Nicotinamide adenine dinucleotide phosphate
M-CSF	= Macrophage colony-stimulating factor
GM-CSF	= Granulocyte macrophage colony-stimulating factor
IFN-γ	= Interferon gamma
IL	= Interleukin
MMP-9	= Matrix metalloproteinases-9
ECs	= Endothelial cells

SMCs	= Smooth muscle cells
NK cells	= Natural killer cells
MDM	= Monocytes-derived macrophages
KRPG	= Krebs-Ringer phosphate buffer with glucose
PBS	= Phosphate buffer saline solution
TNB	= 1,3,5-trinitrobenzène
DTNB	= 5,5 '-dithiobis(2-nitrobenzoic acid)
PMA	= *phorbol myristate acetate*
CRP	= C-reactive protein
HIV	= Human immunodeficiency virus
ART	= Antiretroviral therapy
HAART	= High active antiretroviral therapy
STI	= Schedule treatment interruption
pVL	= plasma viral load
s-VCAM-1	= soluble cell adhesion molecule-1
MCP-1	= monocyte chemoattractive protein-1
sCD40L	= soluble CD40 ligand
CD	= Cluster of differentiation
PBMCs	= Peripheral mononuclear cells
M	= Median
IQR	= Interquartile range

Contents

CHAPTER II: An increase in viral load during treatment interruptions induces a burst of factors implicated in cardiovascular diseases.

Abstract

We organized the thesis into two chapters:

Chapter I:

To evaluate the synergic effect of IL-12/IL-18 and M-CSF/GM-CSF on monocytes differentiation and their impact on the respiratory burst and cholesterol metabolism of monocytes-derived macrophages, monocytes were differentiated for 7 days in the presence of both granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) or Interleukin-12 (IL-12) and IL-18 to produce respectively M/GM-Φ and IL12/IL18-Φ. As control, monocytes were differentiated only with M-CSF, GM-CSF, IL-12 and IL-18 to produce respectively M-Φ, GM-Φ**,** IL12-Φ and IL18-Φ. Samples analyses of four monocytes donors demonstrated a differential in the cholesterol metabolism and respiratory burst of macrophage subpopulations. Stimulated M/GM-Φ and IL12/IL18-Φ produce high level of H2O2 and myeloperoxidase; and generate significant amount of HOCl in response to PMA. In contrast, they show low levels of anti-oxidant enzyme catalase. Moreover they intensify LDL oxidation and spontaneously accumulate significant amount of cholesterol when incubated with unmodified low-density lipoprotein.

The results suggest that M-CSF/GM-CSF or IL12/IL18 pathways might be some critical synergic signals experienced by monocytes/macrophages during their differentiation *in-vivo*; in which their atherogenic potential or their capacity to oxidize LDL increase.

Chapter II:

Recent studies show that HIV-1 load rebound after long periods of treatment interruptions (TI), results in a burst of coronary artery disease (CAD) biomarkers. We investigate whether short interruptions induce a burst of these biomarkers**,** whether their levels return to the baseline during treatment resumption and if the burst were related to the number of interruptions. CAD biomarkers CRP, CXCL8, D-dimer, MMP-9 and plasma lipids were measured from stored plasma samples of 21 chronically HIV-1 infected subjects enrolled in a study evaluating six cycles of “2 weeks off” / “4 weeks on” antiretroviral therapy. Subjects were clustered into those with a viral load rebound after stopping treatment and those without.

The levels of CRP, MMP-9, CXCL8, D-dimer and triglycerides rose significantly after each TI in subjects with viral load rebound. Changes of means increment in subjects without viral load rebound were too low relative to the baseline and without clinical interest as values stayed between the normal plasma ranges. No times effect was observed during TI except for CRP. All biomarkers return to baseline levels after each treatment resumption.

The results suggest that antiretroviral TI as short as two weeks are associated with a clinically relevant burst of acute CAD biomarkers, that indicating the importance of adhering to treatment.

CHAPTER I

IL-12/IL18 and MCSF/GMCSF trigger two new pathways of pro-oxidant enzymes up-regulation on macrophages.

1 - Introduction

Cardiovascular disease (CVD), particularly atherosclerotic vascular disease, is a leading cause of global mortality with an estimated 17.5 million CVD deaths in 2005, accounting for 30% of all global deaths (WHO). Now widely recognized, inflammatory mechanisms play a vital role in the initiation, maintenance and progression of vascular disease with a strong correlation between inflammatory markers and prognosis in acute and chronic coronary artery disease.

1.1- Inflammation

Is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. The external agent is recognized by resident dendritic cells and macrophages in tissues, induces the local production of quimiocines that facilitate the arrival of others immune cells, leading to tissues infiltration and vascularization.

In addition to the induction of quimiocines, activation of macrophages induces the production of inflammatory cytokines, free radicals and pro-oxidants enzymes [1, 2]. Prolonged inflammation known as *chronic inflammation*, leads to a progressive release of reactive oxygen intermediates at the site of inflammation and is responsible for tissue damage [3]. This type of inflammation leads to a host of diseases, such as rheumatoid arthritis and atherosclerosis [2, 4]. Atherosclerosis is induces by metabolic abnormalities such as increased levels of total and low density lipoprotein (LDL)-cholesterol [5].

1.2. Inflammation and Oxidative Stress

The inflammation and oxidative stress contribute to the pathogenesis of many human diseases including atherosclerosis [6]. The inflammatory process is often associated with free radical damage and oxidative stress which, in some cases, will lead to proteins oxidation; the main process of atherosclerosis [2, 3, 7]. Protein oxidation is

the covalent modification of a protein induced by reactive oxygen intermediates or by-products of oxidative stress [8, 9].

Oxidative stress is a state in which the intracellular oxido-reduction homeostasis of a cell is altered, which occurs because of excessive production of reactive oxygen species (ROS) and/or by reduction in the antioxidants mechanisms, leading to cell damage [3, 10, 11]. ROS include anion superoxide ($O2^{-}$), hydrogen peroxide (H2O2) and hydroxyl radical (•OH) [12-15] (FIG.1).

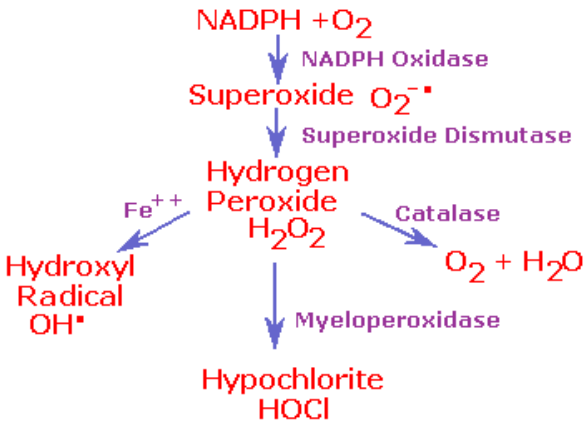

FIG.1. Schematic representation of Oxygen Radicals generation in activated phagocytes.

Cellular use of oxygen generated free radicals permanently. These chemical species are highly unstable and reactive, characterized by the presence of an unpaired electron in the final orbit; and their high reactivity is associated with the need to take electrons to achieve their balance; and they can react with any chemical structure that is within their reach [10]. Usually these structures use by free radical for electronic delivery are macromolecules such as proteins, lipids and even DNA.

A part of oxygen reaching mitochondria can escape complete reduction and therefore, form free oxygen radical. The first is a highly reactive structure called anion superoxide. High production of this anion leads to the activation of some phagocytes (polymorphonuclear cells, monocytes and macrophages). In this case the molecule superoxide becomes H2O2 by the action of the enzyme superoxide dismutase; H2O2 oxidizes chloride to anion hypochlorite by the action of the enzyme myeloperoxidase. These compounds of oxygen, hydrogen peroxide and anion

hypochlorite are strong oxidizing agents [7].

1.3- Enzymes involved in inflammation.

1.3.1- Pro-oxidants enzymes involved in the inflammation: the enzymes of respiratory burst.

1.3.1.1- NADPH oxidase

Granulocytes and macrophages possess an enzyme system of membrane called NADPH oxidase that can produce the radical superoxide from NADPH and oxygen in response to an appropriate stimulus. In fact, it's a phagolysosome membrane enzyme that takes an electron from NADPH and transfers it to O_2, forming the superoxide radical, O_2^-**.** The induction of this enzyme is controlled by the protein kinase C (PKC), enzyme that when activated in vitro by the PMA gives rise to the formation of ROS [2, 15-18]. The superoxide radical is only moderately reactive. However, it is soon converted to hydrogen peroxide (H2O2) by the enzyme superoxide dismutase (FIG.1, and FIG.2).

$$2NADPH + 2O_2 \rightarrow 2NADP^+ + 2O_2^{\bullet -} + 2H^+$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

FIG.2. Simplified Scheme of the catalytic process

1.3.1.2- Myeloperoxidase

Another cell-derived factor that may participate in phagocyte dependent oxidation of LDL is myeloperoxidase (MPO). MPO is an abundant heme protein released by activated neutrophils and monocytes and present in some tissue macrophages such as those in vascular lesions [19-21]. MPO may play a role in monocyte-macrophage oxidation of LDL by a variety of distinct pathways. MPO can act to amplify the

oxidizing potential of H2O2, the dismutation product of O2 by using it as a co-substrate to generate a variety of oxidants, including diffusible radical species, reactive halogens, aldehydes, and nitrating agents[22, 23]. The heme group of MPO is buried deep within a hydrophobic binding pocket and catalyzes the oxidation of a variety of small substrates that then can diffuse away from the enzyme and damage cellular targets. Thus, MPO-mediated oxidation reactions occur in the absence of free transition metal ions and are resistant to inhibition by chelators. Because of its high concentration in biological matrices, chloride is regarded as a major substrate for MPO. MPO catalyzes the two-electron oxidation of chloride forming the powerful oxidant, hypochlorous acid (HOCl) (Eq.1).

Eq.1) $H_2O_2 + Cl^- \xrightarrow{MPO} ClO^- + H_2O$

Exposure of LDL to reagent HOCl results in chlorination and oxidation of protein and lipid constituents of LDL, induces LDL aggregation, and converts the lipoprotein into a high uptake form for macrophages. MPO-generated HOCl also oxidizes free amino acids, abundant nutrients in plasma and extracellular fluids, converting them into aldehydes. MPO-generated aldehydes can then modify nucleophilic targets on LDL protein and lipids. In addition, MPO can catalyze the one-electron oxidation of L-tyrosine, generating the tyrosyl radical [24, 25] (FIG.3). PMA-activated phagocytes can produce tyrosyl radical and initiate LDL lipid peroxidation and dityrosine cross-linking of proteins [21, 26-30].

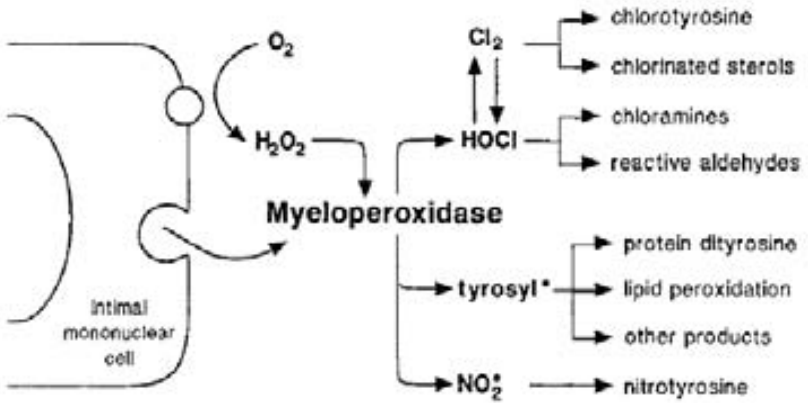

FIG.3. Model of potential pathways available to macrophages for generation of MPO-derived oxidants.

1.3.2- Antioxidants enzymes

Cells throughout the body clearly need protection from the molecules described above. Most of these cells have superoxide dismutase and catalase, which converts the hydrogen peroxide to oxygen and water (FIG.1). In other words, the combination of superoxide dismutase and catalase removes oxygen radicals and protects cells of the body [13, 31].

1.4- Atherosclerosis

1.4.1- Definition of atherosclerosis

The term arteriosclerosis generally refers to the thickening and hardening of the artery wall. It progresses with a gradual build-up of plaque or thickening of the inside of the artery walls, which causes a decrease in the amount of blood flow, and a decrease in the oxygen supply to the vital body organs and extremities [6].

Classically, the lesions of atherosclerosis are listed in order of appearance in fatty streak, fibrous plaques and complicated lesions [32-34] (FIG.4).

The fatty streak mainly consists of foamy appearing macrophage cells, sometimes with some additional T lymphocytes, aggregated platelets and smooth muscle cells in localized regions of the intima. Fatty streaks may be precursor of atheromas and not all fatty streaks are destined to become fibrous plaques. This type of lesion appears at any level of the arterial tree and to all ages, the aorta being the first affected from childhood. The fibrous plaques are areas of thickening of the intima consisting of a core of extracellular lipids and necrotic cell debris, covered by a fibromuscular layer. These plaques appear at first of the abdominal aorta, coronary arteries and carotid arteries between 20 and 30 years and their number increases gradually with age. Finally, the complicated lesion is a calcified fibrous plaque, which contains, in varying degrees, necrosis, thrombosis and ulceration. These lesions are frequently associated with various symptoms [34]. Necrosis can cause rupture of the intima responsible for an

aneurysm or hemorrhage. An arterial embolism may occur when pieces of plaque break off and pass into the lumen. Stenosis and a functional disorder in an organ can occur as a result of gradual occlusion of an artery due to thickening of the plaques and the formation of clots.

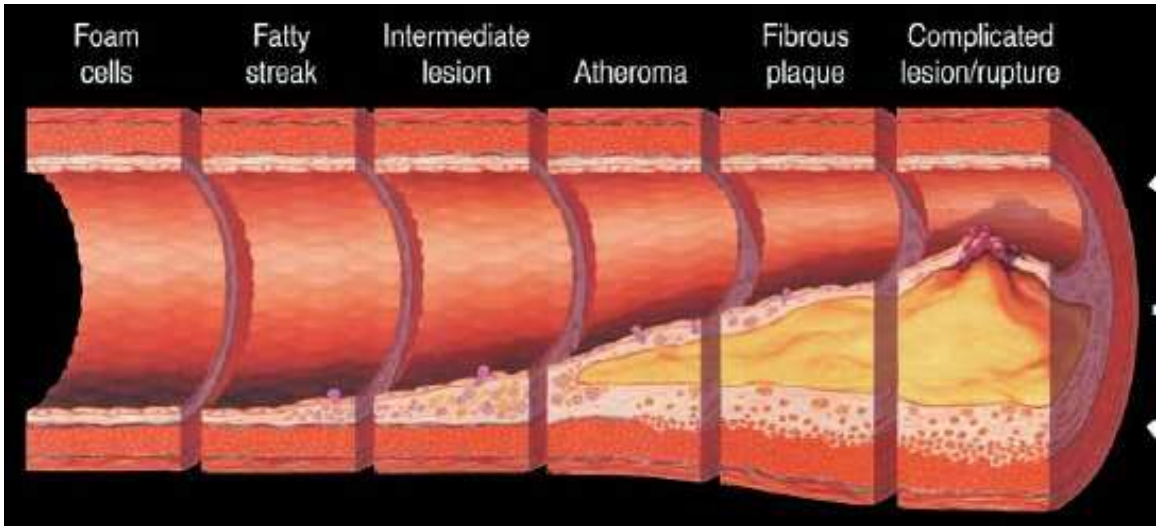

FIG.4. Order of appearance of atherosclerosis lesions. (*Arterioscler Thromb Vasc Biol*. 2007;27:15-26.)

A protective fibrous cap normally forms between the fatty deposits and the artery lining (the intima). These capped fatty deposits (called atheromas) produce enzymes which cause the artery to enlarge over time. As long as the artery enlarges sufficiently to compensate for the extra thickness of the atheroma, then narrowing or stenosis of the opening lumen occurs. The artery becomes expanded and egg shaped, still with a circular opening. If the enlargement of the atheroma thickness is beyond the proportion of the artery, then an aneurysm is created [32, 35].

The muscular portion of artery walls usually remains strong, even after they have remodeled to compensate for the atheromatous plaques. However, atheromas within the vessel wall are soft and fragile with little elasticity. Arteries constantly expand and contract with each heartbeat. In addition, the calcification deposits between the outer

portion of the atheroma and the muscular wall, as they progress, lead to a loss of elasticity and stiffening of the artery [35-37].

If the fibrous cap separating a soft atheroma from the bloodstream within the artery ruptures, atheroma tissue fragments are exposed and released. Atheroma tissue fragments are very clot promoting; they attract blood platelet accumulation and activate the blood clotting system proteins. This leads to a temporary patch covering and narrowing (stenosis) within the artery lumen. Though this is often a repetitive and progressive process over time, it is usually asymptomatic until a severe enough events occurs in a critical enough area [37].

Fibrous cap ruptures usually result in only a partial narrowing, stenosis, of the artery lumen, a narrowing which usually partially re-opens with healing and regrowth of the intimal lining. However, sometimes the combination of atheroma material release, bleeding into the atheroma bed, platelet accumulation and accumulation of blood clotting proteins suddenly builds to the point of creating a complete, or near complete obstruction [35]. The obstruction, either at the site of rupture, or as a result of debris sent downstream, prevents adequate blood flow to cells downstream. Cells starved for adequate blood supply are injured and may die [37].

1.4.2- Macrophages and the development of atherosclerosis.

Atherosclerosis is characterized by intimal accumulation of lipids, mainly cholesterol and cholesterol esters, and the infiltration of inflammatory cells, particularly macrophages and T cells, in addition to migration and proliferation of medial smooth-muscle cells. Macrophages are the most prominent cell type in atherosclerotic lesions and are associated with two hallmarks of the disease; lipid deposition and inflammation [6, 32, 38]. Recruitment of circulating monocytes to the subendothelial space is one of the earliest events in atherogenesis. Within tissues, monocytes differentiate into macrophages. The macrophages ingest oxidized cholesterol, slowly turning into large "foam cells" – so described because of the appearance numerous vesicles take on to accommodate their high lipid content. The early stages are called fatty streaks. Foam cells eventually die, and further propagate the inflammatory process. Intracellular microcalcification deposits form within vascular smooth muscle cells of the surrounding

muscular layer, specifically in the muscle cells adjacent to the atheromas. In time, as cells die, this leads to extracellular calcium deposits between the muscular wall and outer portion of the atheromatous plaques. Connective and elastic tissue materials also accumulate there, as may cell debris, cholesterol crystals, and calcium. This accumulation of fat-laden cells, smooth muscle cells, and other materials forms a patchy deposit called an atheroma or atherosclerotic plaque [32].

Cholesterol is delivered into cell by LDL particles [39]. To attract and stimulate macrophages, LDL must be in the oxidized form, a key step in the ongoing inflammatory process. Additionally, macrophages must be able to remove excess cholesterol by their functioning HDL particles (high density lipoprotein) to avoid becoming foam cells and dying [5, 40]. To date, the only known mechanism by which macrophages can export excess lipid is into HDL particles [41].

LDL receptor is the primary receptor for binding and internalization of plasma-derived LDL cholesterol and regulates plasma LDL concentration [42]. LDL receptor activity is normally under tight metabolic control via a feedback system that is dependent on intracellular cholesterol concentration [40, 41, 43, 44]. This system maintains a constant level of cholesterol in hepatocytes and other cells by controlling both the rate of cholesterol uptake from LDL via LDL receptor and the rate of de novo cholesterol synthesis [5, 45, 46].

Oxidative modification of low density lipoprotein (LDL) plays a major role in the pathogenesis of atherosclerosis [47-51]. It is well-known that the macrophage scavenger receptors play an important role in the internalization of chemically modified lipoproteins, such as oxidized low-density lipoprotein (oxLDL) by macrophages, leading to the transformation of macrophages into lipid-laden foam cells [5, 52-56]. Subsequently, LDL is oxidatively modified by free radicals that are either secreted from cells within lesions or generated extracellular in the arterial wall [57]. Oxidatively modified LDL (oxLDL) induces a multitude of cellular responses which lead to vascular dysfunction [30, 58].

1.5- Macrophages subpopulations and inflammatory profile

The inflammatory profile of each macrophages subpopulation depends on the type of cytokine present in the environment where the monocytes were differentiated into macrophages. Macrophages have been identified as heterogeneous cells with a variety of physiological and pathophysiological functions. Investigators have hypothesized that monocytes will differentiate into specific macrophage subpopulations in response to alternative cytokine environments [59]. Because of this, human monocytes entering the vessel wall may be capable of differentiating into alternative lineage-determined macrophage subpopulations in response to the local cytokine environment.

However, macrophages that have acquired a proinflammatory profile at the end of their differentiation will in parallel present a respiratory burst more effective than the cells that have acquired an anti-inflammatory profile [59, 60]. In this study, the cytokines use to differentiate the monocytes to monocytes-derived macrophages were:

1.5.1-M-CSF

The main function of macrophage colony-stimulating factor (M-CSF) is to ensure the survival and differentiation of mononuclear phagocytes including among others monoblasts, promonocytes, monocytes, macrophages and osteoclasts. MCSF also increases the cytotoxicity, phagocytosis, chemotaxis and cytokine production in monocytes and macrophages [61, 62]. The M-CSF main producing cells are monocytes and macrophages but it is also expressed by vascular endothelium and smooth muscle cells. In contrast to others macrophages subpopulation, M-CSF macrophages are elongated and spindle shaped; they present enhance expression of the proinflammatory marker CD14 [59] and produce high level of H_2O_2 [60, 63].

1.5.2-GM-CSF

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a differentiation factor whose main function is the stimulation of progenitor cells of granulocytes and

macrophages for their survival, proliferation and differentiation [60, 62, 64]. Its main functions on monocytes include the increased of cell survival, adhesion and induction of MHC-II expression. GM-CSF is produced by different cell types such as fibroblasts, epithelial, endothelial, stromal and monocytes. Macrophages and intimal smooth muscle cells also secrete GM-CSF within atherosclerotic lesions [65]. However, some studies indicate that monocytes differentiated with GM-CSF have High Basal and inducible levels of catalase Activity [63] and therefore associated with the antioxidant system.

1.5.3-IL-12

Is a potent regulator of cell mediated immune responses and it induces IFN-γ production by NK and T cells. It is produced by activated monocytes/macrophage cells, neutrophils, B lymphocytes and connective tissue type mast cells. Among its biological activities IL-12 promotes the growth and activity of activated NK, CD4+ and CD8+ cells and induces the development of IFN-γ producing Th1 cells [66].

1.5.4-IL-18

IL-18, a pleiotropic proinflammatory cytokine, is produce by endothelial cells and activated macrophages. As suggested by its name, IL-18/IGIF (interferon-gamma inducing factor) was initially recognized as a potent inducer of IFN-gamma production by T-cells [60, 67, 68] and NK cells [69]. In synergy with IL-12, the effects of IL-18, through its induction of IFN-gamma, can lead to a rapid activation of the monocyte/macrophage system with an upregulation of these cell's innate immune capabilities [70, 71]. Furthermore, IL-18 not only promotes IFN-gamma synthesis, but also likely participates in its overall activities [67].

Beyond induction of IFN-γ with subsequent promotion of Th1 immune response, IL-18 enhances the expression of matrix metalloproteases (MMPs) and these two abilities of IL-18 characterize it as a crucial and potent mediator of atherosclerotic plaque destabilization and vulnerability. Increased expression of IL-18 in human atherosclerotic plaque has been shown, especially in lesions prone to rupture, where it is localized mainly in plaque macrophages [32, 72].

1.5.5-IL-12/IL-18

The combination of interleukins IL-12/IL-18 leads to the differentiation of monocytes into macrophages. These two cytokines act synergistically on monocytes/ macrophages system by increasing their survival, their activation and they induce the production of inflammatory cytokines IFN-γ and CXCL8 once the monocytes differentiated into macrophages [73]. Furthermore, monocytes differentiated with the combination of IL-12 and IL-18 give rise to aggregated macrophages like those observed in atherosclerotic plaques.

1.6- Inflammatory cytokines and markers of coronary artery disease.

As inflammatory markers implicated in CAD, in our study we analyze:

1.6.1-IL-6

Is a 26-kDa single chain glycoprotein, produced by many cell types including activated monocytes/macrophages and endothelial cells, as well as by adipose tissue. IL-6 is able to stimulate macrophages to secrete MCP-1 and participates in the proliferation of SMCs. In addition, ECs stimulated by IL-6, express intercellular adhesion molecule-1
(ICAM-1). IL-6 also promotes production of hepatic acute-phase reactants, including CRP [60]. IL-6 is expressed at the shoulder region of atherosclerotic plaques and may increase plaque instability by driving expression of matrix metalloproteinases, MCP-1, and tumor necrosis factor (TNF)-α [72]. Furthermore, IL-6 represents the principal procoagulant cytokine, but its most important function is the amplification of the inflammatory cascade through which IL-6 at least in part might exerts its direct proatherogenic effects in the arterial wall. Indeed, large amounts of IL-6 have been found in human atherosclerotic plaque[74].

1.6.2-IL-8/CXCL8

Is a proinflammatory CXC chemokine of 8.4 kDa that can signal through the CXCR1 and CXCR2 receptors. It is secreted by monocytes, macrophages and endothelial cells. IL-8 has been implicated in a number of inflammatory diseases including atherosclerosis. IL-8 stimulates HIV-1 replication, attracts neutrophils and T cells, stimulates the adhesion of monocytes, and contributes to angiogenesis [75].

1.6.3-IL-10

Unlike the previous cytokines, interleukin-10 (IL-10) is an anti-inflammatory cytokines of 18.6 kDa secreted by activated monocytes/macrophages and lymphocytes. It has multifaceted anti-inflammatory properties including inhibition of the prototypic proinflammatory transcription factor nuclear-factor-kB, leading to suppressed cytokine production, inhibition of matrix-degrading metalloproteinases, reduction of tissue factor expression, inhibition of apoptosis of macrophages and monocytes following infection, and promotion of the phenotypic switch of lymphocytes into the Th2 phenotype. All of these inflammatory mechanisms play a pivotal role for atherosclerotic lesion development and progression, suggesting a potential regulatory role of IL-10 [60, 74].

1.6.4-Matrix Metalloproteinases-9

Matrix metalloproteinases-9 (MMP-9) belongs to a family of multidomain zinc-dependent endopeptidases that promote degradation of all protein and proteoglycan-core-protein components of the extracellular matrix. MMP-9 is widely expressed in monocytes/ macrophages, ECs and SMCs and fibroblasts. MMPs are involved in the embryonic development and morphogenesis, wound healing and tissue resorption. On the other hand, MMP-9 might be implicated in vascular and cardiac remodeling as a result of dysregulated activation of these enzymes. Recently, several lines of evidences have demonstrated that MMP-9 play an important role in atherogenesis [32, 76]. Most importantly, MMP-9 is highly expressed in macrophage-rich areas of the atherosclerotic

plaque, especially at the shoulder region of the cap, which might promote weakening of the fibrous cap and subsequent destabilization of atherosclerotic lesions.

2 - Hypothesis

M-CSF and GM-CSF are two factors of differentiation of monocytes. IL-12 and IL-18 together but not alone, induce monocytes differentiation and the production of inflammatory cytokines. M-CSF is expressed by vascular endothelium, smooth muscle cells and macrophages; and intimal smooth muscle cells also secrete GM-CSF. IL-12 and IL-18 are both produced by activated macrophages; granulocytes are also a source of IL-12 while endothelial cells also produce IL-18.

We hypothesized that: the autocrine and/or paracrine action of both IL-12/IL18 or M-CSF/GM-CSF on monocytes and macrophages may trigger the respiratory burst and contribute to the atherosclerotic process.

3- Objectives

The objectives of this study were to:

1. Determine the capacity of IL-12/IL-18 and M-CSF/GM-CSF to induce monocytes differentiation based on changes in cells morphology and distribution.

2. Assess the synergic effect of IL-12/IL-18 and M-CSF/GM-CSF on the respiratory burst of macrophages by:
   - 2.1 quantifying the activity of antioxidant enzyme catalase
   - 2.2 quantifying the release of the pro-oxidant product H2O2
   - 2.3 quantifying the activity of pro-oxidant enzyme myeloperoxidase (MPO)

3. Assess the atherogenic potential of monocytes differentiated with IL-12/IL-18 or M-CSF/GM-CSF based on:
   - 3.1 the ability to produce oxidants (HOCl)
   - 3.2 the ability of activated macrophages to oxidized LDL-C

4. Determine the cholesterol accumulation potential of monocytes differentiated with IL-12/IL-18 or M-CSF/GM-CSF by quantifying the intracellular accumulation of cholesterol

5. Assess the regulatory properties of IL-10 in the atherogenesis by analyzing:
   - 5.1 the protecting role of IL-10 in the inhibition of foam cells formation
   - 5.2 the induction of CAD biomarkers release by oxLDL

4-Material and methods

4.1- Monocytes isolation and differentiation

4.1.1 Monocytes isolation from peripheral blood.

Peripheral blood was obtained from healthy donors of the Transfusion Centre and Tissues Bank. Mononuclear cells of peripheral blood or PBMC were obtained by the Ficoll gradient centrifugation (Lymphoprep, Axis-Shield, Norway) from peripheral blood. Once the PBMCs obtained, monocytes were purified by positive selection (Automacs,) using magnetic spheres coated with antibodies against the CD14 (Miltenyi Biotech SL, Spain).

To verified the purity of the selection, cells were labelled with antibodies against CD3 (Markers for T cells), anti-CD19 (Markers for B cells), anti-CD56 (Markers for NK cells) and anti-CD14 (Markers for monocytes) conjugated to fluochromes (BD, Spain). The values of purity by positive selection were greater than 97% as determined by flow cytometry.

4.1.2- Culture of monocytes

Once isolated, monocytes were cultured at a concentration of 10^6 cells / ml in 24-well plates, for 7 days in an incubator at 37 º C under 95% air and 5% CO2. Monocytes in culture were subjected to the following conditions: M-CSF (100 ng / ml, PeproTech, UK), GM-CSF (1,000 U / ml, PeproTech, UK) or both cytokines (M-CSF / GM-CSF); IL-12 (100 ng / ml, PeproTech, UK), IL-18 (100 ng / ml, MBL, Japan) or both cytokines (IL-12/IL-18). The culture medium used in all cases was the complete medium RPMI-1640 (Gibco, Invitrogen, Spain) supplemented with 20% fetal bovine serum (FBS, Gibco, Invitrogen, Spain) and in presence of 100 U / ml penicillin and 100 µg / ml streptomycin, medium called R20.

4.1.3- Differentiation of monocytes

Monocytes were differentiated for 7 days in the presence of alternative macrophage development cytokines: M-CSF to produce M-CSF macrophages (M-Φ), GM-CSF to produce GM-CSF macrophages (GM-Φ) or with both cytokines to produce

M-CSF/GM-CSF macrophages (M/GM-Φ); we also use IL-12 to produce IL-12 macrophages (IL12-Φ), IL-18 to produce IL-18 macrophages (IL18-Φ) or with both cytokines to produce IL-12/IL18 macrophages (IL12/IL18-Φ).

The correct differentiation of cells was assessed by microscopic observations, base on the morphology and distribution of cells in the day seventh of culture.

4.2- Assessment of Respiratory burst in MDM

4.2.1- H_2O_2 production by macrophages

Cellular release of H2O2 was detected using the protocol devised by Mohanty and colleagues [77]. In brief, cultured MDM were rinsed with phosphate-buffered saline. After the washing step, cells were resuspended in Krebs-Ringer phosphate buffer with glucose (KRPG) (145 mM NaCl, 4.86 mM KCl, 0.54 mM CaCl2, 1.22 mM MgSO4, 5.7 mM sodium phosphate and 5.5 mM glucose). $8x10^5$ cells were then added to 100 µl of reaction mixture (50 µM Amplex^R Red reagent and 0.1 U/mL HRP in KRPG) in 96-well flat-bottom tissue culture plates. Immediately, after the addition of 100 ng/ml phorbol myristate acetate (PMA) (Sigma, Spain), the plate was placed in a fluorometer (Titertek Fluoroskan II; Flow Laboratories Inc.), and fluorescence was recorded for each well (0–120 min) at 37 °C. H2O2 release was calculated from the increase of fluorescence and by using purified H2O2 as standard.

4.2.2- MPO and Catalase Activity in macrophages

Monocyte-derived macrophages were harvested with buffer A [(10 mmol/L phosphate buffer containing phenylmethylsulfonyl fluoride (1 mmol/L), leupeptin (100 mmol/L), pepstatin A (1 mmol/L), and cetyltrimethylammonium bromide (0.5%) pH =7.0]. The cells were lysated by multiples cycles of freezing in liquid nitrogen and thawing in ice bath. The cell lysate was centrifuged at 14500 RPM for 5 minutes at 5ºC and the supernatant was used for MPO and Catalase activity assays. Proteins content was measured in the total cell lysate by the BCA method using the kit of Pierce. Peroxidation activity of MPO in the cell lysate was measured by the EnzChek® Myeloperoxidase Activity Assay Kit (Molecular probes Invitrogen, Spain) and MPO activity was calculated by the increase of fluorescence; due to the oxidation of

nonfluorescent Amplex® UltraRed reagent by H2O2-generated redox intermediates MPO-I and MPO-II; and using purified MPO as a standard. Furthermore Catalase activity was assess by the Amplex® Red Catalase Assay Kit (Molecular probes Invitrogen, Spain) and Catalase activity was calculated by the production of the highly fluorescent oxidation product, resorufin; using purified Catalase as a standard.

4.3- HOCL release and the oxidation reactions.

4.3.1- HOCl Production and MPO Degranulation from Macrophages

HOCl production from macrophages was determined by the TNB method [22]. MDM at the concentration of 4.10^6 cells/mL were stimulated with 100 ng/mL PMA in 1 mL of Krebs-Henseleit buffer solution (pH 7.4) containing taurine (20 mmol/L) at 37°C for 2 hours. After the incubation, reactions were stopped by creating cell pellets (by centrifugation [2000*g*] at 4°C) and the concentration of HOCl-mediated product, taurine chloramine, in the incubation medium was determined by adding the Ellman's Reagent TNB (250 µM) to the entire mixture and the absorbance recorded at 412 nm (Ultrospec 3000, Pharmacia Biotech). 50 µg of Catalase (type C-40, 12,000 U/mg, Sigma Chemical Co.) was added to all samples before the addition of TNB to reduce residual H202, due to the ability of high concentrations of H202 to slowly oxidize TNB.

Preparation of TNB.

TNB was prepared by reducing DTNB (Sigma Chemical Co.) as described by Lisa M. Landino and colleagues [78]. DTNB (0.5 g) in 25 ml 0.5 M Tris-HCl pH 8.8 was treated with 2.5 ml β-mercaptoethanol (Sigma Chemical Co.). The pH of the solution was adjusted to 1.5 with 6 M HCl. Orange crystals of TNB formed after 6–8 hours at 4 ° C. The crystals were filtered and washed with cold 0.1 M HCl. Solid TNB was stable at RT indefinitely. A fresh stock solution of TNB was prepared in 0.10 M phosphate buffer (PB) pH 7.4, and its concentration determined from its absorbance at 412 nm assuming an extinction coefficient ε_{412} = 14,150 M^{-1} cm^{-1}.

4.3.2- Oxidation of LDL-C by activated macrophages (*Oxidation Reactions)*

Monocytes-derived Macrophages (MDM) subpopulations (4.10^6 cells/mL) obtained after differentiation of monocytes, were washed two times with PBS and incubated at 37°C in Krebs-Henseleit buffer solution (pH 7.4) in presence of 0.3 mg/mL LDL-C (Intracel, USA). MDM were activated with 100 ng/mL phorbol myristate acetate (PMA) and maintained in suspension by intermittent inversion (Complete System). After 2 hours, reactions were stopped by creating cell pellets (by centrifugation [2000*g*] at 4°C) and adding 0.25 mM EDTA to the supernatants. The analysis of LDL oxidation products in samples supernatants were assessed by electrophoresis on agarose gel. LDL oxidation products were then assayed in supernatants using Lipidophor agarose-gel electrophoresis kits (Technoclone GmbH, Austria). We mix 50 µL of supernatant with 50 µL of prewarmed (50°C) agar. We then put 20 µL of this mixture into the prepared cavities of the gel plates. The electrophoresis runs for 80 min at 70 Volt and 15 mAmp.

4.4- Cholesterol accumulation by macrophages and Lipid staining (Oil-Red-O)

4.4.1- Cholesterol accumulation assay

MDM subpopulations were incubated with 2 mg/ml of low-density lipoprotein (LDL) and the appropriate differentiation cytokines for 24 hours. After incubation, the cells were washed two times with cold PBS, and lipids were extracted from the cells with 250 µl of hexane/isopropyl alcohol (3:2, v/v) for 20 min at room temperature; dried under N_2 and the extract was reconstituted in 30 µl of 2-propanol-containing 10% Triton X-100 and was used as a sample solution. Cholesterol content in cells was determined fluorometrically (FIG.5) [79] using Amplex® Red Cholesterol Assay Kit (Invitrogen, Spain). After lipid extraction, the remaining cellular protein was dissolved in 0.1 N NaOH for protein determination. Protein was determined by the BCA method using a kit obtained from Pierce.

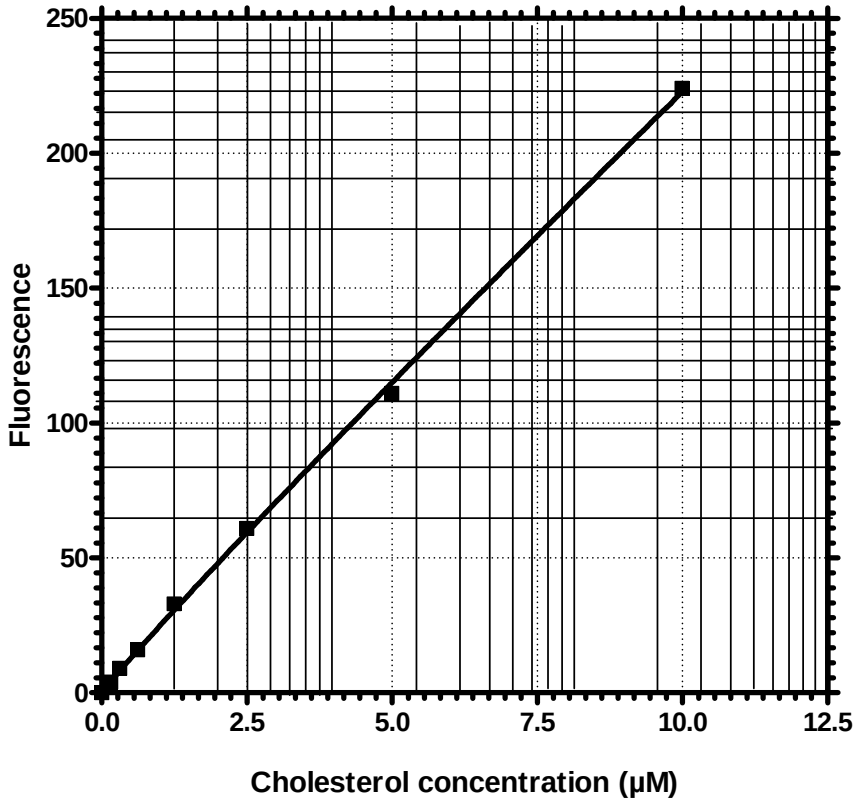

FIG.5. Detection of cholesterol using the Amplex® Red reagent–based assay. Each reaction contained 150 µM Amplex® Red reagent, 1 U/mL HRP, 1 U/mL cholesterol oxidase, 0.1 µM cholesterol esterase and the indicated amount of the cholesterol in 1X reaction buffer. Samples were incubated at 37°C for for 1h. Fluorescence was measured with a fluorescence microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm.

4.4.2- Oil-Red-O staining

After incubation with native LDL as indicated in the above conditions, the cells were washed twice in ice-cold PBS, followed by formaldehyde fixation (10% in PBS) for 15 min at room temperature. Neutral lipids were stained using 0.5% Oil-Red-O (Sigma) in isopropanol for 15 min; cells smear were washed with running water and counterstained with Vector® Hematoxylin QS (Vector Laboratories, Spain). The Oil-Red-O-stained lipids were evaluated morphologically by microscopy.

4.5- Regulatory properties of IL-10

4.5.1- ox-LDL-mediated foam cell formation Assays

MDM subpopulations from 4 healthy donors were incubated for 24 h in the presence of oxLDL alone (25 µg/ml) or oxLDL+ IL-10 (25 µg/ml, 25 ng/ml respectively). Foam cells formation and oxLDL accumulation were assessed by lipid mass quantification assay as described in (4.4.1).

4.5.2- CAD biomarkers analyses

The concentration of IL-6, IL-8, INF-γ, IL-10, MMP-9 in samples supernatants after incubation of MDM subpopulations with 25 µg/ml oxLDL or without oxLDL were determined using the Luminex analysis (Millipore, Spain)

4.6- Statistical Analysis

Data in figures represent the means ± SD of four monocytes donors. The difference between two mean values was analyzed using a paired two-tailed *t*-test. A value of $P < 0.05$ was considered statistically significant.

5 -Results

This study focuses on the combined action of cytokines MCSF / GMCSF and IL-12/IL-18 on monocytes of peripheral blood and assesses the respiratory burst of these cells once differentiate into macrophages, as well as the production of inflammatory cytokines and chemokines associated with atherosclerosis.

5.1 Differentiation of monocytes with MCSF/GMCSF and IL-12/IL-18 induces changes in morphology and distribution of MDM in culture.

We cultured monocytes at a concentration of 10^6 cells/ml in the R20 with M-CSF/GM-CSF, IL-12/IL-18. As control we cultured monocytes with M-CSF, GM-CSF, IL-12 and IL-18. Cultures were maintained for 7 days to ensure the correct differentiation, during which we made microscopic observations to determine the distribution and morphology of each MDM subpopulation in culture.

The addition of M-CSF/GM-CSF and IL-12/IL-18 causes a change in the distribution of cells in culture, as indicated by the results we obtained from microscopy. Indeed, in cultures of monocytes stimulated with IL-12 (FIG.6 -B) or IL-18 (FIG.6 -C), some cells die and disappear from the cultures; leaving the possibility that they were cleared by the remaining cells in culture. Monocytes differentiated with IL-12 have a more or less rounded shape, whereas those differentiated with IL-18 are somewhat more or less elongated.

Human monocytes (FIG.6- A) differentiated in the presence of GM-CSF for 7 days maintained the rounded shape of the monocytes precursors resembling the fried egg phenotype described in previous studies (FIG.6- D). The same human monocytes differentiated in the presence of M-CSF for 7 days produced macrophages with an elongated shape and numerous vacuoles (FIG.6- E). In contrast, if the stimulus comes from the combination of M-CSF/GM-CSF (FIG.6- F) or IL-12/IL-18 (FIG.6- G) monocytes adhere totally to the plate culture and the cells appear aggregated and have a fibroblastic appearance.

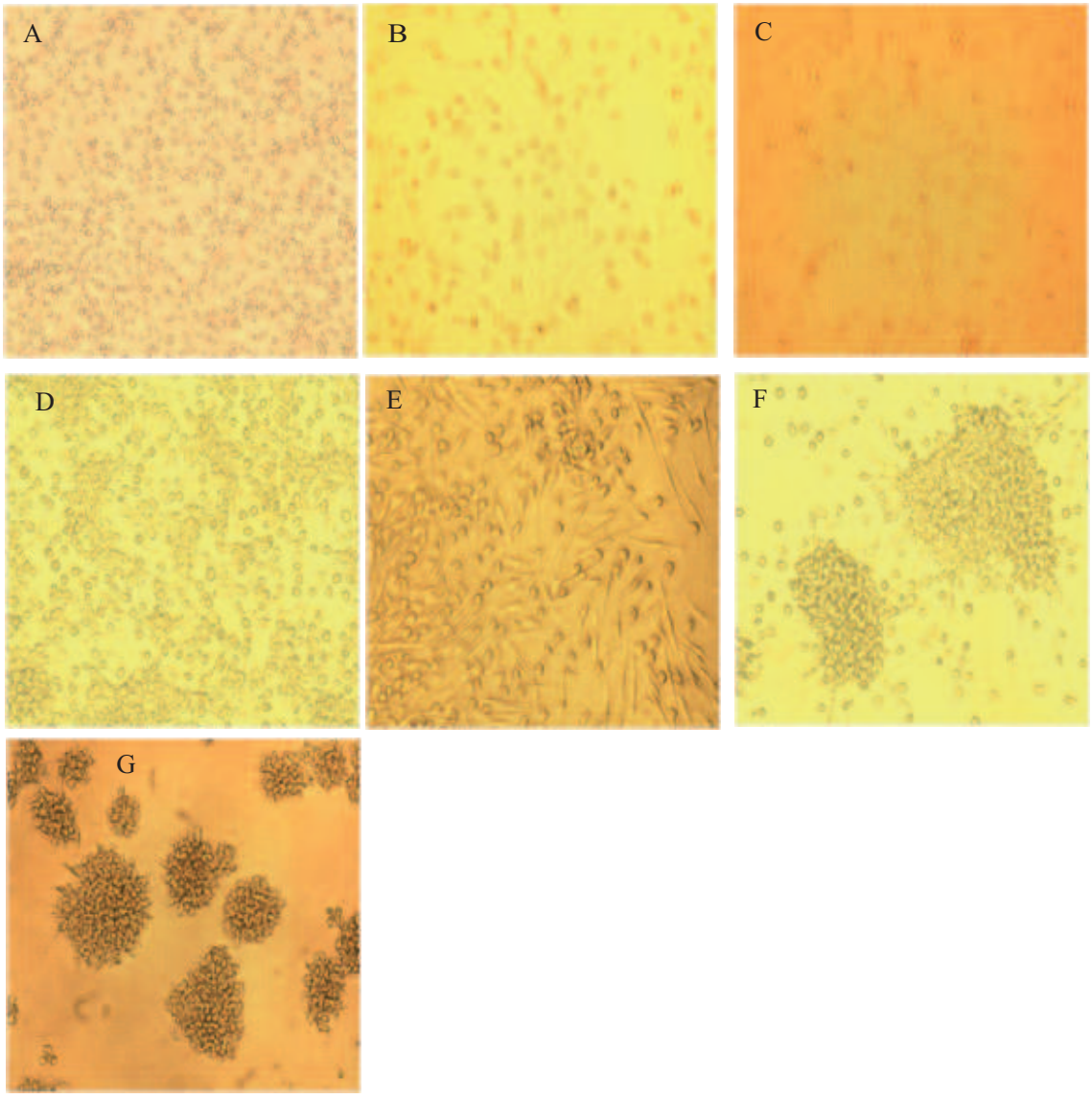

FIG.6. Morphology of monocytes cultures. The images show the in-vitro cultures of (A) freshly isolated monocytes or stimulated during one week with (B) IL-12, (C) IL-18, (D) GM-CSF, (E) M-CSF, (F) M-CSF/GM-CSF and (G) IL-12/IL-18.

5.2 The synergic effect of IL-12/IL18 and M-CSF/GM-CSF on the respiratory burst of macrophages

The ability of MDM to generate ROS was assessed by quantifying the production of antioxidant enzyme catalase and pro-oxydants enzymes MPO and NADPH oxydase (H2O2).

5.2.1 Intracellular *Catalase* activity in macrophages

Catalase activity in GM- Φ lysates after 7 days of culture was about 8 units/mg protein, whereas those in all others macrophages subpopulations were less than 3 units/mg protein (all P < 0,05) (FIG.7-A). In agreement with this enzyme activity results, the above findings suggest that expression of catalase gene is quite different between GM- Φ and others macrophages subpopulations.

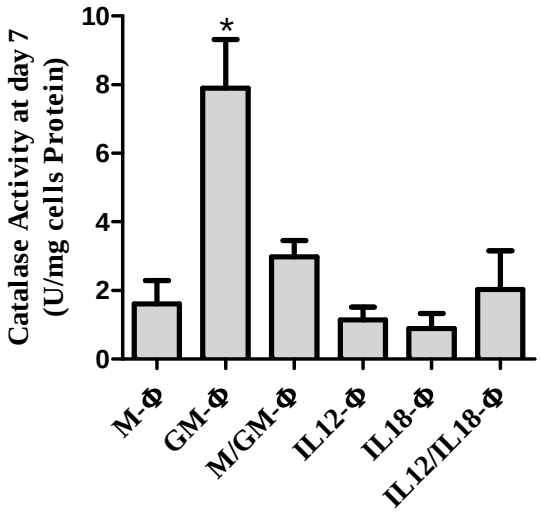

FIG.7-A. Activities of intracellular catalase in monocyte-derived macrophages subpopulations. At day 7 of differentiation, MDM were lysated in buffer A. Proteins content was measured in the total cell lysate by the BCA method. Catalase activities in cells lysate were then analyzed using a fluorimetric assay. Statistical analysis was performed between samples using a paired two-tailed *t*-test. GM-Φ showed high catalase activities than others macrophages subpopulations (all P < 0.05).

5.2.2 H2O2 release by macrophages

PMA has a marked ability to stimulate the release of ROS in phagocytes. MDM were incubated for 2h in Krebs-Ringer phosphate buffer containing PMA.

M-Φ, M/GM-Φ and IL12/IL18- MΦ release a large amount of H2O2, but GM-Φ inhibits H2O2 release by stimulation with PMA (FIG.7-B). As demonstrated above, GM- Φ expresses high levels of catalase activity. These findings suggest the possibility

that GM-Φ, but not others macrophages populations, has a marked ability to scavenge H2O2. Furthermore, IL12-Φ and IL18-Φ H2O2 release were very low and these two results show a possibility that these cytokines when alone in culture can't maintain or ensure the survival and viability of MDM.

As shown in (FIG.7-B), when macrophages were stimulated with 100 ng/ml PMA for 60 min; M-Φ, M/GM-Φ and IL12/IL18-Φ release 2,133 ± 1,007 nM/ml H2O2, 3,264 ±1,733 nM/ml H2O2 and 2,246 ± 0,488 nM/ml H2O2, respectively. In contrast GM-Φ, IL12-Φ and IL18-Φ release only 0,476 ± 0,325 nM/ml H2O2, 0,109 ± 0,088 nM/ml H2O2 and 0,084 ± 0,026 nM/ml H2O2, respectively. Unstimulated MDM subpopulations did not produce H2O2. These findings suggest that M-Φ, M/GM-Φ and IL12/IL18-Φ release large amount of H2O2, unlike GM-Φ, through their distinct regulation of catalase activities.

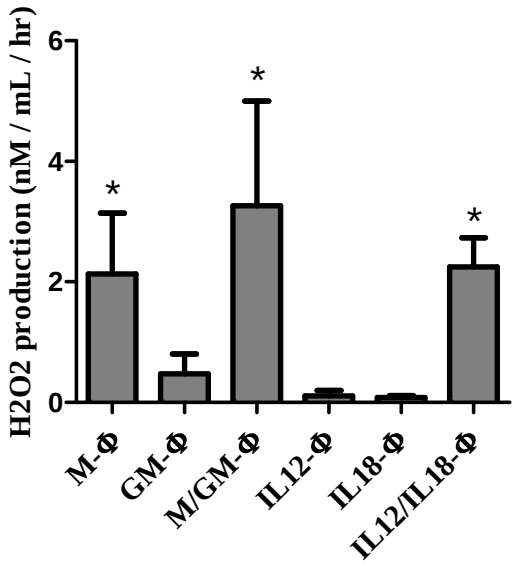

FIG.7-B. Stimulant-induced H2O2 release in monocyte-derived macrophages subpopulations. Cultured MDM were rinsed in PBS and resuspended in Krebs-Ringer phosphate buffer with glucose (KRPG). The reaction mixture containing 100 ng/ml PMA was dispensed into wells containing MDM subpopulations. The cellular release of H2O2 was recorded for 2h at 37 °C using a fluorometer. Statistical analysis was performed between samples using a paired two-tailed *t*-test.

5.2.3 Myeloperoxidase activity in MDM subpopulations

We used cultured human monocytes to explore mechanisms that might regulate MPO expression in macrophages. Recent studies show that GM-CSF preserved MPO activity during the 7-day culture period, whereas M-CSF was inactive in this regard

[65]. In our study, the combination of either M-CSF/GM-CSF or IL12/IL18 induces the up-regulation of MPO activity (FIG.7-C). GM- Φ also shows high MPO activity but M-Φ, IL12- Φ and IL18- Φ were inactive in this regard (FIG.7-C). MPO activity in M-Φ was very low comparing with GM-Φ or M/GM-Φ (P= 0,013 and P = 0,032 respectively). Similarly, MPO activity in IL12/IL18-Φ was statiscally significant than in IL12-Φ or IL18-Φ (P = 0,049 and P = 0,045 respectively) .These results indicate that GM-CSF selectively regulates MPO levels during differentiation of circulating monocytes into macrophages. These results also show that the combination of GM-CSF with M-CSF or IL-12 with IL-18 improves the regulation of MPO expression in macrophages. Furthermore, these results indicate that only the differentiation of monocytes with M-CSF/GM-CSF or IL-12/IL-18 give rise to $^{MPO+\ H2O2+}$ macrophages phenotype.

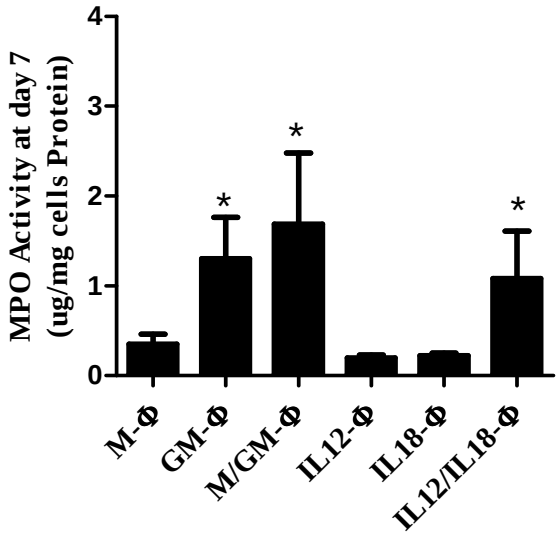

FIG.7-C. Activities of intracellular myeloperoxidase in monocyte-derived macrophages subpopulations. At day 7 of differentiation, MDM were lysated in buffer A. Proteins content was measured in the total cell lysate by the BCA method. Myeloperoxidase activities in cells lysate were then analyzed using a fluorimetric assay. Statistical analysis was performed between samples using a paired two-tailed *t*-test. GM-Φ, M/GM- Φ and IL12/IL18- Φ show high myeloperoxidase activities than others macrophages subpopulations (all $P < 0.05$).

5.3 Atherogenic potential of monocytes differentiated with M-CSF/GM-CSF and IL-12/IL-18

5.3.1 Ability of activated M/GM-Φ and IL12/IL18-Φ to produce HOCl.

Taurine chloramine generation by the stimulated macrophages is mediated by HOCl. But firstly for HOCl production the system will require H202, myeloperoxidase, and Cl^-. In 4 experiments 4 X 10^6 PMA-stimulated GM-Φ, M/GM- Φ and IL12/IL18- Φ chlorinated extracellular taurine and generated respectively 15,92 ± 3,37; 27,55 ± 11,17; 20,98 ± 3,87 nM chloramine during a 2h incubation, whereas M-Φ, IL12-Φ and IL18-Φ induce only low levels of chloramines generation (all P< 0,05) (FIG.8-A). HOCL generation was proportional to the amount of H2O2 and MPO in each MDM phenotype. These results indicate that some macrophages phenotypes can produce significant amounts of HOCl when activated.

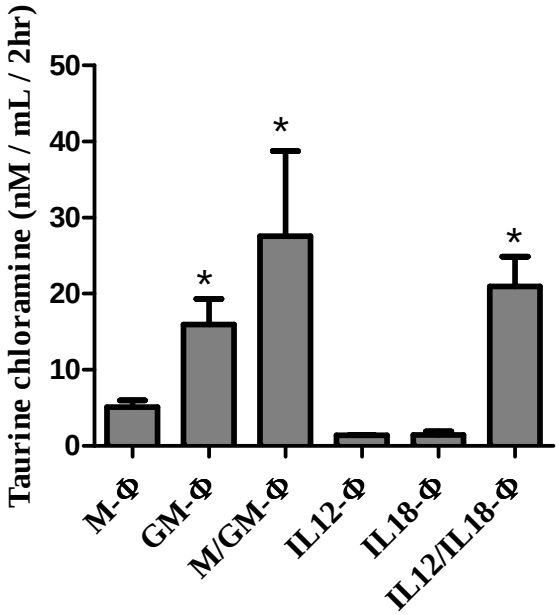

FIG.8-A. Taurine chloramine formation by macrophages. 4.10^6 of each MDM phenotype were incubated with 20 mM taurine for 2h at 37°C in 1 ml of Krebs-Henseleit buffer solution (pH 7.4). Cells were incubated in the presence of 100 ng/ml of PMA. Taurine chloramine formation was quantitated by the TNB method. Results are expressed as the mean± SD of four monocytes donors.

5.3.2 LDL oxidation by activated M/GM-Φ and IL12/IL18-Φ.

MDM were activated in Krebs-Henseleit buffer solution containing LDL with PMA, and the extent of LDL modification by reactive ROS species was determined by electrophoresis. Under these conditions, GM- Φ, M/GM-Φ and IL12/IL18-Φ activation promote protein chlorination or lipids oxidation of LDL. While M-Φ failed to do it. During oxidation of LDL, its electronegativity increases, and therefore electrophoretic mobility on agarose gel is a good indicator of the extent of oxidation
[80, 81]. Under the conditions as described in the Methods section, native LDL and LDL incubated with M-Φ migrate only slowly (FIG.8-B). Electrophoretic mobility of LDL incubated with GM-Φ, M/GM-Φ and IL12/IL18-Φ increases, but the extent of mobility was higher in M/GM-Φ and IL12/IL18-Φ than in GM-Φ.

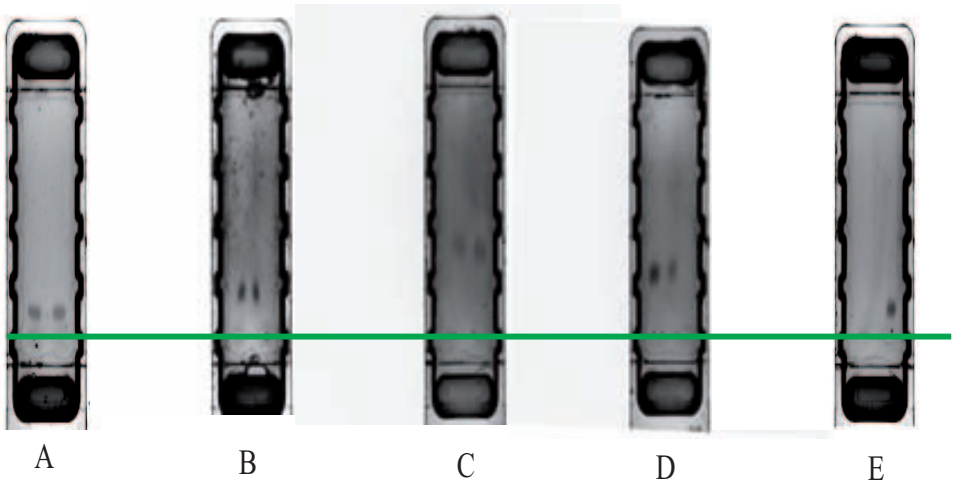

FIG.8-B. Oxidation of LDL by PMA activated macrophages via the MPO/H2O2 system. MDM (4.10^6 cells/mL) were incubated at 37°C in Krebs-Henseleit buffer solution (pH 7.4) in presence of LDL (0.3 mg/mL). Cells were activated with 100 ng/mL PMA and maintained in suspension by intermittent inversion (Complete System) for 2 hours. Reactions were stopped by removing cells through centrifugation. LDL oxidation products were then analyzed by electrophoresis as described in materials and methods. Electrophoretic mobility increases significantly after oxidative modification of the lipoproteins, indicating enhanced negative charge. (A) M-Φ, (B) GM-Φ, (C) M/GM-Φ, IL12/IL18- MΦ and (E) native LDL.

5.4 Cholesterol Accumulation potential in M/GM-Φ and IL12/IL18-Φ.

The differential expression of genes related to cholesterol homeostasis prompted further investigation of the cholesterol accumulation potential in macrophages subpopulations. We analyzed this potential in six macrophages phenotypes. Macrophages subpopulations from four monocytes donors were incubated with 2 mg/ml of native LDL. FiG.9-A summarizes the spontaneous cholesterol accumulation in these macrophages subpopulations. M-Φ, M/GM-Φ and IL12/IL18-Φ spontaneously accumulated respectively more than 2, 1.76 and 1.36-fold LDL-derived cholesterol relative to GM-Φ (all P< 0.05). Similarly, IL12/IL18-Φ accumulated more than twice LDL-derived cholesterol than IL12-Φ and IL18-Φ (all P< 0.05). Oil-Red-O staining images as illustrated in FIG.9-B also show the same results and demonstrate that all macrophages subpopulations are capable to accumulate spontaneously LDL in vitro but that cholesterol homeostasis in macrophages subpopulations is different.

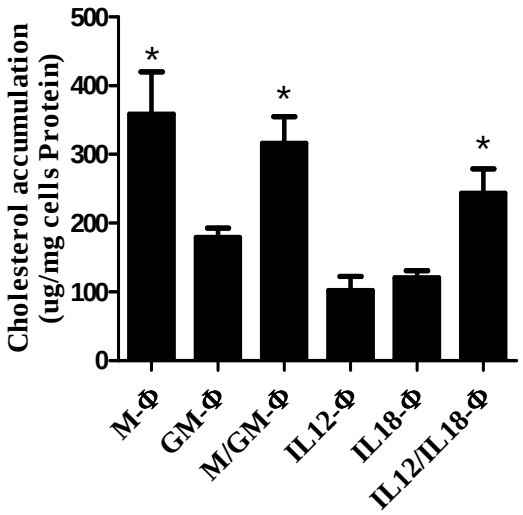

FIG.9-A. Cholesterol accumulation of differentiated macrophages. Cultured MDM of four monocytes donors were incubated with 2 mg/ml LDL for 24 hours and their cholesterol and protein contents were determined. Shown is the cholesterol accumulation of macrophages subpopulations incubated with native LDL for the indicated conditions. M-Φ, M/GM-Φ and IL12/IL18-Φ spontaneously accumulated significant quantities of LDL-derived cholesterol relative to GM-Φ, IL12-Φ and IL18-Φ (all $P < 0.05$).

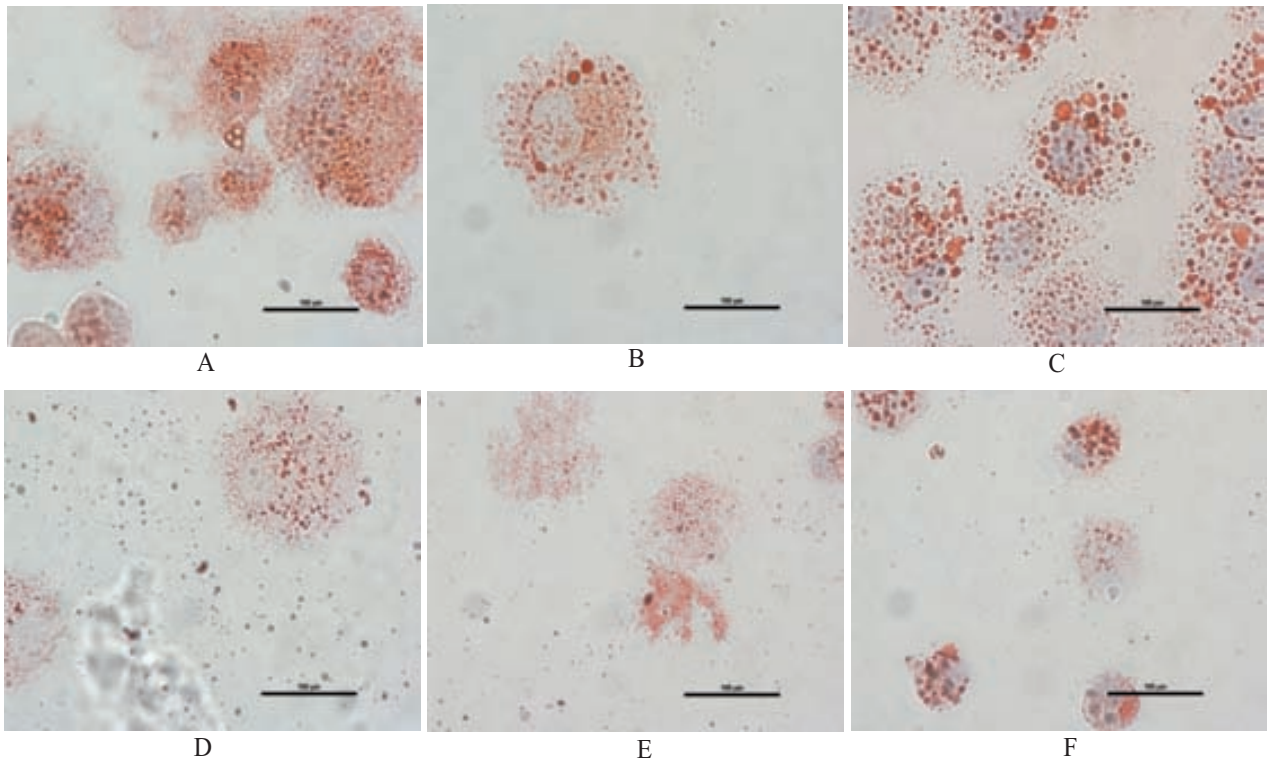

FIG.9-B. Oil-Red-O staining. Cultured MDM of four monocytes donors were incubated with 2 mg/ml LDL for 24 hours. Cells were then stain by the Oil-Red-O technique as described in material and methods. (A) M-Φ, (B) GM-Φ, (C) M/GM-Φ, (D) IL12-Φ, (E) IL18-Φ and (F) IL12/IL18-Φ.

5.5-Regulatory properties of IL-10 in atherogenesis

5.5.1- The protecting role of IL-10 in the inhibition of foam cells formation

Macrophages were incubated for 24 hours with oxLDL alone or in combination with IL-10. Our results show that the addition of oxLDL (25 µg/mL) leads to lipids loading in macrophages subpopulations. However, we observed that this accumulation was still higher in MDM subpopulations that have acquired a great oxidant potential (FIG.10-A)

We also found that IL-10 (25 ng/ml) significantly mediated decrease of neutral lipids accumulation in oxLDL-treated macrophages after incubation for 24 h, as measured by lipid mass quantification (FIG.10-A). Oil-Red-O staining images show that oxLDL induces foam cells formation in all macrophages subpopulations (Figures not show). However, with microscopic observations in cells cultures (FIG.10-B and

FIG.10-B´), we observed that IL-10 significantly reduces oxLDL-induced foam cells formation in macrophages. When IL-10 was add in oxLDL-treated macrophages, cellular morphology observation reveals that cells remain activated in cultures meanwhile those cells treated without IL-10 take a foam cells like appearance. Moreover we observed that the addition of oxLDL induces a change of distribution in all MDM phenotypes in cultures.

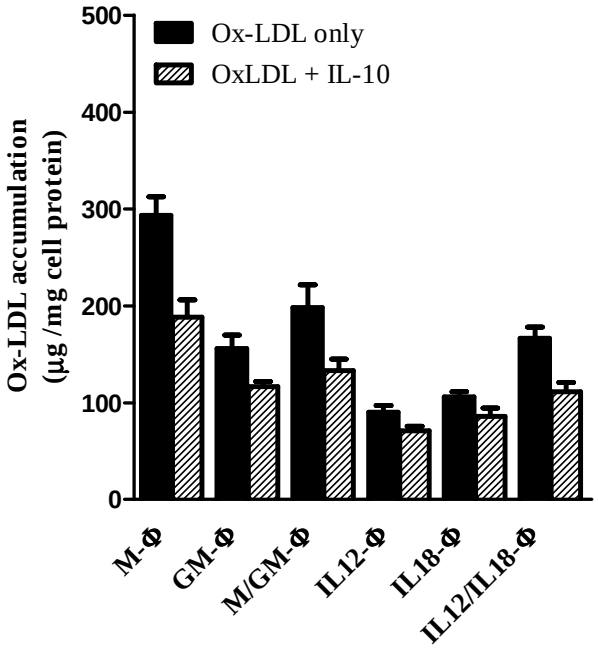

FIG.10-A. IL-10 inhibits ox-LDL-induced lipid loading and foam cells formation in monocyte-derived macrophages. Monocyte-derived macrophages from four monocytes donors were incubated for 24 h in the presence of oxLDL (25 µg/ml) or oxLDL (25 µg/ml) + IL-10 (25 ng/ml), and accumulation of neutral lipid was quantified fluorimetrically; furthermore we make an microcopy observation to assess foam cell formation after Oil-Red-O staining. IL-10 decreases the lipid filling in all macrophages subpopulation.

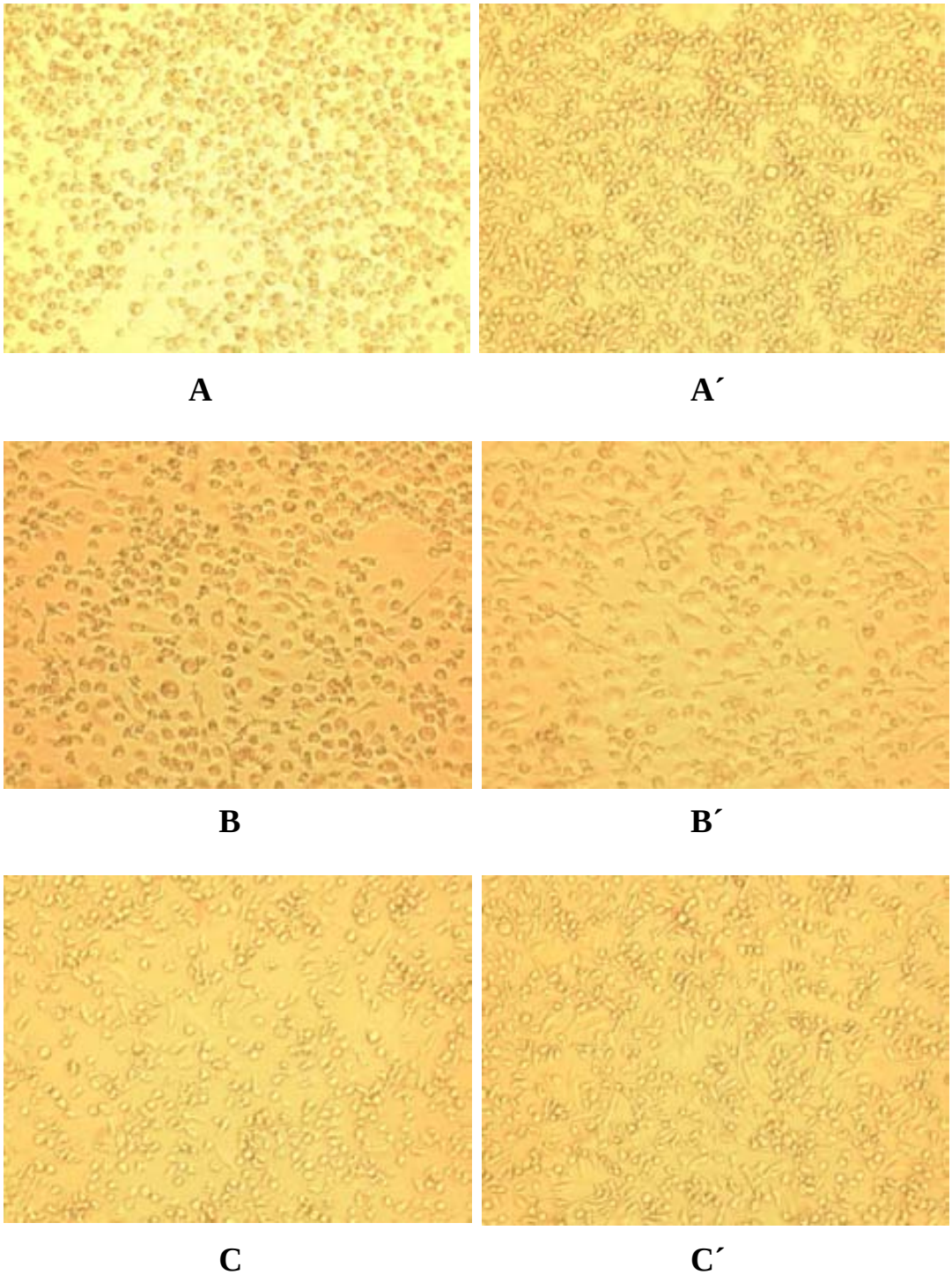

FIG.10-B) IL-10 inhibits oxLDL-induced foam cells formation in monocyte-derived macrophages. Cultured MDM of four monocytes donors were incubated with 25 µg/ml oxLDL or (25 µg/ml oxLDL + 25 ng/ml IL-10) for 24 hours. Cells were then observed by microscopy using a 10X magnification. In (A) M-Φ, (B) GM-Φ, (C) M/GM-Φ; MDM were treated with oxLDL only. In (A´, B´, and C´) the same MDM subpopulations were treated with the combination of oxLDL + IL-10.

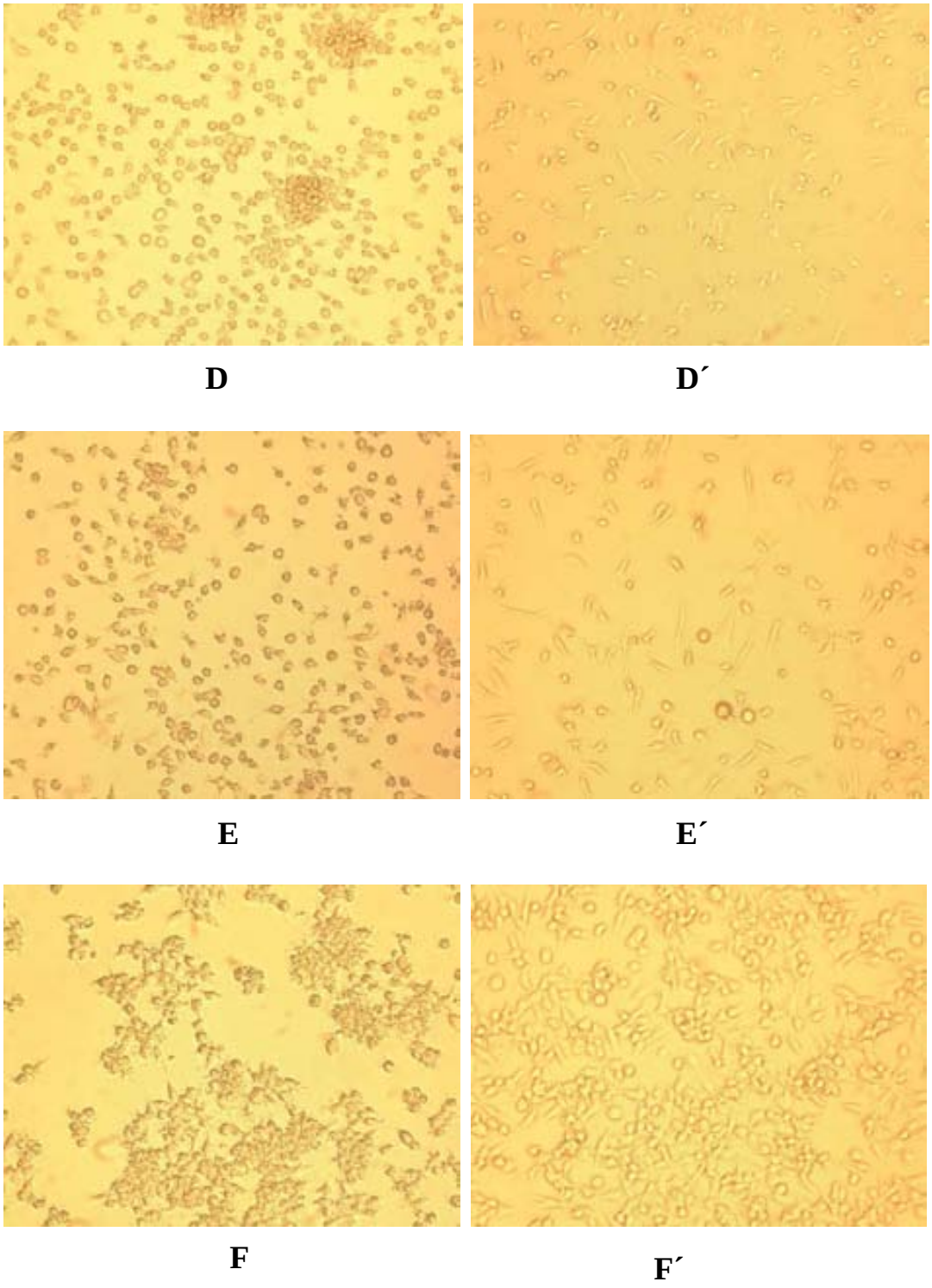

FIG.10-B´. IL-10 inhibits oxLDL-induced foam cells formation in monocyte-derived macrophages. Cultured MDM of four monocytes donors were incubated with 25 μg/ml oxLDL or (25 μg/ml oxLDL + 25 ng/ml IL-10) for 24 hours. Cells were then observed by microscopy using a 10X magnification. In (D) IL12-Φ, (E) IL18-Φ and (F) IL12/IL18-Φ; MDM were treated with oxLDL only. In (D´, E´, and F´) the same MDM subpopulations were treated with the combination of oxLDL + IL-10.

5.5.2 ox-LDL induces the release of CAD biomarkers by macrophages.

Our results show that the incubation of macrophages with oxidized cholesterol triggers the release of pro-inflammatory cytokines IL-6, chemokine IL-8 and protease MMP-9 (FIG.10-C). oxLDL also triggers the release of INF-γ in IL12/IL18-Φ. Unlike the previous results, we found that oxLDL inhibits the expression of anti-inflammatory cytokine IL-10 in macrophages. These results clearly show that oxLDL activate the mechanisms or pathways of inflammation in macrophages on the one hand; on the other hand it contributes to the inhibition of anti-inflammatory mechanisms. Furthermore, we observed that in all these macrophages subpopulations, there was no relationship between the amount of intracellular lipid accumulation and the production of inflammatory products.

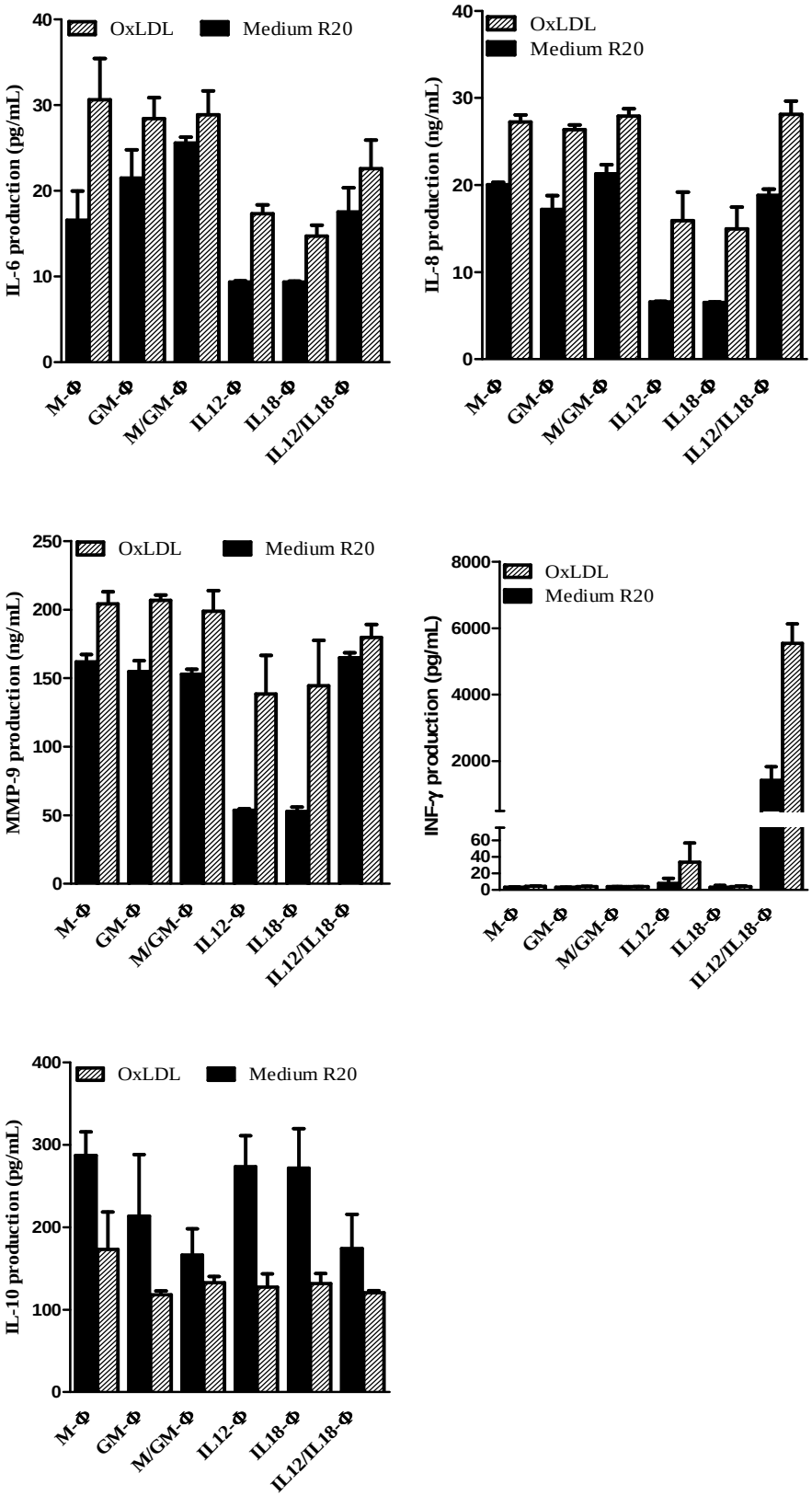

FIG.10-C. oxLDL mediated release of CAD makers by MDM. The concentration of CAD biomakers in samples supernatants after incubation of MDM subpopulations with 25 μg/ml oxLDL or without oxLDL was determined using the Luminex analysis.

6 -Discussion

The recruitment of monocytes and macrophages aggregation in the arterial wall is one of the factors favorable to inflammation and the development of atherosclerotic lesions. However, macrophages aggregation during the *in-vitro* differentiation of monocytes with IL-12/IL-18 has already been described by our group [73].

The present study evaluated the synergic effect and the atherogenic potential of human monocyte-derived macrophage subpopulations obtain after differentiation of monocytes by M-CSF/GM-CSF or IL-12/IL-18.

We demonstrate that human monocytes can be differentiated into divergent macrophage phenotypes (ie, M-Φ, GM-Φ, M/GM-Φ, IL12-Φ, IL-18-Φ, and IL12/IL18-Φ) with potentially alternative functions in human atherosclerotic disease. Our data underscore macrophages heterogeneity [82, 83]. In fact, in the experiments we performed in vitro, we found that the combination of cytokines M-CSF / GM-CSF or IL12/IL18 has an important effect on the morphology and distribution of monocytes from peripheral blood. These two MDM phenotypes have formed aggregates or small colonies in cultures while the rest of the four macrophages subpopulations that we have studied (ie, M-Φ, GM-Φ, IL12-Φ and IL-18-Φ) have shown a distribution more or less dispersed in cultures. The increased expression of the adhesion molecule ICAM-1 was demonstrated by our group as a factor responsible for this aggregation [73]. In this study, we investigated the impact that macrophages aggregation may have in lipid accumulation and deposition as well as in LDL oxidation.

The mechanisms contributing to LDL oxidation in vivo require an important respiratory burst from macrophages [10, 84-87]. That is, an active source of H_2O_2 and MPO. Increased production of ROS by activated macrophages is the result of synthesis of increased amounts of NADPH oxidase [3, 9, 10, 87].

In our study we found that the activity of catalase enzyme in GM- Φ was higher than in others MDM subpopulations. Furthermore, GM-Φ release low levels of H_2O_2 when activated with PMA; leaving a possibility that GM-Φ, but not others macrophages phenotypes, has a marked ability to scavenge H_2O_2 through their inducible level of

catalase. With these results we were able to confirm that GM-CSF promotes antioxidant mechanisms in MDM [59, 63]. However, our results also show that GM-CSF selectively regulates the expression of the pro-oxidant enzyme MPO in MDM [65]. But giving that an important part of H2O2 might be consumed by GM-Φ through their high catalase activity, the impact of high MPO expression on HOCl production in this macrophages subpopulation will be reduce; as some amount of H2O2 require for HOCl formation will be scavenge by the enzyme catalase [26, 63, 88].

In contrast, M-Φ phenotype shows high levels of H2O2 when activated with PMA. However, this result contrasts with their low levels of MPO and catalase activities. These observations help us to conclude that M-Φ phenotype promotes oxidative mechanisms but don't have enough MPO to form the oxidant HOCl [59, 89]. Nevertheless, the augmentation of H202 release in activated M-Φ illustrates the role they can play in lipoproteins oxidation *in-vivo*; as their release may be use by some MPO-containing macrophages to produce HOCl [28].

However, M/GM-Φ and IL12/IL18-Φ phenotypes produce significant levels of H2O2 when activated with PMA. The same macrophages subpopulations show higher MPO activity than others. Furthermore, this high pro-oxidant profile contrast with their low antioxidant potential; as they show low catalase activity relative to the level that we observed in GM-Φ.

These findings clearly show that the combine action of cytokines M-CSF /GM-CSF or IL12/IL18 on monocytes produces $^{MPO + H2O2 +}$macrophages phenotypes, while GM-CSF and M-CSF when use each alone only produce MPO-positive or H2O2-positive macrophages phenotypes respectively. With these observations we were able to confirm that M-CSF/GM-CSF induces a synergic effect on monocytes/macrophages as both factors improve the respiratory burst of macrophages. With one of these factors, macrophages are able to produce either MPO or H2O2. In contrast, IL12/IL18-Φ need dual signals (ie both IL-12 and IL-18) to produce either MPO or H2O2.

Thus, the combination of cytokines M-CSF/GM-CSF or IL12/IL18 selectively regulates MPO and NADPH oxidase (H2O2 production) expression in macrophages. H2O2 production in IL12/IL18-Φ might be a result of high INF-γ release by these macrophages subpopulation [73, 90], while this production in M/GM-Φ comes from M-

CSF [63]. With these result we can suggest that the type and the number of cytokines in a given environment *in-vivo* are factors that determine the pro or anti-oxidant profile of macrophages [59].

HOCl is a powerful oxidant capable of reacting with a host of biologically important molecules [91, 92]. Studies designed to determine the requirements for taurine chloramine generation by the macrophages clearly indicated a pivotal role for H202, myeloperoxidase, and Cl^- [22]. GM-Φ, M/GM-Φ and IL12/IL18-Φ generate significant amount of taurine chloramine when activated with PMA. These data indicate that macrophages phenotypes with the capacity to release both MPO and H2O2 can generate oxidant capable of attacking the amine group of taurine to form the N-Cl derivative [93]. We obtained the identical results when the same macrophages phenotypes initiated LDL oxidation when incubated with native LDL [94, 95]. Furthermore, M/GM-Φ and IL12/IL18-Φ induce high LDL oxidation and therefore demonstrate the atherogenic potential that these macrophages phenotypes might play in human atherosclerotic lesions. The formation of HOCl by activated GM-Φ, M/GM-Φ and IL12/IL18-Φ also suggests that they are able to promote inflammation, development or maintenance of atherosclerotic lesions *in-vivo* [59].

The activation of H2O2-MPO-containing macrophages and subsequent degranulation might contribute to the transition to acute complications of atheroma [65]. However, For H2O2-MPO-positive macrophages to realize their respiratory burst capacity and produce HOCL in the arterial wall, they must release H2O2 and MPO into the extracellular space. The stimuli for and mechanisms of this degranulation remain unknown. In our study we use PMA as stimuli. The present findings that PMA (a potent PKC activator) induced the HOCl production from H2O2-MPO-positive macrophages *in vitro* suggest possible signaling pathways. Thus, all these observations support the concept that local accumulation of H2O2-MPO producing macrophages in plaques could influence atheroma stability.

We also observed that all macrophages phenotypes were capable of accumulating lipid and forming foam cells *in-vitro*. However; only M-Φ, M/GM-Φ and IL12/IL18-Φ take up great amount of LDL by a spontaneous mechanism and accumulate cholesterol when incubated with native LDL [42]. These results show that these three macrophages

phenotypes have acquired a great cholesterol accumulation during differentiation. In addition these results also confirm that cholesterol metabolism is very different in macrophages subpopulations [59]. Research suggests that the regulation of macrophage cholesterol metabolism may be an extension of the inflammatory process because similar transcription factors affect both systems [96, 97]. This suggests that macrophages phenotypes with a great cholesterol accumulation potential will also promote inflammation [47].

When comparing cholesterol accumulation potential in each macrophages phenotype, we were able to suggest that M/GM-Φ subpopulation during the *in-vitro* differentiation has acquired an intermediate cholesterol metabolism profile between M-Φ and GM-Φ subpopulations; while IL12/IL18-Φ has acquired a new metabolic profile between IL12-Φ and IL-18-Φ subpopulations. This indicates that the presence of M-Φ, M/GM-Φ or IL12/IL18-Φ subpopulations in atherosclerotic lesions will promote both lipids deposition and foam cell formation in the arterial wall. Moreover, cholesterol accumulation potential in M-Φ, M/GM-Φ and IL12/IL18-Φ paralleled with their respiratory burst potential; indicating the role that these macrophages phenotypes may play in LDL oxidation *in-vivo*.

IL-10 is proposed to have a therapeutic potential in various inflammatory diseases, including atherosclerosis [60, 74, 98]. Macrophages are central for both inflammation and lipid deposition during atherogenesis [34, 38, 59], and we therefore used a part of our study to investigate if IL-10 inhibit macrophages foam cell formation. We were able to demonstrate that IL-10 decreases lipid accumulation in oxLDL-stimulated MDM. This decrease was accompanied by a reduction of foam cells formation as observed in microscopic images. This finding suggests that IL10 has a remarkable therapeutic potential by protecting macrophages to become foaming through the inhibition of lipid accumulation [98-104].

In contrast, the incubation of MDM with only oxLDL leads to lipid filling and foam cells formation of macrophages. M-Φ, M/GM-Φ and IL12/IL18-Φ show higher oxLDL uptake than others MDM subpopulations; indicating that macrophages subpopulations with a great respiratory burst are the first to become foam cells and thus,

they may be more implicated in atherosclerotic plaques formation than others macrophages phenotypes.

Moreover, oxLDL-mediated lipid filling was accompanied by an increase or spontaneous release of IL-6, CXCL8, INF-γ and MMP-9 by macrophages. These results indicate that the presence of oxLDL in artery wall will promote inflammation, activate others immune cells, induce monocytes recruitment and atherosclerotic plaques instability [4, 68, 74, 75, 105].

In contrast, we also observed that oxLDL inhibits the expression of anti-inflammatory cytokine IL-10 in MDM. With the previous results, we were able to suggest that oxLDL is a stimulant agent implicated in the activation of inflammatory pathways of macrophages on the one hand, on the other hand it contributes to the inhibition of anti-inflammatory mechanisms [74].

In summary our work, regarding to the combine action of IL-12 and IL-18 shows that for the activation of macrophages a two signal is needed. IL-12 and IL-18 are from a two very different families (Immunoglobulin and TLR pathways, and the activation pathways respectively). This activation is needed to control the immune system and not to produce inflammation, but only when is needed. The effect of IL-12 and IL-18 is not additive, but is similar to the TCR and CD28 signal needed for activation of antigen specific cell. In contrast the effect of both M-CSF and GM-CSF is additive. As these cytokines are present in the atherosclerotic lesion they might contribute to control this process.

7 -Conclusions

In this present study we can give the following conclusions:

1. The synergic effect of IL-12/IL18 and M-CSF/GM-CSF on monocytes promotes survival, differentiation to macrophages and clustering of monocytes, while with each cytokine alone the cells remain evenly dispersed in the cultures.

2. The differentiation of monocytes with IL-12/IL-18 and M-CSF/GM-CSF triggers the respiratory burst of macrophages by decreasing the expression of anti-oxidant catalase (<3 units/mg), and increasing the expression of H2O2 and MPO in macrophages.

3. The differentiation of monocytes with IL-12/IL-18 and M-CSF/GM-CSF increases the capacity of macrophages to generate the oxidant HOCl in response to exogenous stimuli.

4. The synergic effect of IL-12/IL-18 or M-CSF/GM-CSF on monocytes favors cholesterol accumulation potential who is associated to the respiratory burst.

5. IL-10 inhibits oxLDL-induced lipid loading and foam cells formation in macrophages.
   5.1 oxLDL promotes both inflammation and intracellular cholesterol deposition with formation of foam cells by macrophages.
      5.1.1 Macrophages subpopulations with a pro-oxidant profile have high affinity to oxLDL than others macrophages phenotypes.
   5.2 oxLDL is source of IL-6, IL-8, INF-γ and MMP-9 production by macrophages.

8 -References

1. Myers MA, McPhail LC, Snyderman R. Redistribution of protein kinase C activity in human monocytes: correlation with activation of the respiratory burst. J Immunol **1985** Nov;135(5):3411-6.
2. Cathcart MK. Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. Arterioscler Thromb Vasc Biol **2004** Jan;24(1):23-8.
3. Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med **1992** Jun;119(6):598-620.
4. Rader DJ. Inflammatory markers of coronary risk. N Engl J Med **2000** Oct 19;343(16):1179-82.
5. Rose H, Hoy J, Woolley I, et al. HIV infection and high density lipoprotein metabolism. Atherosclerosis **2008** Jul;199(1):79-86.
6. Berliner JA, Navab M, Fogelman AM, et al. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation **1995** May 1;91(9):2488-96.
7. Halliwell B, Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J **1984** Apr 1;219(1):1-14.
8. Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem J **1996** Jan 1;313 (Pt 1):17-29.
9. Fridovich I. Oxygen toxicity: a radical explanation. J Exp Biol **1998** Apr;201(Pt 8):1203-9.
10. Gille G, Sigler K. Oxidative stress and living cells. Folia Microbiol (Praha) **1995**;40(2):131-52.
11. Farber JL, Kyle ME, Coleman JB. Mechanisms of cell injury by activated oxygen species. Lab Invest **1990** Jun;62(6):670-9.
12. McCord JM. Superoxide radical: controversies, contradictions, and paradoxes. Proc Soc Exp Biol Med **1995** Jun;209(2):112-7.
13. Crawford DR, Davies KJ. Adaptive response and oxidative stress. Environ Health Perspect **1994** Dec;102 Suppl 10:25-8.
14. Boveris A. Biochemistry of free radicals: from electrons to tissues. Medicina (B Aires) **1998**;58(4):350-6.
15. Musson RA, Shafran H, Henson PM. Intracellular levels and stimulated release of lysosomal enzymes from human peripheral blood monocytes and monocyte-derived macrophages. J Reticuloendothel Soc **1980** Sep;28(3):249-64.
16. Takeya R, Sumimoto H. Molecular mechanism for activation of superoxide-producing NADPH oxidases. Mol Cells **2003** Dec 31;16(3):271-7.
17. Babior BM. NADPH oxidase. Curr Opin Immunol **2004** Feb;16(1):42-7.
18. Kitagawa S, Takaku F, Sakamoto S. A comparison of the superoxide-releasing response in human polymorphonuclear leukocytes and monocytes. J Immunol **1980** Jul;125(1):359-64.
19. Hazen SL. Myeloperoxidase and plaque vulnerability. Arterioscler Thromb Vasc Biol **2004** Jul;24(7):1143-6.

20. Reiss M, Roos D. Differences in oxygen metabolism of phagocytosing monocytes and neutrophils. J Clin Invest **1978** Feb;61(2):480-8.
21. Domigan NM, Charlton TS, Duncan MW, Winterbourn CC, Kettle AJ. Chlorination of tyrosyl residues in peptides by myeloperoxidase and human neutrophils. J Biol Chem **1995** Jul 14;270(28):16542-8.
22. Weiss SJ, Klein R, Slivka A, Wei M. Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. J Clin Invest **1982** Sep;70(3):598-607.
23. Yang CY, Gu ZW, Yang M, et al. Selective modification of apoB-100 in the oxidation of low density lipoproteins by myeloperoxidase in vitro. J Lipid Res **1999** Apr;40(4):686-98.
24. Hazen SL, Crowley JR, Mueller DM, Heinecke JW. Mass spectrometric quantification of 3-chlorotyrosine in human tissues with attomole sensitivity: a sensitive and specific marker for myeloperoxidase-catalyzed chlorination at sites of inflammation. Free Radic Biol Med **1997**;23(6):909-16.
25. Hazen SL, Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J Clin Invest **1997** May 1;99(9):2075-81.
26. Daugherty A, Dunn JL, Rateri DL, Heinecke JW. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. J Clin Invest **1994** Jul;94(1):437-44.
27. Malle E, Marsche G, Arnhold J, Davies MJ. Modification of low-density lipoprotein by myeloperoxidase-derived oxidants and reagent hypochlorous acid. Biochim Biophys Acta **2006** Apr;1761(4):392-415.
28. Jacob JS, Cistola DP, Hsu FF, et al. Human phagocytes employ the myeloperoxidase-hydrogen peroxide system to synthesize dityrosine, trityrosine, pulcherosine, and isodityrosine by a tyrosyl radical-dependent pathway. J Biol Chem **1996** Aug 16;271(33):19950-6.
29. Hazen SL, Zhang R, Shen Z, et al. Formation of nitric oxide-derived oxidants by myeloperoxidase in monocytes: pathways for monocyte-mediated protein nitration and lipid peroxidation In vivo. Circ Res **1999** Nov 12;85(10):950-8.
30. Stocker R. Lipoprotein oxidation: mechanistic aspects, methodological approaches and clinical relevance. Curr Opin Lipidol **1994** Dec;5(6):422-33.
31. Yu BP. Cellular defenses against damage from reactive oxygen species. Physiol Rev **1994** Jan;74(1):139-62.
32. Koenig W, Khuseyinova N. Biomarkers of atherosclerotic plaque instability and rupture. Arterioscler Thromb Vasc Biol **2007** Jan;27(1):15-26.
33. Thiebaut R, Daucourt V, Mercie P, et al. Lipodystrophy, metabolic disorders, and human immunodeficiency virus infection: Aquitaine Cohort, France, 1999. Groupe d'Epidemiologie Clinique du Syndrome d'Immunodeficience Acquise en Aquitaine. Clin Infect Dis **2000** Dec;31(6):1482-7.
34. Sanz J, Fayad ZA. Imaging of atherosclerotic cardiovascular disease. Nature **2008** Feb 21;451(7181):953-7.
35. Bonithon-Kopp C, Touboul PJ, Berr C, Magne C, Ducimetiere P. Factors of carotid arterial enlargement in a population aged 59 to 71 years: the EVA study. Stroke **1996** Apr;27(4):654-60.

36. Braunwald E. Unstable angina: an etiologic approach to management. Circulation **1998** Nov 24;98(21):2219-22.
37. Bots ML, Hoes AW, Hofman A, Witteman JC, Grobbee DE. Cross-sectionally assessed carotid intima-media thickness relates to long-term risk of stroke, coronary heart disease and death as estimated by available risk functions. J Intern Med **1999** Mar;245(3):269-76.
38. Boyle JJ. Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. Curr Vasc Pharmacol **2005** Jan;3(1):63-8.
39. Brown MS, Goldstein JL. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc Natl Acad Sci U S A **1999** Sep 28;96(20):11041-8.
40. Griffith RL, Virella GT, Stevenson HC, Lopes-Virella MF. Low density lipoprotein metabolism by human macrophages activated with low density lipoprotein immune complexes. A possible mechanism of foam cell formation. J Exp Med **1988** Sep 1;168(3):1041-59.
41. Leventhal AR, Chen W, Tall AR, Tabas I. Acid sphingomyelinase-deficient macrophages have defective cholesterol trafficking and efflux. J Biol Chem **2001** Nov 30;276(48):44976-83.
42. Liu B, Xie C, Richardson JA, Turley SD, Dietschy JM. Receptor-mediated and bulk-phase endocytosis cause macrophage and cholesterol accumulation in Niemann-Pick C disease. J Lipid Res **2007** Aug;48(8):1710-23.
43. Valledor AF, Ricote M. Nuclear receptor signaling in macrophages. Biochem Pharmacol **2004** Jan 15;67(2):201-12.
44. Goldstein JL, Brown MS. The LDL receptor and the regulation of cellular cholesterol metabolism. J Cell Sci Suppl **1985**;3:131-7.
45. Mujawar Z, Rose H, Morrow MP, et al. Human immunodeficiency virus impairs reverse cholesterol transport from macrophages. PLoS Biol **2006** Oct;4(11):e365.
46. Vermeer BJ, Reman FC. Cholesterolester accumulation. Arch Dermatol Res **1980**;268(3):321-7.
47. Ye Q, Chen Y, Lei H, et al. Inflammatory stress increases unmodified LDL uptake via LDL receptor: an alternative pathway for macrophage foam-cell formation. Inflamm Res **2009** Nov;58(11):809-18.
48. Witztum JL. The role of oxidized LDL in atherosclerosis. Adv Exp Med Biol **1991**;285:353-65.
49. Rong JX, Rangaswamy S, Shen L, et al. Arterial injury by cholesterol oxidation products causes endothelial dysfunction and arterial wall cholesterol accumulation. Arterioscler Thromb Vasc Biol **1998** Dec;18(12):1885-94.
50. Jessup W, Kritharides L, Stocker R. Lipid oxidation in atherogenesis: an overview. Biochem Soc Trans **2004** Feb;32(Pt 1):134-8.
51. Toshima S, Hasegawa A, Kurabayashi M, et al. Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. Arterioscler Thromb Vasc Biol **2000** Oct;20(10):2243-7.
52. Steinbrecher UP, Zhang HF, Lougheed M. Role of oxidatively modified LDL in atherosclerosis. Free Radic Biol Med **1990**;9(2):155-68.
53. Feng J, Han J, Pearce SF, et al. Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-gamma. J Lipid Res **2000** May;41(5):688-96.

54. Nicholson AC, Febbraio M, Han J, Silverstein RL, Hajjar DP. CD36 in atherosclerosis. The role of a class B macrophage scavenger receptor. Ann N Y Acad Sci **2000** May;902:128-31; discussion 31-3.
55. Krieger M, Abrams JM, Lux A, Steller H. Molecular flypaper, atherosclerosis, and host defense: structure and function of the macrophage scavenger receptor. Cold Spring Harb Symp Quant Biol **1992**;57:605-9.
56. Podrez EA, Febbraio M, Sheibani N, et al. Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. J Clin Invest **2000** Apr;105(8):1095-108.
57. Chisolm GM, 3rd, Hazen SL, Fox PL, Cathcart MK. The oxidation of lipoproteins by monocytes-macrophages. Biochemical and biological mechanisms. J Biol Chem **1999** Sep 10;274(37):25959-62.
58. Ehara S, Ueda M, Naruko T, et al. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation **2001** Apr 17;103(15):1955-60.
59. Waldo SW, Li Y, Buono C, et al. Heterogeneity of human macrophages in culture and in atherosclerotic plaques. Am J Pathol **2008** Apr;172(4):1112-26.
60. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev **2006** Apr;86(2):515-81.
61. Chitu V, Stanley ER. Colony-stimulating factor-1 in immunity and inflammation. Curr Opin Immunol **2006** Feb;18(1):39-48.
62. Comalada M, Xaus J, Sanchez E, Valledor AF, Celada A. Macrophage colony-stimulating factor-, granulocyte-macrophage colony-stimulating factor-, or IL-3-dependent survival of macrophages, but not proliferation, requires the expression of p21(Waf1) through the phosphatidylinositol 3-kinase/Akt pathway. Eur J Immunol **2004** Aug;34(8):2257-67.
63. Komuro I, Keicho N, Iwamoto A, Akagawa KS. Human alveolar macrophages and granulocyte-macrophage colony-stimulating factor-induced monocyte-derived macrophages are resistant to H2O2 via their high basal and inducible levels of catalase activity. J Biol Chem **2001** Jun 29;276(26):24360-4.
64. Fleetwood AJ, Lawrence T, Hamilton JA, Cook AD. Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. J Immunol **2007** Apr 15;178(8):5245-52.
65. Sugiyama S, Okada Y, Sukhova GK, Virmani R, Heinecke JW, Libby P. Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. Am J Pathol **2001** Mar;158(3):879-91.
66. Gately MK, Desai BB, Wolitzky AG, et al. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). J Immunol **1991** Aug 1;147(3):874-82.
67. Okamura H, Tsutsi H, Komatsu T, et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature **1995** Nov 2;378(6552):88-91.
68. Micallef MJ, Ohtsuki T, Kohno K, et al. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. Eur J Immunol **1996** Jul;26(7):1647-51.

69. Tsutsui H, Nakanishi K, Matsui K, et al. IFN-gamma-inducing factor up-regulates Fas ligand-mediated cytotoxic activity of murine natural killer cell clones. J Immunol **1996** Nov 1;157(9):3967-73.
70. Billiau A. Interferon-gamma: biology and role in pathogenesis. Adv Immunol **1996**;62:61-130.
71. Docke WD, Randow F, Syrbe U, et al. Monocyte deactivation in septic patients: restoration by IFN-gamma treatment. Nat Med **1997** Jun;3(6):678-81.
72. Armstrong EJ, Morrow DA, Sabatine MS. Inflammatory biomarkers in acute coronary syndromes: part IV: matrix metalloproteinases and biomarkers of platelet activation. Circulation **2006** Mar 7;113(9):e382-5.
73. Coma G, Pena R, Blanco J, et al. Treatment of monocytes with interleukin (IL)-12 plus IL-18 stimulates survival, differentiation and the production of CXC chemokine ligands (CXCL)8, CXCL9 and CXCL10. Clin Exp Immunol **2006** Sep;145(3):535-44.
74. Fichtlscherer S, Heeschen C, Zeiher AM. Inflammatory markers and coronary artery disease. Curr Opin Pharmacol **2004** Apr;4(2):124-31.
75. Lane BR, Lore K, Bock PJ, et al. Interleukin-8 stimulates human immunodeficiency virus type 1 replication and is a potential new target for antiretroviral therapy. J Virol **2001** Sep;75(17):8195-202.
76. Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res **2002** Feb 22;90(3):251-62.
77. Mohanty JG, Jaffe JS, Schulman ES, Raible DG. A highly sensitive fluorescent micro-assay of H2O2 release from activated human leukocytes using a dihydroxyphenoxazine derivative. J Immunol Methods **1997** Mar 28;202(2):133-41.
78. Landino LM, Mall CB, Nicklay JJ, Dutcher SK, Moynihan KL. Oxidation of 5-thio-2-nitrobenzoic acid, by the biologically relevant oxidants peroxynitrite anion, hydrogen peroxide and hypochlorous acid. Nitric Oxide **2008** Feb;18(1):11-8.
79. Mizoguchi T, Edano T, Koshi T. A method of direct measurement for the enzymatic determination of cholesteryl esters. J Lipid Res **2004** Feb;45(2):396-401.
80. Steinbrecher UP, Witztum JL, Parthasarathy S, Steinberg D. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. Arteriosclerosis **1987** Mar-Apr;7(2):135-43.
81. Steinbrecher UP. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. J Biol Chem **1987** Mar 15;262(8):3603-8.
82. Libby P, Geng YJ, Aikawa M, et al. Macrophages and atherosclerotic plaque stability. Curr Opin Lipidol **1996** Oct;7(5):330-5.
83. van der Wal AC, Das PK, Tigges AJ, Becker AE. Macrophage differentiation in atherosclerosis. An in situ immunohistochemical analysis in humans. Am J Pathol **1992** Jul;141(1):161-8.
84. Klebanoff SJ. Oxygen metabolism and the toxic properties of phagocytes. Ann Intern Med **1980** Sep;93(3):480-9.
85. Goss SP, Hogg N, Kalyanaraman B. The effect of nitric oxide release rates on the oxidation of human low density lipoprotein. J Biol Chem **1997** Aug 22;272(34):21647-53.
86. Hogg N, Darley-Usmar VM, Wilson MT, Moncada S. The oxidation of alpha-tocopherol in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide. FEBS Lett **1993** Jul 12;326(1-3):199-203.

87. Nakagawara A, Nathan CF, Cohn ZA. Hydrogen peroxide metabolism in human monocytes during differentiation in vitro. J Clin Invest **1981** Nov;68(5):1243-52.
88. Kettle AJ, Winterbourn CC. Assays for the chlorination activity of myeloperoxidase. Methods Enzymol **1994**;233:502-12.
89. Guo Z, Van Remmen H, Yang H, et al. Changes in expression of antioxidant enzymes affect cell-mediated LDL oxidation and oxidized LDL-induced apoptosis in mouse aortic cells. Arterioscler Thromb Vasc Biol **2001** Jul;21(7):1131-8.
90. Nathan CF, Horowitz CR, de la Harpe J, et al. Administration of recombinant interferon gamma to cancer patients enhances monocyte secretion of hydrogen peroxide. Proc Natl Acad Sci U S A **1985** Dec;82(24):8686-90.
91. Albrich JM, McCarthy CA, Hurst JK. Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. Proc Natl Acad Sci U S A **1981** Jan;78(1):210-4.
92. Selvaraj RJ, Zgliczynski JM, Paul BB, Sbarra AJ. Chlorination or reduced nicotinamide adenine dinucleotides by myeloperoxidase: a novel bactericidal mechanism. J Reticuloendothel Soc **1980** Jan;27(1):31-8.
93. Lampert MB, Weiss SJ. The chlorinating potential of the human monocyte. Blood **1983** Sep;62(3):645-51.
94. Hazen SL, Hsu FF, Gaut JP, Crowley JR, Heinecke JW. Modification of proteins and lipids by myeloperoxidase. Methods Enzymol **1999**;300:88-105.
95. Savenkova ML, Mueller DM, Heinecke JW. Tyrosyl radical generated by myeloperoxidase is a physiological catalyst for the initiation of lipid peroxidation in low density lipoprotein. J Biol Chem **1994** Aug 12;269(32):20394-400.
96. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nat Med **2003** Feb;9(2):213-9.
97. Joseph SB, Bradley MN, Castrillo A, et al. LXR-dependent gene expression is important for macrophage survival and the innate immune response. Cell **2004** Oct 15;119(2):299-309.
98. Halvorsen B, Waehre T, Scholz H, et al. Interleukin-10 enhances the oxidized LDL-induced foam cell formation of macrophages by antiapoptotic mechanisms. J Lipid Res **2005** Feb;46(2):211-9.
99. Pinderski Oslund LJ, Hedrick CC, Olvera T, et al. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. Arterioscler Thromb Vasc Biol **1999** Dec;19(12):2847-53.
100. Pinderski LJ, Fischbein MP, Subbanagounder G, et al. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. Circ Res **2002** May 31;90(10):1064-71.
101. Wang P, Wu P, Siegel MI, Egan RW, Billah MM. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. J Biol Chem **1995** Apr 21;270(16):9558-63.
102. Mallat Z, Besnard S, Duriez M, et al. Protective role of interleukin-10 in atherosclerosis. Circ Res **1999** Oct 15;85(8):e17-24.

103. Heeschen C, Dimmeler S, Hamm CW, et al. Serum level of the antiinflammatory cytokine interleukin-10 is an important prognostic determinant in patients with acute coronary syndromes. Circulation **2003** Apr 29;107(16):2109-14.
104. Anguera I, Miranda-Guardiola F, Bosch X, et al. Elevation of serum levels of the anti-inflammatory cytokine interleukin-10 and decreased risk of coronary events in patients with unstable angina. Am Heart J **2002** Nov;144(5):811-7.
105. Persson J, Nilsson J, Lindholm MW. Cytokine response to lipoprotein lipid loading in human monocyte-derived macrophages. Lipids Health Dis **2006**;5:17.

CHAPTER II

An increase in viral load during treatment interruptions induces a burst of factors implicated in cardiovascular diseases.

1 - Introduction

1.1 HIV infection and Cardiovascular disease

Cardiovascular disease is a major cause of morbidity and mortality in HIV-1-infected untreated subjects. HIV-1+ subjects are at a higher risk of suffering cardiovascular disease than HIV-1-negative subjects of the same age, gender and with similar cardiovascular risk factors [1, 2]. In subjects who have never been treated with antiretrovirals, chronic inflammation derived from HIV-1 replication accelerates the development of atherosclerosis. Initiation of antiretroviral therapy (ART) rapidly reduces the cardiovascular risk associated with active HIV-1 replication [3, 4].

1.2 Antiretroviral therapy and metabolic disorders

Treatment of HIV infection with a protease inhibitor (PI)-containing regimens causes severe dyslipidemia, which could be a key contributor to the elevated risk of coronary artery diseases (CAD) in HIV-infected patients [5-8]. The proposed mechanisms mainly deal with elevation of total and low density lipoprotein cholesterol (LDL-C) levels; however, other pathways of lipoprotein metabolism may also contribute to the significant rise in cardiovascular risk in HIV-infected patients. High density lipoprotein (HDL) metabolism is also affected in such patients, as HIV-induced dyslipidemia includes low levels of HDL cholesterol (HDL-C) and high levels of triglycerides [1, 9]. Such metabolic changes increase, in turn, the risk of cardiovascular complications in the long term. The proatherogenic profile associated with ART leads to cholesterol accumulation in the vascular wall through passive (injuries of endothelium) or active mechanisms (scavenger receptors) and the formation of atherosclerotic plaques. Recruitment of monocytes and macrophages into the atherosclerotic plaque is responsible for the activation of enzymes and release of free radicals that cause cytolysis, further endothelial injury and LDL oxidation[10].

1.3 Treatment interruptions and risks of CAD

There is no doubt that continuous treatment of HIV-infected persons with combinations of three or more antiretroviral drugs reduces morbidity and mortality from HIV-1 infection [11-17]. Nevertheless, growing concern about the long-term toxicity of antiretroviral drugs, the prevalence of HIV-1 drug resistance in patients receiving therapy [18, 19], and the substantial cost of continuous treatment has focused interest on postponing therapy or administering it only intermittently [20-28]. Potential benefits of intermittent therapy include (*i*) augmentation of HIV-specific immunity through ‘‘autovaccination’’ [29, 30] from recrudescent viremia that occurs during treatment interruptions; (*ii*) lowering of drug exposure to decrease toxicity [31-33]; and (*iii*) reduction in the cost of antiretroviral therapy [34].

The association of treatment interruptions and CAD is based in the large interruptions studies SMART [35] and STACCATTO [3]. In the first study a total of 5.472 participants with a CD4 count above 350 cells/mm^3 at baseline were randomly assigned to two strategy arms. In the drug conservation (DC) arm, patients stayed off therapy while CD4 count was above 350 cells/mm^3 and resumed when it fell to 250 cells/mm^3. Those in the viral suppression (VS) arm received continuous therapy throughout the study. Among the factors studied the SMART study found that interleukin-6 (IL-6) and D-dimer levels rose significantly after 1 month of treatment interruption in HIV-1-infected subjects and showed a positive correlation with viral load and death in the interruption group unlike the steady-therapy group [35]. Similarly, a sub-analysis of the STACCATO study [3] analysed activation markers in subjects who did or did not interrupt therapy. In contrast with the SMART study, they did not find a link between viral load and IL-6. Nevertheless they found an association with the levels of soluble cell adhesion molecule-1 (s-VCAM-1) and monocyte chemoattractive protein-1 (MCP-1), two pro-inflammatory markers of endothelial dysfunction, increased after treatment interruption and decreased again upon treatment reinitiating. These changes were associated with changes in viral load.

The pathological substrate of acute coronary artery disease (CAD) is plaque rupture leading to thrombus formation and tributary tissue ischaemia or necrosis [36]. Recruitment and activation of monocytes and macrophages into the atherosclerotic

plaque is responsible for the activation of enzymes and release of free radicals that cause cytolysis, further endothelial injury and LDL cholesterol (LDL-C) oxidation [37] as well as the production of cytokines IL-1, IL-6 and IL-8 [38]. This pro-inflammatory cascade increases C-reactive protein (CRP) that oxidize and opsonize LDL-C, facilitating its uptake by macrophages through their acetyl-LDL receptors [36, 37].

1.4 Inflammatory makers of coronary artery disease

1.4.1 IL-6

Is a 26-kDa single chain glycoprotein, produced by many cell types including activated monocytes/macrophages and endothelial cells, as well as by adipose tissue. IL-6 is able to stimulate macrophages to secrete MCP-1 and participates in the proliferation of SMCs. In addition, ECs stimulated by IL-6, express intercellular adhesion molecule-1 (ICAM-1). IL-6 also promotes production of hepatic acute-phase reactants, including CRP. IL-6 is expressed at the shoulder region of atherosclerotic plaques and may increase plaque instability by driving expression of matrix metalloproteinases, MCP-1, and tumor necrosis factor (TNF)-α [39]. Furthermore, IL-6 represents the principal procoagulant cytokine, but its most important function is the amplification of the inflammatory cascade through which IL-6 at least in part might exerts its direct proatherogenic effects in the arterial wall. Indeed, large amounts of IL-6 have been found in human atherosclerotic plaque[36].

1.4.2 IL-8/CXCL8

Is a proinflammatory CXC chemokine of 8.4 kDa that can signal through the CXCR1 and CXCR2 receptors. It is secreted by monocytes, macrophages and endothelial cells. IL-8 has been implicated in a number of inflammatory diseases including atherosclerosis. IL-8 stimulates HIV-1 replication, attracts neutrophils and T cells, stimulates the adhesion of monocytes, and contributes to angiogenesis [38].

1.4.3 CRP

Is synthesized by hepatocytes and its production is under transcriptional control of several cytokines, with interleukin (IL)-6 being a primary stimulus. However, recent evidence has suggested that CRP may be also produced locally in vascular smooth muscle cells (SMCs) and macrophages of atherosclerotic lesions [40, 41]. In vivo data support the concept that CRP is able to bind to lipids [42], opsonize native LDL for macrophages [43], present in atherosclerotic plaques[44] and, therefore, play a direct role in atherogenesis. A recent post-mortem study further confirmed a potential pathogenic role of CRP in atheromatous plaque vulnerability,[45] demonstrating that higher CRP concentrations strongly correlated with increased numbers of thin cap fibroatheromas. Functionally, CRP has several effects that may influence progression of vascular disease, including activation and chemoattraction of circulating monocytes, mediation of endothelial dysfunction, induction of a prothrombotic state, increase of cytokine release, activation of the complement system, facilitation of extracellular matrix remodeling as well as lipid-related effects[46].

1.4.4 D-dimer

Both coagulation and fibrinolytic systems play an important role in the clinically silent evolution and progression of atheroma and in events that follow the rupture of the plaque resulting in clinical symptoms [47].Thrombus formation in a disrupted atherosclerotic plaque triggers most of the cardiovascular ischemic events. As the thrombus is dissolved by the fibrinolytic system, researchers hypothesized that a decrease in fibrinolytic activity could be a risk factor for ischemic events [48-50]. D-Dimer fragments are produced when plasmin, an enzyme that activates fibrinolytic system[51], degrades fibrin to remove it from blood vessels, ducts and organic fluids. When the conversion of fibrinogen in fibrin takes place, the mechanism that keeps the hemostatic balance is activated, with the conversion of plasminogen to plasmin, for fast removal of fibrin, preventing thrombotic complications. D-Dimer plasma levels evaluate not only the activation of the fibrinolytic system, but also the severity of a hypercoagulability condition[52].

In the SMART Study[35], a large international treatment strategy trial comparing CD4 cell-guided structured treatment interruption versus continuous antiretroviral

therapy, researchers found that the level of IL-6 increased by about 30% and D-dimer increased by about 15% in the treatment interruption arm, while remaining stable in the continuous therapy arm. Both markers increased progressively as HIV viral load rose after stopping treatment. Interestingly, increased levels of IL-6 and D-dimer were more strongly associated with all-cause mortality than with heart-related death specifically, indicating that their detrimental effects likely extend beyond the cardiovascular system. Researchers concluded that HIV infection results in activation of coagulation and inflammatory pathways that may impact multiple organs." They suggested that HIV viraemia may directly affect the vascular endothelium, or lining of the blood vessels, resulting in increased coagulation.

1.4.5 Matrix Metalloproteinases-9

Matrix metalloproteinases-9 (MMP-9) belongs to a family of multidomain zinc-dependent endopeptidases that promote degradation of all protein and proteoglycan-core-protein components of the extracellular matrix. MMP-9 is widely expressed in monocytes/ macrophages, ECs and SMCs and fibroblasts. MMPs are involved in the embryonic development and morphogenesis, wound healing and tissue resorption. On the other hand, MMP-9 might be implicated in vascular and cardiac remodeling as a result of dysregulated activation of these enzymes. Recently, several lines of evidences have demonstrated that MMP-9 play an important role in atherogenesis [53]. Most importantly, MMP-9 is highly expressed in macrophage-rich areas of the atherosclerotic plaque, especially at the shoulder region of the cap, which might promote weakening of the fibrous cap and subsequent destabilization of atherosclerotic lesions.

1.4.6 Soluble CD40 Ligand

CD40 and CD40L (CD 154), both members of the TNF superfamily, are coexpressed by all major cells implicated in atherosclerosis, namely activated T-lymphocytes, vascular ECs, SMCs, and monocytes/macrophages. Both, the receptor and the ligand are functional and CD40/CD40L interactions enhance the expression of various proatherogenic molecules like adhesion molecules, various chemokines (eg, MCP-1), cytokines, growth factors, and MMPs. In addition, CD40L mediated functions include prothrombotic actions by enhancing the expression of tissue factor and diminishing the expression of thrombomodulin. OxLDL may play a role as an initial trigger of CD40/CD40L expression. Platelet activation induced by plaque rupture

results in increased surface expression of CD40L, which is then cleaved. Circulating soluble (s) CD40L may activate ECs and CD40 expressed in other cells constitutive for the atherosclerotic plaque and induce a proinflammatory cascade in the vessel wall. Human studies indicated that sCD40L is associated with high intraplaque lipid content in patients[53].

2 - Hypothesis

Recent studies show that HIV-1 load rebound after long periods of antiretroviral therapy (ART) treatment interruptions, results in burst of coronary artery disease biomarkers.

We hypothesized that treatment interruptions as short as two weeks might also trigger a rebound of viral load and induce a burst of inflammatory mediators implicated in the instability of atherosclerotic plaque.

3 - Objectives

The objectives of this study were to:

1) Investigate whether in the early phases of interruption (2 weeks), an increase in viral load rebound resulted in an increase in the levels of inflammatory cytokines and other CAD associated factors.
   1.1) Assess whether the levels of all these mediators of atherosclerosis only depend on the plasma viral load.

2) Assess whether short ART treatment interruption induce major changes in lipids profile

3) Investigate whether during the periods of treatment resumption the levels of these factors returned to basal levels

4) Investigate whether the burst of inflammatory factors were related to the number of interruptions over time.

4-Material and methods

4.1 Study design.

This was a retrospective reanalysis of stored plasma samples (collected from 1998 to 2000) from a prospective, randomized, controlled study designed to assess the virological and immunological effect of a fixed schedule of antiretroviral treatment interruptions in chronically HIV-1-infected subjects ("2x4 Study"). The procedures followed in the study were in accordance with the Helsinki Declaration in 1975, as revised in 1983, were approved by the Clinical Research Ethical Committee of the Hospital Universitary Germans Trias i Pujol, and all patients gave verbal informed consent.

The present sub-analysis sought to investigate whether repeated ART interruptions and an increase in viral load were associated with rapid increases in CAD-related biomarkers.

4.2 Study population.

The parent "2x4 Study" included 21 HIV-1-infected subjects over 18 years of age, with a plasma viral load <20 copies/ml for at least the previous 2 years, a CD4/CD8+ cell ratio > 0.7 during the previous 6 months and a CD4+ count at nadir of ≥ 400 cells/ml, who did not show any contraindication for receiving antiretroviral treatment and had no AIDS-related illness or severe clinical events.

4.3 Intervention and sampling.

In the parent study, all 21 participants underwent six cycles of a "2 weeks off" / "4 weeks on" antiretroviral treatment interruption schedule. Plasma samples were drawn and cryopreserved at –80 ° C on the day when subjects interrupted and reinitiated therapy. In total, 12 serial plasma samples per patient were tested for the presence and levels of CAD biomarkers.

These subjects were divided in two groups; those that had a viral rebound during these two weeks of interruption and those that the viral load remained undetectable. We compared a comprehensive set of inflammatory mediators associated with acute CAD (CXCL8, IL-1β, TNF-α , IL-6), CRP [38] metalloproteinase-9 (MMP-9) [54], soluble CD40 ligand (sCD40L), as well as the lipidic profile of these subjects as the levels of plasma lipids also contribute to the pathology of the disease including (total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), and low density lipoprotein cholesterol (LDL-C).

4.4 Laboratory methods

CD4+ T cell counts were determined in whole blood by flow cytometry. Plasma HIV-1 RNA levels (pVL) were quantified by PCR (Roche Amplicor HIV-1 Monitor assay, Roche, Barcelona, Spain). Plasma inflammatory markers CXCL8, CRP, IL-1β, TNF-α and IL-6 (LabClinics S.A., Barcelona, Spain), D-dimer (American Diagnostica Inc. Stamford, CT, USA), MMP-9 and sCD40L (LabClinics S.A.) were measured by highly sensitive ELISA with commercial kits according to the manufacturer's instructions. Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), and LDL-C were measured by standard enzymatic methods (Biochemistry laboratory). LDL cholesterol was evaluated according to the Friedewald equation: LDL-C = TC - (HDL-C + TG/ 5) when triglycerides were no higher than 5.75 mmol/ml.

4.5 Statistical analysis.

Differences between subjects with pVL rebound and those without rebound were assessed using nonparametric tests (Mann-Whitney U). The study of the changes in CAD markers over time involves the analysis of repeated measures corresponding to markers measured at different times for each subject. The analysis of this longitudinal data was performed with a linear mixed-model[55] , which provides the average change trajectory for each CAD marker. Spearman’s test was used to assess the correlation between cardiovascular markers and pVL. Two-tailed p values ≤ 0.05 were considered

statistically significant. Statistical analyses were conducted using SPSS Version 15.0 software and GraphPad PRISM Version 4.00 for Windows.

The results are expressed as mean ± SE unless stated otherwise.

5 -Results

5.1 Kinetics of T lymphocytes and HIV-1 RNA

Of the 21 subjects enrolled in the study through six cycles of STI, 13 suffered from a significant pVL rebound in the period of treatment interruption in each cycle (Group A) while in the other 8 subjects the pVL remained constant (Group B). In group A, the rebound of pVL was accompanied by a drop in CD4+ T cell levels that was not seen in the other group (data not shown). Finally, the repeated interruptions had a significant time effect on pVL as the mean increments of pVL tended to decrease over time with each cycle.

5.2 Baseline values

At baseline, all 21 subjects had an undetectable viral load and CD4+ T cell counts > 400 cells/µl (Table 1). Of the 21 subjects, 19 had levels of total, LDL and HDL cholesterol above normal levels (≤ 5.2, < 3.36, < 1.45 mmol/ml respectively). Only 5 subjects in which 3 was in Group A were considered hypertriglyceridemic as TG levels were higher than normal (≤ 2 mmol/ml). There were no statistically significant differences in the basal values of the two groups of patients for any of the parameters studied between the group A and group B (Table 1).

	Group A (n = 13)	**Group B (n = 8)**
Women	3	3
Men	10	5
Mean age	37.5± 6.3	40± 4.5
CD4+ T cell counts (Median, IQR)	901(695-1150)	863(705-1050)
CD4+ cell nadir in cells/ml	≥400	≥400
Median Log_{10} HIV RNA (copies/ml)	<20	<20
CXCL8 in pg/ml (M, IQR)	6.75 (6.23-7.91)	7.71 (6.78-8.20)
CRP in µg/ml (M,IQR)	0.2 (0.13-0.27)	0.13 (0.04-0.23)
D-dimer in ng/ml (M, IQR)	39.33 (30.98-50.75)	44.08 (26.29-47.13)
MMP-9 in ng/ml (M, IQR)	66.11 (53.75-74.33)	66.95 (56.51-69.09)
sCD40L in ng/ml (M, IQR)	0.95 (0.55-1.28)	0.84 (0.31-1.13)
T. Cholesterol in mmol/ml (M, IQR)	5.7 (4.7-7.4)	6.9 (5.75-7.45)
LDL Cholesterol in mol/ml (M, IQR)	4.5 (3.42-4.91)	4.89 (4.01-5.1)
HDL Cholesterol mmol/ml (M, IQR)	1.17 (0.88-1.37)	1.1 (1.04-1.2)
Triglycerides in mmol/ml (M, IQR)	1.9 (1.4-2.35)	1.55 (1.03-2.01)

Table 1. Characteristics of the subjects at baseline.

Group A represent subjects with plasma viral load rebound after each interruption and Group B subjects without plasma viral rebound throughout the “2X4” Study.

5.3 Induction of inflammatory cytokines

We then compared changes in the levels of inflammatory cytokines (CXCL8, IL-1β, IL-6 and TNFα.) during the periods of interruption. The levels of IL-1β, TNFα and IL-6 were undetectable in all except two patients, but we observed a sharp increase in CXCL8 in each period of interruption followed by a reduction in CXCL-8 during the periods of treatment (Table 2). That increment was higher in Group A than in Group B (13.19 ± 2.35 pg/mL; P= 0.001 vs. 2.51 ± 0.81 pg/mL; P = 0.015). Furthermore, no significant time effect was observed in any of the groups as the mean increments were similar over time. Interestingly during the 4 weeks of reintroduction of HAART, we observed a reduction in both viral load and in CXCL8 levels.

5.4 Effect of pVL rebound on other cardiovascular disease biomarkers

We then investigated whether the increase in viral load had any effect on the levels of CRP (a protein that has the ability to oxidize and opsonize LDL-C, facilitating its uptake by macrophages). In this study, we observed cyclical changes in CRP plasma concentration with peaks in periods of ART interruption in subjects of Group A (Table 2). Nevertheless, a significant effect of time was observed as the mean increment in CRP tended to decrease over time (1.14 ± 0.07 µg/mL; P < 0.0001). In those subjects without rebound (Group B), although the mean increment in CRP was significant, it was 4-fold lower than that in the former group (0.32 ± 0.06 µg/mL; P = 0.0001). No statistically significant changes were observed over time.

In a similar way we observed a significant increase in the mean increment of plasma D-dimer (a fibrin degradation product present in plasma after a blood clot is degraded by fibrinolysis) and of MMP-9 (a protease that contributes to plaque instability and rupture) in patients from Group A and Group B (D-dimer: 62.9 ± 8.1 ng/mL; P<0.0001 vs. 16.7 ± 3.5 ng/mL; P = 0.016; MMP9: 122.3 ± 20.8 ng/mL; P < 0.0001 vs. 12.1 ± 2.1 ng/mL; P < 0.0001) (Table 2). Overall, the mean increment in D-dimer and MMP-9 levels closely paralleled viral load levels. The mean increments at each interruption were similar and did not vary over time.

In contrast there was no parallelism between changes in pVL and sCD40L (the sCD40 ligand can activate CD40 expressed on endothelial cells and, thereby, induce a proinflammatory cascade in the vessel wall [36]). There was a drop in sCD40L levels during treatment interruption in subjects with or without pVL rebound, although it did not reach statistical significance. Nevertheless the increase in the levels of sCD40L during treatment reinitiation seemed to be associated with an increase in the CD4+ T cell count.

5.5 Plasma lipids

The group of subjects with pVL rebound (Group A) had abnormally high levels of TG when ART was interrupted (2.65 ± 0.71; P = 0.002) when compared with the group of subjects without viral rebound (Group B), in which no significant increment in TG levels was observed (-0.01 ± 0.12 mmol/mL; P = 0.999).

Moreover, the concentration of HDL-C remained unchanged in both groups while there was a reduction in LDL-C levels in all subjects. No significant time effect was observed for TG, HDL-C or LDL-C in either group. Interestingly, there was no parallelism between LDL-C levels and pVL, suggesting that other factors such as HAART administration may play a critical role in lipid disorders caused by LDL-C in HIV-1-infected subjects.

		VIRAL REBOUND				WITHOUT VIRAL REBOUND			
Markers	**units**	**MEAN**	**SE**	**P**	**T. E**	**MEAN**	**SE**	**P**	**T. E**
CXCL8	pg/ml	13.19	2.35	0.001	NO	2.51	0.81	0.015	NO
CRP	µg/ml	1.14	0.07	<0.0001	YES,↓	0.32	0.06	0.0001	NO
D-dimer	ng/ml	62.97	8.12	<0.0001	NO	16.76	3.54	0.016	NO
MMP9	ng/ml	122.36	20.83	<0.0001	NO	12.06	2.18	<0.0001	NO
sCD40	ng/ml	-0.27	0.17	0.142	NO	-0.58	0.24	0.076	NO
HDL	mmol/ml	0.026	0.15	0.859	NO	-0.11	0.03	0.100	NO
LDL	mmol/ml	-0.367	0.03	<0.0001	NO	-0.38	0.06	<0.0001	NO
TRIG	mmol/ml	2.65	0.71	0.002	NO	-0.01	0.12	0.999	NO

Table 2. Changes in CAD makers levels during six cycles of structured treatment interruption. CAD markers levels were measured during six cycles of a "2 weeks off" and "4 weeks on" ART interruption schedule in 21 chronically HIV-1-infected subjects. Thirteen patients had a VL rebound following treatment interruption. Data are presented as Mean and Standard Error. TE: time effect.

6 -Discussion

Cardiovascular complications have become one of the prominent causes of mortality and morbidity in HIV-1 subjects [1, 4-6, 56-59]. The risk of having a cardiovascular event increases with treatment interruptions [60, 61], and although STI are not currently recommended, they form a model for subjects that do not adhere to treatment. The SMART study [35] has shown that higher levels of IL-6 and D-dimer in plasma were strongly associated with an increase risk of death. In STACCATO study [3], soluble cell adhesion molecule-1 (s-VCAM-1) and monocyte chemoattractive protein-1 (MCP-1) increased after treatment interruption followed by a decrease after treatment reinitiation, but the did not found changes in IL-6.

The design of our study differs from the others in that, all 21 participants underwent six cycles of a “2 weeks off” / “4 weeks on” antiretroviral treatment interruption schedule and allowed us to compare those that had a rebound of viral load and those that did not at the early events of STI . It also allowed us to investigate the time effect of repeated cycles of interruptions on the different markers.

Furthermore, at early stages of interruption, we could not detect any IL-1, IL-6 or TNF except for two patients that were positive for IL-6. It is possible that the levels of circulating cytokines in plasma were too low to be detectable with our assays. In contrast, after only 2 weeks of interruption we already found high level of IL-8 in the patients in group A, a cytokine / chemokine that stimulates HIV-1 replication, attracts neutrophils and T cells, stimulates the adhesion of monocytes, and contributes to angiogenesis [38]. Furthermore it’s produced by macrophages and endothelial cells. Interestingly, once treatment was reinitiated, the level of these markers decreased to basal levels including viral load in only 4 weeks. the same ratio that viral load returned to negative levels, reinforcing the hypothesis that the activation induced by viral load might explain our observations.

At the same time the levels of CRP, D-dimer, MMP9 and TG mean also incremented rapidly in the subjects from that group A and all play an important role in the formation and instability of the atheroma plaque. [35, 36, 38, 40, 53, 54, 62-65]. It is unlikely that the changes observed in the levels of these markers are due to the local

production of inflammatory cytokines or from the plaques themselves as they will get diluted in the plasma at very low levels. A plausible explanation for the increased risk of acute CAD after STIs is that the rebound of HIV replication following ART interruption could lead to a burst of inflammatory mediators that, in turn, could induce instability in a previously stable atherosclerotic plaque [3, 66, 67].

In group B we also observed statistically significant change in the levels of CRP, MMP9 during the interruption periods ($p<0.0001$) but these changes were not clinically relevant as their values were always between the normal plasmatic levels. These results also show that very low or undetectable levels of viral load rebound may induces baseline changes of some CAD markers. In this study, and as an exception, sCD40L expression was not only dependent on pVL during the "off" treatment periods but was correlated with the expansion and activation of CD4 lymphocytes during treatment reintroduction [68] .

Less is known about the lipid profile in HIV subjects; the positive correlation between viral load and triglycerides that we found in this study revealed that subjects without an adequate virological response often suffer from hypertriglyceridemia associated with hypoalphalipoproteinemia [1, 58, 59, 61]. Interestingly, hypertriglyceridemia may depend on, or mirror, the severity of the infection, as those subjects with high CD4+ T cell counts did not suffer from hypertriglyceridemia [1], whereas patients with low CD4+ T cell counts sometimes might [60, 69, 70]. No changes were observed in the lipidic profile of HDL in any of the two groups and no changes were observed between the two stages of the interruption and reinitiation of the treatment. In contrast, big changes in LDL cholesterol were observed in both groups supporting the idea that antiretroviral drugs might influence the levels of LDL. Furthermore in agreement with recent studies [61] , we found that STI contributes to the baseline reduction in high LDL-C levels.

Thus, although STI reduces the risk of toxicity associated with long-term treatment with ART, short and repeated interruptions do not prevent the increase in mediators of atherosclerosis. The upregulation and down regulation of these factors are very quick as in only two weeks we can have major changes in these inflammatory markers. In a similar way in only 4 weeks the levels of these markers can return to the normal. Changes in biomarkers plasma levels are not related to the number of interruptions.

Furthermore, we found that STI induces hypertriglyceridemia associated with a reduction in HDL-C levels in subjects without control of pVL rebound. Repeated interruptions change the lipid profile towards a less atherogenic profile due to the progressive reduction in LDL-C. Thus these data emphasize the importance of adhering to treatment to avoid the risk of cardiovascular complications.

7 -Conclusions

In this present study we can give the following conclusions:

1. Short periods of ART treatment interruptions do not prevent the increase in mediators of atherosclerosis and are associated with a clinically relevant burst of CRP, D-dimer, MMP-9, and CXCL8 in subjects without control of pVL rebound.

    1.1. The levels of these mediators of atherosclerosis mainly depend on the level of plasma viral load while that of sCD40L is more influenced by the level of CD4+ T cells.
    1.2. Undetectable levels of viral load rebound may induce baseline changes of some CAD markers during ART treatment interruptions.

2. Short ART treatment interruptions induce significant changes in plasma triglycerides and LDL levels.
    2.1. Hypertriglyceridemia increases with the rebound of viral load
    2.2. LDL-C plasma level is not influenced by the viral load but rather by antiretroviral drugs administration.
    2.3. Lipid profile change towards a less atherogenic profile due to the progressive reduction in LDL-C level.

3. In short interruptions of ART the burst of CAD biomarkers is reversible after antiretroviral treatment resumption.

4. Changes in biomarkers plasma levels are not related to the number of interruptions.

    4.1. As an exception, the number of interruptions might influenced plasma viral load and CRP as both paralleled throughout the interrupted cycles.

8 -References

1. Rose H, Hoy J, Woolley I, et al. HIV infection and high density lipoprotein metabolism. Atherosclerosis **2008** Jul;199(1):79-86.
2. Neuhaus J, Angus B, Kowalska JD, et al. Risk of all-cause mortality associated with nonfatal AIDS and serious non-AIDS events among adults infected with HIV. AIDS Mar 13;24(5):697-706.
3. Calmy A, Gayet-Ageron A, Montecucco F, et al. HIV increases markers of cardiovascular risk: results from a randomized, treatment interruption trial. AIDS **2009** May 15;23(8):929-39.
4. Grinspoon S, Carr A. Cardiovascular risk and body-fat abnormalities in HIV-infected adults. N Engl J Med **2005** Jan 6;352(1):48-62.
5. Sabin CA, Worm SW, Weber R, et al. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIV-infected patients enrolled in the D:A:D study: a multi-cohort collaboration. Lancet **2008** Apr 26;371(9622):1417-26.
6. Friis-Moller N, Reiss P, Sabin CA, et al. Class of antiretroviral drugs and the risk of myocardial infarction. N Engl J Med **2007** Apr 26;356(17):1723-35.
7. Andersen O, Haugaard SB, Andersen UB, et al. Lipodystrophy in human immunodeficiency virus patients impairs insulin action and induces defects in beta-cell function. Metabolism **2003** Oct;52(10):1343-53.
8. Friis-Moller N, Sabin CA, Weber R, et al. Combination antiretroviral therapy and the risk of myocardial infarction. N Engl J Med **2003** Nov 20;349(21):1993-2003.
9. Mujawar Z, Rose H, Morrow MP, et al. Human immunodeficiency virus impairs reverse cholesterol transport from macrophages. PLoS Biol **2006** Oct;4(11):e365.
10. Chisolm GM, 3rd, Hazen SL, Fox PL, Cathcart MK. The oxidation of lipoproteins by monocytes-macrophages. Biochemical and biological mechanisms. J Biol Chem **1999** Sep 10;274(37):25959-62.
11. Palella FJ, Jr., Delaney KM, Moorman AC, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N Engl J Med **1998** Mar 26;338(13):853-60.
12. Ananworanich J, Siangphoe U, Hill A, et al. Highly active antiretroviral therapy (HAART) retreatment in patients on CD4-guided therapy achieved similar virologic suppression compared with patients on continuous HAART: the HIV Netherlands Australia Thailand Research Collaboration 001.4 study. J Acquir Immune Defic Syndr **2005** Aug 15;39(5):523-9.
13. Selik RM, Byers RH, Jr., Dworkin MS. Trends in diseases reported on U.S. death certificates that mentioned HIV infection, 1987-1999. J Acquir Immune Defic Syndr **2002** Apr 1;29(4):378-87.
14. Crum NF, Riffenburgh RH, Wegner S, et al. Comparisons of causes of death and mortality rates among HIV-infected persons: analysis of the pre-, early, and late HAART (highly active antiretroviral therapy) eras. J Acquir Immune Defic Syndr **2006** Feb 1;41(2):194-200.
15. d'arminio Monforte A, Cozzi-Lepri A, Phillips A, et al. Interruption of highly active antiretroviral therapy in HIV clinical practice: results from the Italian Cohort of Antiretroviral-Naive Patients. J Acquir Immune Defic Syndr **2005** Apr 1;38(4):407-16.

16. d'Arminio Monforte A, Sabin CA, Phillips A, et al. The changing incidence of AIDS events in patients receiving highly active antiretroviral therapy. Arch Intern Med **2005** Feb 28;165(4):416-23.
17. Perelson AS, Essunger P, Cao Y, et al. Decay characteristics of HIV-1-infected compartments during combination therapy. Nature **1997** May 8;387(6629):188-91.
18. Finzi D, Blankson J, Siliciano JD, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med **1999** May;5(5):512-7.
19. Lori F, Jessen H, Lieberman J, et al. Treatment of human immunodeficiency virus infection with hydroxyurea, didanosine, and a protease inhibitor before seroconversion is associated with normalized immune parameters and limited viral reservoir. J Infect Dis **1999** Dec;180(6):1827-32.
20. Havlir DV. Structured intermittent treatment for HIV disease: Necessary concession or premature compromise? Proc Natl Acad Sci U S A **2002** Jan 8;99(1):4-6.
21. Lori F, Lisziewicz J. Structured treatment interruptions for the management of HIV infection. JAMA **2001** Dec 19;286(23):2981-7.
22. Hogg RS, Havlir D, Miller V, Montaner JS. To stop or not to stop: that is the question, but what is the answer? AIDS **2002** Mar 29;16(5):787-9.
23. Foli A, Maserati R, Barasolo G, et al. Strategies to decrease viral load rebound, and prevent loss of CD4 and onset of resistance during structured treatment interruptions. Antivir Ther **2004** Feb;9(1):123-32.
24. Maggiolo F, Ripamonti D, Gregis G, Quinzan G, Callegaro A, Suter F. Effect of prolonged discontinuation of successful antiretroviral therapy on CD4 T cells: a controlled, prospective trial. AIDS **2004** Feb 20;18(3):439-46.
25. Cardiello PG, Hassink E, Ananworanich J, et al. A prospective, randomized trial of structured treatment interruption for patients with chronic HIV type 1 infection. Clin Infect Dis **2005** Feb 15;40(4):594-600.
26. Danel C, Moh R, Minga A, et al. CD4-guided structured antiretroviral treatment interruption strategy in HIV-infected adults in west Africa (Trivacan ANRS 1269 trial): a randomised trial. Lancet **2006** Jun 17;367(9527):1981-9.
27. Mocroft A, Phillips AN, Soriano V, et al. Reasons for stopping antiretrovirals used in an initial highly active antiretroviral regimen: increased incidence of stopping due to toxicity or patient/physician choice in patients with hepatitis C coinfection. AIDS Res Hum Retroviruses **2005** Sep;21(9):743-52.
28. Carr A, Cooper DA. Adverse effects of antiretroviral therapy. Lancet **2000** Oct 21;356(9239):1423-30.
29. Lori F, Lewis MG, Xu J, et al. Control of SIV rebound through structured treatment interruptions during early infection. Science **2000** Nov 24;290(5496):1591-3.
30. Abbas UL, Mellors JW. Interruption of antiretroviral therapy to augment immune control of chronic HIV-1 infection: risk without reward. Proc Natl Acad Sci U S A **2002** Oct 15;99(21):13377-8.
31. Gonzalez de Requena D, Nunez M, Jimenez-Nacher I, Soriano V. Liver toxicity caused by nevirapine. AIDS **2002** Jan 25;16(2):290-1.
32. Qurishi N, Kreuzberg C, Luchters G, et al. Effect of antiretroviral therapy on liver-related mortality in patients with HIV and hepatitis C virus coinfection. Lancet **2003** Nov 22;362(9397):1708-13.

33. Mocroft A, Soriano V, Rockstroh J, et al. Is there evidence for an increase in the death rate from liver-related disease in patients with HIV? AIDS **2005** Dec 2;19(18):2117-25.
34. Dybul M, Chun TW, Yoder C, et al. Short-cycle structured intermittent treatment of chronic HIV infection with highly active antiretroviral therapy: effects on virologic, immunologic, and toxicity parameters. Proc Natl Acad Sci U S A **2001** Dec 18;98(26):15161-6.
35. Kuller LH, Tracy R, Belloso W, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. PLoS Med **2008** Oct 21;5(10):e203.
36. Fichtlscherer S, Heeschen C, Zeiher AM. Inflammatory markers and coronary artery disease. Curr Opin Pharmacol **2004** Apr;4(2):124-31.
37. Bukrinsky M, Sviridov D. Human immunodeficiency virus infection and macrophage cholesterol metabolism. J Leukoc Biol **2006** Nov;80(5):1044-51.
38. Lane BR, Lore K, Bock PJ, et al. Interleukin-8 stimulates human immunodeficiency virus type 1 replication and is a potential new target for antiretroviral therapy. J Virol **2001** Sep;75(17):8195-202.
39. Armstrong EJ, Morrow DA, Sabatine MS. Inflammatory biomarkers in acute coronary syndromes: part IV: matrix metalloproteinases and biomarkers of platelet activation. Circulation **2006** Mar 7;113(9):e382-5.
40. Calabro P, Willerson JT, Yeh ET. Inflammatory cytokines stimulated C-reactive protein production by human coronary artery smooth muscle cells. Circulation **2003** Oct 21;108(16):1930-2.
41. Yasojima K, Schwab C, McGeer EG, McGeer PL. Generation of C-reactive protein and complement components in atherosclerotic plaques. Am J Pathol **2001** Mar;158(3):1039-51.
42. de Beer FC, Soutar AK, Baltz ML, Trayner IM, Feinstein A, Pepys MB. Low density lipoprotein and very low density lipoprotein are selectively bound by aggregated C-reactive protein. J Exp Med **1982** Jul 1;156(1):230-42.
43. Zwaka TP, Hombach V, Torzewski J. C-reactive protein-mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis. Circulation **2001** Mar 6;103(9):1194-7.
44. Torzewski J, Torzewski M, Bowyer DE, et al. C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. Arterioscler Thromb Vasc Biol **1998** Sep;18(9):1386-92.
45. Burke AP, Tracy RP, Kolodgie F, et al. Elevated C-reactive protein values and atherosclerosis in sudden coronary death: association with different pathologies. Circulation **2002** Apr 30;105(17):2019-23.
46. Verma S, Devaraj S, Jialal I. Is C-reactive protein an innocent bystander or proatherogenic culprit? C-reactive protein promotes atherothrombosis. Circulation **2006** May 2;113(17):2135-50; discussion 50.
47. Salomaa V, Rasi V, Kulathinal S, et al. Hemostatic factors as predictors of coronary events and total mortality: The FINRISK '92 Hemostasis Study. Arterioscler Thromb Vasc Biol **2002** Feb 1;22(2):353-8.
48. Danesh J, Whincup P, Walker M, et al. Fibrin D-dimer and coronary heart disease: prospective study and meta-analysis. Circulation **2001** May 15;103(19):2323-7.

49. Thompson SG, Kienast J, Pyke SD, Haverkate F, van de Loo JC. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. N Engl J Med **1995** Mar 9;332(10):635-41.
50. Salomaa V, Stinson V, Kark JD, Folsom AR, Davis CE, Wu KK. Association of fibrinolytic parameters with early atherosclerosis. The ARIC Study. Atherosclerosis Risk in Communities Study. Circulation **1995** Jan 15;91(2):284-90.
51. Syrovets T, Simmet T. Novel aspects and new roles for the serine protease plasmin. Cell Mol Life Sci **2004** Apr;61(7-8):873-85.
52. Matsuo T, Kobayashi H, Kario K, Suzuki S. Fibrin D-dimer in thrombogenic disorders. Semin Thromb Hemost **2000**;26(1):101-7.
53. Koenig W, Rothenbacher D, Hoffmeister A, Griesshammer M, Brenner H. Plasma fibrin D-dimer levels and risk of stable coronary artery disease: results of a large case-control study. Arterioscler Thromb Vasc Biol **2001** Oct;21(10):1701-5.
54. Gough PJ, Gomez IG, Wille PT, Raines EW. Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice. J Clin Invest **2006** Jan;116(1):59-69.
55. Singer J, Willet J. Applied Longitudinal Data Analysis. Oxford University Press, NY **2003**.
56. SMART. T, INSIGHT., groups. atDs. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIV-infected patients. AIDS **2008** 2008 September 12;22: F17-24.
57. Fox Z, Phillips A, Cohen C, et al. Viral resuppression and detection of drug resistance following interruption of a suppressive non-nucleoside reverse transcriptase inhibitor-based regimen. AIDS **2008** Nov 12;22(17):2279-89.
58. Lampe FC, Duprez DA, Kuller LH, et al. Changes in lipids and lipoprotein particle concentrations after interruption of antiretroviral therapy. J Acquir Immune Defic Syndr Jul 1;54(3):275-84.
59. Duprez DA, Kuller LH, Tracy R, et al. Lipoprotein particle subclasses, cardiovascular disease and HIV infection. Atherosclerosis **2009** Dec;207(2):524-9.
60. Ananworanich J, Gayet-Ageron A, Le Braz M, et al. CD4-guided scheduled treatment interruptions compared with continuous therapy for patients infected with HIV-1: results of the Staccato randomised trial. Lancet **2006** Aug 5;368(9534):459-65.
61. Seoane E, Resino S, Micheloud D, et al. Lipid and apoprotein profile in HIV-1-infected patients after CD4-guided treatment interruption. J Acquir Immune Defic Syndr **2008** Aug 1;48(4):455-9.
62. Inoue T, Kato T, Uchida T, et al. Local release of C-reactive protein from vulnerable plaque or coronary arterial wall injured by stenting. J Am Coll Cardiol **2005** Jul 19;46(2):239-45.
63. Gunther K, Dhlamini B. D-dimer levels are markedly raised in HIV-related thrombotic thrombocytopenic purpura. AIDS **2007** May 11;21(8):1063-4.
64. Tanaka A, Shimada K, Sano T, et al. Multiple plaque rupture and C-reactive protein in acute myocardial infarction. J Am Coll Cardiol **2005** May 17;45(10):1594-9.
65. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. N Engl J Med **2002** Nov 14;347(20):1557-65.

66. Papasavvas E, Azzoni L, Pistilli M, et al. Acute viral rebound as a result of structured treatment interruptions in chronically HIV-1-infected subjects: a pathway to cardiovascular risk? Program and Abstracts of the 15th Conference on Retroviruses and Opportunistic Infections (CROI); Boston, Massachusetts, Abstract #964 **2008**.

67. Nakajima K, Nakajima Y, Takeichi S, Fujita MQ. Plasma remnant-like lipoprotein particles or LDL-C as major pathologic factors in sudden cardiac death cases. Atherosclerosis **2008** May;198(1):237-46.

68. Sipsas NV, Sfikakis PP, Kontos A, Kordossis T. Levels of soluble CD40 ligand (CD154) in serum are increased in human immunodeficiency virus type 1-infected patients and correlate with CD4(+) T-cell counts. Clin Diagn Lab Immunol **2002** May;9(3):558-61.

69. El-Sadr WM, Lundgren JD, Neaton JD, et al. CD4+ count-guided interruption of antiretroviral treatment. N Engl J Med **2006** Nov 30;355(22):2283-96.

70. Lundgren JD, Babiker A, El-Sadr W, et al. Inferior clinical outcome of the CD4+ cell count-guided antiretroviral treatment interruption strategy in the SMART study: role of CD4+ Cell counts and HIV RNA levels during follow-up. J Infect Dis **2008** Apr 15;197(8):1145-55.

Publications

1. **Distribution of CD31 on CD4 T-Cells from Cord Blood, Peripheral Blood and Tonsil at Different Stages of Differentiation.** ***The Open Immunology Journal,*** **2010, *3,* 19-26.**
Raul Ruiz-Hernandez, Antoni Jou, Cecilia Cabrera, Ferdinand Noukwe, Josep deHaro, Francesc Borras, Julia Blanco, Marco Fernandez, Bonaventura Clotet, Lidia Ruiz and Margarita Bofill.

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Distribution of CD31 on CD4 T-Cells from Cord Blood, Peripheral Blood and Tonsil at Different Stages of Differentiation

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Abstract: CD31+ is a marker for recent thymic emigrants. Nevertheless it is present in a proportion of memory cells. We looked at the distribution of CD31 on CD4 T-cell subpopulations. In cord blood, CD31 was present in the majority of the CD45RA^{high} and 60% of the CD45RA^{low} cells, and in adults over 70% of "true" naive and in 5–10% of all memory subpopulations (central memory, effector memory, follicular helper and T regulatory cells). No major differences were seen in the distribution of chemokine receptors between CD31+ and CD31– populations within the naive cells nor the memory populations except for CCR3 and CCR9, which were preferentially expressed in the CD31+ memory cells. The CD31 distribution and cytokine receptors was similar between HIV negative and positive individuals, and between adult blood and tonsils. There was a correlation between the levels of TRECs and the percentages of CD31 in all samples studied.

Keywords: HIV, CD31, recent thymic emmigrants, thymus, TRECS, chemokine receptors, cord blood, tonsil.

INTRODUCTION

CD31, also known as the Platelet cell adhesion molecule 1 (PECAM 1), is a surface glycoprotein of 130 kDa that belongs to the immunoglobulin super family [1]. This molecule is involved in the migration of cells through the endothelium cell junction and mediates cell-to-cell adhesion [2]. CD31 is expressed on a diversity of cell types including thymocytes, lymphocytes, endothelial cells, circulating monocytes, and granulocytes [1].

Because CD31 is expressed in the majority of thymic cells and on 85% of CD4 cells from cord blood [3,4] it has been suggested that CD31 is a useful marker for recent thymic emigrants. Besides, the CD31+ populations in adults contain very high levels of T-Cell Receptor Excision Circles (TRECs) an episomal DNA produced during the rearrangement of the TCR in the thymus (where the level of TRECs is very high). The episomal DNA is diluted during T cell division and accordingly, naïve T cells have higher levels of TRECs that memory cells. In adults, CD31 is expressed in CD45RA+ subpopulation of CD4+T-cells that contains the majority of TRECs detected on CD4 T-cells [4].

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This population has been named as ^{Thymic}naïve CD4 T cells [5], a cell population that expands in the periphery under homeostatic stimuli. The CD45RA+ CD31 - cell population has been called ^{central} naïve and it expands through TcR engagement [5]. CD31 is also expressed on about 10% of CD4 memory T-cells [3, 4], and accordingly, small amounts of TRECs have also been observed in the memory population.

The expression of CD45RA and CD45RO, two isoforms of the CD45 family, has classically been used to define naïve and memory CD4 T. Nevertheless, CD4 T cells are functionally and phenotypically heterogeneous depending on the antigenic stimuli, the environment where the cells are located, the state of differentiation of the cells, etc [6]. In this study, we have compared the distribution of CD31 on a antigen naïve and antigen primed environment by studying CD4 T cells from cord blood and peripheral blood from adult. Cord blood CD4 T cells do not usually express CD45RO. However, there are two CD45RA populations regarding the intensity of expression of CD45RA: a CD45RA ^{high} and a CD45RA^{low}, the latter being virtually absent in adult PBMCs [7]. We then compared the levels of TRECs within these populations and observed that the levels of TRECs were higher in the CD45RA high than the CD45RA^{low} CD4 T-cells. Second, in order to see whether the 4 major CD31+ and CD31 – naïve and memory population had different expression of chemokine receptors, we compared their distribution of CXCR3, CXCR5, CCR4, CCR5, CCR6, CCR3

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and CCR9 from peripheral blood and tonsils. Third, as the naïve and memory populations are heterogeneous we expanded these studies by looking the expression of CD31 within the following "naïve" and "memory" CD4 subpopulation: CD45RA+, CD45RO-, CCR7+ CD127+ CCR7+cells ("true" naïve populations), CD45RA-, CD45RO+ CCR7-+ (central memory), CD45RO+CCR7- CD4 T CD45RA+ (effector memory [8]), CD45RA- CD45RO+ CXCR5+ (follicular helper) [9] the CD45RA- CD45RO+ CD25 bright (T regs) [10] populations, and the CD45RA+ CD45RO+, CD27-, CCR7- and CD62L-reverted memory population.

Finally because HIV infection can have a dramatic effect on CD4 T cells [11] we compared the correlation of TRECs and CD31 and the expression of chemokine receptors between healthy donors and HIV+ patients to check how HIV could affect $CD4^{thymic}$ naïve populations.

MATERIALS AND METHODOLOGY

Subjects and Samples

Blood samples from healthy adult volunteers, buffy coats and cord blood were provided by the Centre de Transfusions i Banc de Teixits (Valle Hebron Hospital, Barcelona, Spain). Peripheral blood from 34 HIV-infected volunteers that attended the VIH clinic was provided by the Hospital Universitari Germans Trias I Pujol. Twelve out of the 34 patients had never received antiretroviral therapy. Tonsils (n = 15) and peripheral blood were obtained from patients who underwent routine tonsillectomy at the Hospital Municipal de Badalona (Spain). The procedures followed in the study in accordance with the Helsinki Declaration in 1975, as revised in 1983, were approved by the local Ethical Committee and all patients gave informed consent.

The tonsils were disaggregated with forceps and the tonsillar cells were stained by immunofluorescence.

Isolation of CD4 T Subsets

PBMCs from buffy coats, peripheral blood, and tonsil suspensions were isolated by density-gradient centrifugation on Ficoll-Paque gradient (Atom Reactiva, Barcelona, Spain), and resuspended in culture medium.

Isolated CD4/CD45RA+ or CD45RO+ cells were obtained by negative selection by magnetic purification (StemSep™, StemCell Technologies Inc, Vancouver, Canada) with a purity greater than 95% and further enriched for CD31+ (90- 99 %) and CD31- (1- 10 %) with magnetic beads (EasySep™, StemCell Technologies Inc, Vancouver, Canada). Alternatively, T CD4+ lymphocytes were isolated by immunomagnetic separation columns (StemSep) (purity greater 95%) and subsequently stained with CD45RA and CD31 immunofluorescent antibodies and sorted with purity >98% in both CD31+ and CD31- fractions.

Viral Load

Plasma HIV-1 RNA was measured in 0.5 mL of plasma using the Amplicor HIV-1 Monitor ultrasensitive test (Roche Diagnostics, Barcelona, Spain) a reverse transcription-PCR-based assay with a limit of detection of 50 HIV-1 RNA copies per milliliter.

Flow Cytometry Analysis

Phenotypic analysis was performed on whole blood or tonsillar cells by direct immunofluorescence using four fluorochrome combinations of reagents and monoclonal antibodies to CD4, CD45RA, CD45RO, CD62L, CD27, CD31 (Becton Dickinson, Pharmingen, Madrid, Spain), CD127, CD25, and CD69-FITC (DakoCytomation, Barcelona, Spain). Staining of CCR7 was carried out by a primary IgM mouse anti-human CCR7 antibody, isotype IgM (BD Pharmingen), followed by a secondary goat antibody, anti-mouse IgM FITC (BD Pharmingen). The isotype antibodies were used as negative controls. Stained samples were acquired using a FACScalibur flow cytometer (BD).

Quantification of T-Cell Receptor Excision Cycles

Genomic DNA was extracted from pellets of PBMCs tonsillar cells and isolated subsets using QIAamp DNA Blood Mini or Micro Kit (QIAgen, Valencia, CA) according to the manufacturer's instructions. 2.5.

Quantification of TREC and CCR5 was carried out by Real-Time quantitative PCR, performed in a spectrofluorometric thermal cycler (ABI PRISM 7000; Applied Biosystems, Foster City, CA). CCR5 has been used as a reference gene [12] as it is known that it only has two copies of CCR5 per cell [13]. The thermal cycling conditions were 2 min at 50 °C, 10 min at 95 °C, and 50 cycles each at 95 °C for 15 sec and 60 °C for 1 min. The PCR primer and probe sequences were: TREC: forward 5'–CACATCCCTT TCAA CCATGCT- 3' and reverse 5'–GCCAGCTGCAGG GTTTA GG–3' and the fluorogenic probe was FAM - 5' -AC ACCT CTGGTTTTTGTAAAGGTGCCCACT-3' - TAMRA. CCR5 coding sequence was used to measure cell equivalents in the input DNA. CCR5 primers were: forward 5'–TCATTACAC CTGCAGCTCTCATTT- 3' and reverse 5'–ACACCGAAG CAGAGTTTTTAGGAT- 3', and the fluorogenic probe was VIC -5'-CTGGTCCTGCCGCTGCT TGTCA-3' -TAMRA. (Applied Biosystem, Warrington, Cheshire, UK).

Each 50 µl reaction mixture contained 25 µl Taqman Universal PCR Master Mix (PE Applied Biosystems, Foster City, CA), or 250 nM of each TREC primer and 160 nM TREC probe or 300 nM CCR5 forward primer, 900 nM CCR5 reverse primer and 150 nM CCR5 probe and 5 µl of DNA sample.

Standard serial dilutions (from 40 000 to 10 copies for TRECs, and 10^6 to 10^2 copies for CCR5) of plasmid DNA containing the signal-joint TREC or CCR5 fragment in the pGEM T Easy Vector (Promega, Barcelona, Spain) were used to quantify TRECs and the number of cells in each sample. Samples were analysed in triplicate. Values with a deviation higher than 0.3 Ct were discarded and the results obtained were averaged. Results were expressed as TREC copies/million cells (2 X CCR5 copies) [12, 13].

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 4.0 software (GraphPad Software Inc, San Diego, CA). When comparing two groups we used the Mann-Whitney test followed by the Wilcoxon test if the results were obtained from the same samples. Non-parametric distribution was assumed and significance was set at $p<0.01$.

RESULTS AND DISCUSSION

Expression of CD31 and TRECs on CD4 T-Cells from Cord Blood

In order to study the expression of CD31 in an antigen-free environment, we analysed the expression of CD31 on CD4 T-cell subsets from cord blood, where the fetal immune system is unlikely to have been in contact with foreign antigens. Accordingly, the majority of cord blood CD4 T-cells are CD45RO– [7], the latter being a marker that is used to detect memory cells in adults (Fig. **1**). In contrast, the expression of CD45RA was heterogeneous (Fig. **1a**), with cells that expressed high to negligible levels of CD45RA. Over 85% of CD45RA^{high} cells expressed CD31 while the CD45RA^{low} population contained only 60% of cells positive for CD31. Furthermore, the CD45RA^{high} population contained two to three times higher levels of TRECs than the CD45RA ^{low} (27.5 ± 5.1 versus 13.3 ± 4.05 copies x 10^6 cells; ($p = 0.02$) (Fig. **2**) suggesting a possible pathway of differentiation from thymic medullary T-cells to recent thymic emigrants (CD45RA^{high}/CD31+), to a CD45RA^{low} CD31+ and a CD45RA ^{low} CD31– population in cord blood. In adults, and after antigenic stimulation, this population might give rise to two adult populations: CD45RO^{high}, CD45RA ^{low}CD31+ and CD45RO^{high}, CD45RA ^{low} CD31–. This hypothesis is supported by the fact that after TcR activation *in vitro* the CD4+CD45RA^{high}. T-cells down-regulate the level of CD45RA and up-regulate CD45RO, indicating that antigenic challenge might be responsible for the induction of CD45RO in adults [14]. From that point of view it is possible that CD45RA^{low} cells in cord blood are the equivalent of memory cells from antigen-exposed individuals.

Distribution of CD31+ Cells in the CD4 Populations from Peripheral Blood and Tonsils from Adults

Naive and memory cells have different re-circulation patterns: while naïve T-cells traffic from the peripheral blood to the lymph nodes through the high endothelium venules, memory T-cells travel from the peripheral tissue to the lymph nodes *via* the afferent lymph [15]. As CD31 is involved in the transmigration of lymphoid cells through high endothelial venules, we compared the populations from peripheral blood and tonsils obtained from the same subjects in order to see whether any of the populations were selectively retained in the tissues. Furthermore we compared the chemokine receptors profiles on these populations.

As shown in Table **1**, the homeostatic receptors CCR7 and CXCR4 were present in both positive and negative CD31 naïve memory subsets. The inflammatory chemokine receptors (CXCR3, CXCR5, CCR4, CCR5, CCR6, CCR3 and CCR9) were restricted to the two positive and negative memory CD31 populations. We did not find any major

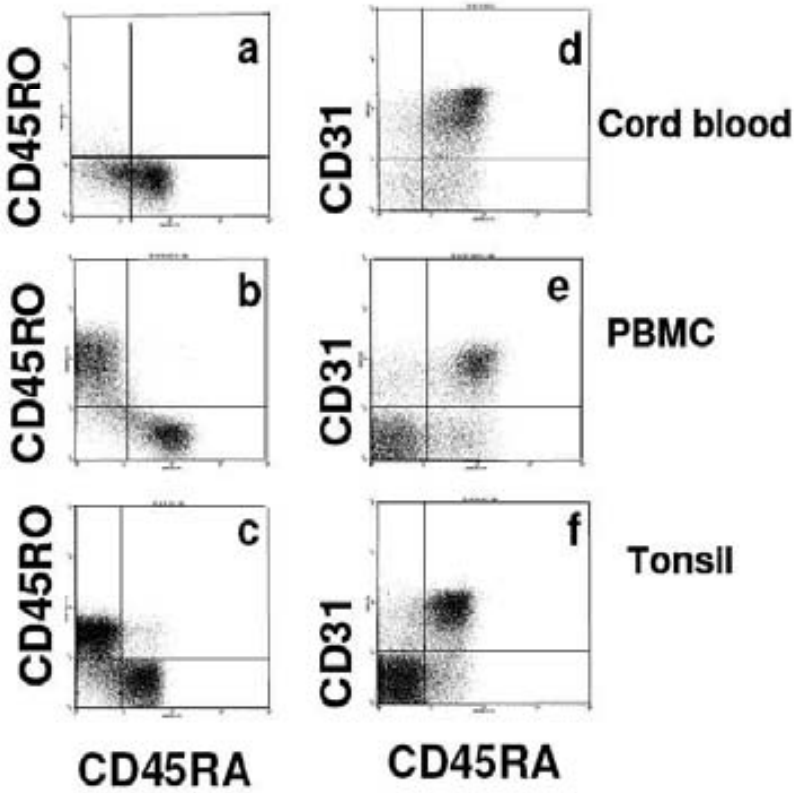

Fig. (1). Percentage of CD31 + CD4 T-cells and levels of TRECs in the different CD4 T-cell populations from cord blood. Mononuclear cells from cord blood, peripheral blood and tonsil were stained with antibodies to CD4, CD45RA, CD45RO and CD31. The figure shows the expression of CD45RA and CD45RO (a-c) or CD45RA and CD31 (d-f) on CD4-gated T-cells in one representative experiment. Fig. (a) shows a very low expression of CD45RO in cord blood and the presence of a CD45RA^{low} CD45RO^{low} population present in cord blood but in very in low numbers in adults.

Table 1. Expression of the Percentage CD31+ Cells in Naïve and Memory CD4 T-Cells in Adults

		PBMC (HIV neg) N = 14	PBMC (HIV +) N = 12	Tonsil N = 11
NAIVE	**CD45RA+RO-**	72.4 ± 2.3	77.37 ± 2.9	87.5 ± 1.9
True naive	**CD45RA+CCR7+**	72.7 ± 4.4	76.9 ± 2.9	83.5 ± 1.7
	CD45RA+CD62L+	73.6 ± 4.7	76.9 ± 2.9	88.8 ± 1.4
	CD45RA+CD27+	72.9 ± 4.6	78.4 ± 2.8	84.6 ± 1.5
	CD45RA+CD127+	71.4 ±5.3	74.9 ±2.9	81.9 ± 1.8
Naive T regs	**CD45RA+CD127-**	56.2 ± 6.7	51.2 ± 5.6	73.4 ± 2.2
MEMORY	**CD45RO+RA-**	6.6 ± 0.4	7.2 ± 0.4	11.4 ± 0.7
Central memory	**CD45RA-CCR7+**	12.1 ± 1.6	17.5 ± 1.7	4.3 ± 1.6
Effector memory	**CD45RA-CCR7-**	4.7 ± 0.4	6.4 ± 1.4	4.1 ± 0.7
Follicular helper	**CD45RA-CXCR5**	7.0 ± 0.8	5.9 ± 1.8	4.9 ± 0.3
T regulatory	**CD45RA-CD127-**	6.8 ± 1.0	7.8 ± 1.6	6.5 ± 0.9
REVERTED MEMORY				
	CD45RA+CCR7-	35.4 ± 7.3	21.5 ± 6.6	84.0 ± 2.8
	CD45RA+CD62L-	29.3 ± 5.2	15.8 ± 3.8	71.3 ± 3.4
	CD45RA+CD27-	12.3 ± 3.5	7.7 ± 1.6	85.5 ± 2.5

The data is expressed as Mean and Standard Error of Mean. PBMC = peripheral blood mononuclear cells.

differences between the percentages of these cytokine receptors between CD31 positive and CD31 negative cells from peripheral blood or tonsillar cells, except for the presence of CCR3 and CCR9, which were more numerous in the CD31+ than in the CD31– cells. The ligands for these two receptors attract cells to the mucosal sites [16, 17]. These populations were nevertheless absent from tonsillar cells, indicating that the cells in mucosal tissues might have a different circulation pathway compared to inflammatory cells.

We then compared the presence of TRECs in the PBMCs and tonsillar cells to elucidate the rate of expansion of the naïve cells at both sites. As mentioned above, the loss of CD45RA was closely mirrored by a decrease in the percentage of CD31+ cells. The majority of TRECs were found in the CD45RA+ CD31+ cells compared to the CD45RA+ CD31– cells and memory populations, and the levels of TRECs present in these populations in blood and tonsils were very similar except for the CD45RA+ CD31– population, in which the levels of TRECs in the tonsils were twice as high as that in peripheral blood (Fig. **2b**).

We then looked at the distribution of CD31 in the CD4 T-cell subpopulations shown in Table **1**. The percentage of CD31 on true naïve CD4 T-cells was identified by the co-expression of CD45RA and CCR7, CD127, CD62L or CD27. Over 90% of CD45RA+ CD4 T-cells expressed CCR7, CD62L, CD27 and CD127. CD31 was present in around 70% of the cells in these populations (Table **1**) from both peripheral blood and tonsils.

It has been postulated that a small proportion of the CD45RA+ CD127– population can be identified as a precursor of a subtype of regulatory T-cells [18]. We found that 56.2 ± 6.7% of adult peripheral blood and 73.4 ± 12.2% of tonsillar cells with this phenotype also expressed CD31. These values are intermediate between those observed in the CD45RA+ naïve and CD45RA– memory populations, indicating that CD31 might be lost during differentiation towards regulatory T-cells.

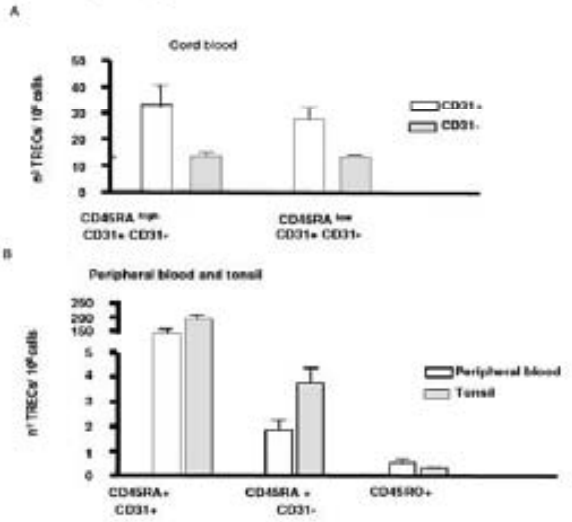

Fig. (2). Percentage of CD31 + CD4 T-cells and levels of TREC in CD4 subsets from cord blood, peripheral blood and tonsil samples. A) Cord blood CD4 CD45RA^{high} and CD45RA^{low} CD4 T-cells were isolated magnetic beads, and further sorted by the expression of CD31. The levels of T-cell receptor excision circles within each fraction were measured as described in the Material and methods. The bars show the number of TRECs x 10^6 cells in the CD45RA^{high} CD31 positive and neg populations and in the CD45RA^{low} CD31+ and CD31 neg populations The data is expressed as mean ± SEM. **B**) The CD45RA+ CD31+, CD45RA+ CD31 negative and CD45RO+ CD4 T-cells from peripheral blood (white bars) and tonsils (grey bars) were isolated as described in material and methods. The levels of T-cell receptor excision circles (TRECs) were measured as described in the Material and methods. The bars show the number of TRECs x 10^6 cells within the different subpopulations. The data is expressed as mean ± SEM (n = 4).

Table 3. Patients Characteristics

Subject	Age (Years)	CD4 Cells/ Cubic mm	Viral Load: (HIV-1 RNA Copies/mL)	Years of Infection	Years on HAART	Years with VL<50 HIV-1 Copies/mL
1	38	401	<50	15	7	5
2	56	377	<50	8	7	6
3	32	264	<50	13	8	2
4	30	651	<50	5	5	4
5	41	340	<50	9	8	6
6	31	194	<50	2	2	1
7	41	322	<50	9	8	4
8	55	446	<50	16	9	0.6
9	67	572	<50	16	11	6
10	40	390	<50	10	9	3
11	18	547	<50	9	8	8
12	31	510	<50	4	4	3
13	51	449	<50	9	8	5
14	46	469	<50	11	10	0.5
15	58	575	<50	18	11	2
16	44	606	<50	9	8	1
17	47	162	<50	9	9	7
18	58	707	<50	11	10	7
19	41	932	<50	17	3	3
20	56	434	<50	20	4	0.5
21	43	585	<50	16	9	3
22	34	575	<50	6	6	1
23	41	661	<50	9	Naive	NA
24	34	429	1100	1	Naive	NA
25	34	468	21000	8	Naive	NA
26	28	355	30000	2	Naive	NA
27	25	1236	33000	1	Naive	NA
28	29	824	6200	4	Naive	NA
29	46	748	15000	1	Naive	NA
30	36	465	79000	2	Naive	NA
31	40	87	<50	1	Naive	NA
32	43	717	<50	1	Naive	NA
33	29	377	11000	1	Naive	NA
34	26	638	21000	1	Naive	NA

CD4 expressed as CD4 cells per cubic millimetre.
VL = Viral Load expressed as HIV-1 RNA copies/millilitre (Amplicor HIV-1 Monitor ultrasensitive test, (Roche, Diagnostics, Barcelona, Spain).
NA = non applicable.
Naive = Subjects that had not received antiretroviral therapy.

We then focussed our attention on the subpopulations of memory CD45RO+ cells (Table **1**). Only 6 to 10% of the CD45RO+ cells were CD31+, but this small percentage of positive cells was also present in all subsets studied, including the follicular helper population (CD45RO+ CXCR5 +) and the regulatory T-cells (CD45RO+ CD127-). There was nevertheless a small but significant difference in the percentage of CD31+ cells between the CCR7– central

and CCR7+ effector memory populations (12.1 ± 1.6 *vs* 4.7 ± 0.4%, p = 0.0001). This difference was not observed in tonsils, where the percentage of CD31 cells was similar to that of peripheral blood effector memory cells (4.7 ± 0.4 *vs* 4.1 ± 0.7).

In sharp contrast, the percentage of CD31+ cells in the peripheral blood reverted memory populations, defined by the expression of CD45RA and lack of expression of CCR7, CD62L or CD27, was very heterogeneous (35.4 ± 7.3%; 29.3 ± 5.2% and 12.3 ± 3.5% respectively) and involved percentages of CD31+ cells that were intermediate between memory and naïve CD4+ T-cells. This suggests that the subpopulations identified by the lack of expression of CCR7, CD62L or CD27 were not identical and that more specific markers are needed to identify this population. In contrast, the percentage of CD31 in the corresponding tonsillar populations (CD45RO+, CCR7–, CD62L– and CD27–) was similar to the levels observed in naïve cells (84.0 ± 2.8%; 71.3 ± 3.4% and 85.5 ± 2.5%, respectively).

HIV Infection

Because HIV infection can damage both thymic and peripheral CD4 T-cells [11], we were interested to know whether HIV infection could influence the expression of CD31. To answer this question we analysed 34 HIV infected subjects from which 12 had not previously received antiretroviral therapy (Table **3**). We first looked at whether CD31 also correlated with the amount of TRECs present in the samples (Fig. **3**), and indeed, we found a positive and significant correlation between the percentage of CD31+ cells and the number of TRECs present in the populations isolated from HIV negative and positive individuals (Fig. **3**) (HIV negative N = : r = 0.3 and HIV positive: r = 0.2; Subjects 1 to 22). As in HIV non-infected subjects, the majority of TRECs were present in the CD4+ CD45RA+ CD31+ cells (data not shown).

Furthermore, we found no statistically significant differences between the percentage of CD31+ cells neither within the CD4 subpopulations (Table **1**) nor in the expression of chemokine receptors (Table **2**) in HIV negative and HIV positive patients who had not received antiretroviral therapy (Patients 23 to 34 from Table **1**). Therefore, CD31 can be used as a marker for recent thymic emigrants in HIV-infected patients. The use of CD31as a marker for recent thymic emigrants has an advantage on the use of the number of TRECs in that the measurement of CD31 is much simpler, quicker and can be carried out at a single cell level in whole blood without the need to isolate the relevant populations.

In summary, we have described a population of cells in cord blood that may be the equivalent of memory cells in an antigen naïve environment. These cells acquire CD45RO and lose CD45RA upon antigenic stimulation *in vitro*.

We have shown the distribution of CD31 among naïve and memory subsets and shown that all memory subpopulations have around 10% of cells that express CD31. Although CD31 is involved in the transmigration of cells we did not find any differences between peripheral blood and tonsillar cells. Finally, we have shown that CD31 also correlates with the number of TRECs in an untreated HIV population and is a marker that can be used to follow recent emigrants in clinical trials.

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ABBREVIATIONS

TRECs	=	T-cell receptor excision circles
HIV	=	Human Immunodeficiency virus
IL	=	Interleukine
T reg	=	T regulatory cells
CD	=	Cluster of differentiation
PECAM-1	=	Platelet cell adhesion molecule 1
Th	=	T helper
PBMCs	=	Peripheral mononuclear cells
MFI	=	Mean fluorescence intensity

REFERENCES

[1] Newman PJ. The biology of PECAM-1. J Clin Invest 1997; 99: 3-8.

[2] Albelda SM, Muller WA, Buck CA, Newman PJ. Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. J Cell Biol 1991; 114: 1059-68.

[3] Stockinger H, Schreiber W, Majdic O, Holter W, Maurer D, Knapp W. Phenotype of human T cells expressing CD31, a molecule of the immunoglobulin supergene family. Immunology 1992; 75: 53-8.

[4] Kimmig S, Przybylski GK, Schmidt CA, *et al*. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. J Exp Med 2002; 195: 789-94.

[5] Kohler S, Thiel A. Life after the thymus: CD31+ and CD31- human naive CD4+ T-cell subsets. Blood 2009; 113: 769-74.

[6] Bofill M, Martinez-Picado J, Ruiz-Hernandez R, *et al*. Naive CD4(+) T cells and recent thymic emigrant levels in treated individuals with HIV: clinical relevance. AIDS Res Hum Retroviruses 2006; 22: 893-6.

[7] Bofill M, Akbar AN, Salmon M, Robinson M, Burford G, Janossy G. Immature CD45RA(low)RO(low) T cells in the human cord blood. I. Antecedents of CD45RA+ unprimed T cells. J Immunol 1994; 152: 5613-23.

[8] Sallusto F, Kremmer E, Palermo B, *et al*. Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. Eur J Immunol 1999; 29: 2037-45.

[9] Aust G, Sittig D, Becherer L, *et al*. The role of CXCR5 and its ligand CXCL13 in the compartmentalization of lymphocytes in thyroids affected by autoimmune thyroid diseases. Eur J Endocrinol 2004; 150: 225-1131.

[10] Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. Eur J Immunol 2001; 31: 1122- 31.

[11] Phillips AN, Sabin CA, Elford J, Bofill M, Janossy G, Lee CA. Acquired immunodeficiency syndrome (AIDS) risk in recent and long-standing human immunodeficiency virus type 1 (HIV-1)-infected patients with similar CD4 lymphocyte counts. Am J Epidemiol 1993; 138: 870-8.

[12] Beloukas A, Paraskevis D, Haida C, Sypsa V, Hatzakis A. Development and assessment of a multiplex real-time PCR assay for quantification of human immunodeficiency virus type 1 DNA. J Clin Microbiol 2009; 47: 2194- 9.

[13] Kostrikis LG, Touloumi G, Karanicolas R, *et al.* Quantitation of human immunodeficiency virus type 1 DNA forms with the second template switch in peripheral blood cells predicts disease progression independently of plasma RNA load. J Virol 2002; 76: 10099-108.

[14] Akbar AN, Terry L, Timms A, Beverley PC, Janossy G. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. J Immunol 1988; 140: 2171-8.

[15] Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. J Exp Med 1990; 171: 801-17.

[16] Pan J, Kunkel EJ, Gosslar U, *et al.* A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. J Immunol 2000; 65: 2943-9.

[17] Campbell JJ, Brightling CE, Symon FA, *et al.* Expression of chemokine receptors by lung T cells from normal and asthmatic subjects. J Immunol 2001; 166: 2842-8.

[18] Valmori D, Merlo A, Souleimanian NE, Hesdorffer CS, Ayyoub M. A peripheral circulating compartment of natural naive CD4 Tregs. J Clin Invest 2005; 115: 1953-62.

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Annex

An increase in viral load during treatment interruptions induces a burst of factors implicated in cardiovascular diseases

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Running head: Coronary disease biomarkers after TI

Abstract

Background: Recent studies show that HIV-1 load rebound after long periods of treatment interruptions (TI), results in burst of coronary artery disease (CAD) biomarkers.

Objective: We investigate whether short interruptions induce a burst of these biomarkers**,** whether their levels return to the baseline during treatment resumption and if the burst were related to the number of interruptions.

Methods: CRP, CXCL8, D-dimer, MMP-9 and plasma lipids were measured from stored plasma samples of 21 chronically HIV-1 infected subjects enrolled in a study evaluating six cycles of "2 weeks off" / "4 weeks on" antiretroviral therapy. Subjects were clustered into those with a viral load rebound after stopping treatment (Group A) and those without (Group B). Plasma measurements were done by ELISA or by enzymatic methods. The analysis of longitudinal data was performed with a linear mixed-model.

Results: The mean increment of CRP (1.1 ± 0.1 µg/mL), MMP-9 (122.3 ± 20.83 ng/mL, CXCL8 (13.1 ± 2.3 pg/mL), D-dimer (62.9 ± 8.1 ng/mL) and triglycerides (2.6 ± 0.1 mmol/ml) was statistically significant higher ($p<0,001$) after each TI in subjects with viral load rebound. Changes of means increment in group B were too low relative to the baseline and without clinical interest as values stayed between the normal plasma ranges. No times effect was observed during TI except for CRP. All biomarkers return to baseline levels after each treatment resumption.

Conclusions: Antiretroviral TI are associated with a clinically relevant burst of acute CAD biomarkers, that indicating the importance of adhering to treatment.

Key words: HIV, Coronary artery disease, Treatment interruptions, CXCL-8, C-reactive protein.

INTRODUCTION

Cardiovascular disease is a major cause of morbidity and mortality in HIV-1-infected untreated subjects [1, 2]. Chronic inflammation derived from HIV-1 replication result in the acceleration of atherosclerosis [3, 4].

The association of treatment interruptions and CAD is based in the large interruptions studies SMART [5] and STACCATTO [3]. In the first study a total of 5.472 participants with a CD4 count above 350 cells/mm^3 at baseline were randomly assigned to two strategy arms. In the drug conservation (DC) arm, patients stayed off therapy while CD4 count was above 350 cells/mm^3 and resumed when it fell to 250 cells/mm^3. Those in the viral suppression (VS) arm received continuous therapy throughout the study. Among the factors studied the SMART study found that interleukin-6 (IL-6) and D-dimer levels rose significantly after 1 month of treatment interruption in HIV-1-infected subjects and showed a positive correlation with viral load and death in the interruption group unlike the steady-therapy group [5]. Similarly, a sub-analysis of the STACCATO study [3] analysed activation markers in subjects who did or did not interrupt therapy. In contrast with the SMART study, they did not find a link between viral load and IL-6. Nevertheless they found an association with the levels of soluble cell adhesion molecule-1 (s-VCAM-1) and monocyte chemoattractive protein-1 (MCP-1), two pro-inflammatory markers of endothelial dysfunction, increased after treatment interruption and decreased again upon treatment reinitiating. These changes were associated with changes in viral load.

The pathological substrate of acute coronary artery disease (CAD) is plaque rupture leading to thrombus formation and tributary tissue ischaemia or necrosis [6]. Recruitment and activation of monocytes and macrophages into the atherosclerotic plaque is responsible for the activation of enzymes and release of free radicals that cause cytolysis, further endothelial injury and LDL cholesterol (LDL-C) oxidation [7] as well as the production of cytokines IL-1, IL-6 and IL-8 [8]. This pro-inflammatory cascade increases C-reactive protein (CRP) that oxidize and opsonize LDL-C, facilitating its uptake by macrophages through their acetyl-LDL receptors [6, 7].

The aim of this study was 1) to investigate whether, in the early phases of interruption, (2 weeks) an increase in viral load rebound resulted in the activation of macrophages and therefore an increase in the levels of inflammatory cytokines and other CAD associated factors. 2) To investigate whether during the periods of treatment the levels of these factors returned to basal levels and 3) whether the burst of inflammatory factors were related to the number of interruptions over time .

In order to solve that question, we used stored plasma samples from subjects who underwent six cycles of a fixed “2 weeks off” / “4 weeks on”, ART interruption schedule (“2x4 Study”) from 1998 to 2000, These patients were divided in two groups; those that had a viral rebound during these two weeks of interruption and those that the viral load remained undetectable. We compared a comprehensive set of inflammatory mediators associated with acute CAD (CXCL8, IL-1β, TNF-α , IL-6), CRP [8] metalloproteinase-9 (MMP-9) [9], soluble CD40 ligand (sCD40L), as well as the lipidic profile of these subjects as the levels of plasma lipids also contribute to the pathology of the disease including (total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), and low density lipoprotein cholesterol (LDL-C).

METHODS

Study design.

This was a retrospective reanalysis of stored plasma samples (collected from 1998 to 2000) from a prospective, randomized, controlled study designed to assess the virological and immunological effect of a fixed schedule of antiretroviral treatment interruptions in chronically HIV-1-infected subjects ("2x4 Study"). The procedures followed in the study were in accordance with the Helsinki Declaration in 1975, as revised in 1983, were approved by the Clinical Research Ethical Committee of the Hospital Universitary Germans Trias i Pujol, and all patients gave verbal informed consent.

The present sub-analysis sought to investigate whether repeated ART interruptions and an increase in viral load were associated with rapid increases in CAD-related biomarkers.

Study population.

The parent "2x4 Study" included 21 HIV-1-infected subjects over 18 years of age, with a plasma viral load <20 copies/ml for at least the previous 2 years, a CD4/CD8+ cell ratio > 0.7 during the previous 6 months and a CD4+ count at nadir of ≥ 400 cells/ml, who did not show any contraindication for receiving antiretroviral treatment and had no AIDS-related illness or severe clinical events.

Intervention and sampling.

In the parent study, all 21 participants underwent six cycles of a "2 weeks off" / "4 weeks on" antiretroviral treatment interruption schedule. Plasma samples were drawn and cryopreserved at –80 ° C on the day when subjects interrupted and reinitiated therapy. In total, 12 serial plasma samples per patient were tested for the presence and levels of CAD biomarkers.

Laboratory methods

CD4+ T cell counts were determined in whole blood by flow cytometry. Plasma HIV-1 RNA levels (pVL) were quantified by PCR (Roche Amplicor HIV-1 Monitor assay, Roche, Barcelona, Spain). Plasma inflammatory markers CXCL8, CRP, IL-1β, TNF-α and IL-6 (LabClinics S.A., Barcelona, Spain), D-dimer (American Diagnostica Inc. Stamford, CT, USA), MMP-9 and sCD40L (LabClinics S.A.) were measured by highly sensitive ELISA with commercial kits according

to the manufacturer's instructions. Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), and LDL-C were measured by standard enzymatic methods (Biochemistry laboratory). LDL cholesterol was evaluated according to the Friedewald equation: LDL-C = TC - (HDL-C + TG/ 5) when triglycerides were no higher than 5.75 mmol/ml.

Statistical analysis. Differences between subjects with pVL rebound and those without rebound were assessed using nonparametric tests (Mann-Whitney U). The study of the changes in CAD markers over time involves the analysis of repeated measures corresponding to markers measured at different times for each subject. The analysis of this longitudinal data was performed with a linear mixed-model[10] , which provides the average change trajectory for each CAD marker. Spearman's test was used to assess the correlation between cardiovascular markers and pVL. Two-tailed p values ≤ 0.05 were considered statistically significant. Statistical analyses were conducted using SPSS Version 15.0 software and GraphPad PRISM Version 4.00 for Windows.

The results are expressed as mean ± SE unless stated otherwise.

RESULTS

Kinetics of T lymphocytes and HIV-1 RNA

Of the 21 subjects enrolled in the study through six cycles of STI, 13 suffered from a significant pVL rebound in the period of treatment interruption in each cycle (Group A) while in the other 8 subjects the pVL remained constant (Group B). In group A, the rebound of pVL was accompanied by a drop in CD4+ T cell levels that was not seen in the other group (data not shown). Finally, the repeated interruptions had a significant time effect on pVL as the mean increments of pVL tended to decrease over time with each cycle.

Baseline values

At baseline, all 21 subjects had an undetectable viral load and CD4+ T cell counts > 400 cells/µl (Table 1). Of the 21 subjects, 19 had levels of total, LDL and HDL cholesterol above normal levels (≤ 5.2, < 3.36, < 1.45 respectively). Only 5 subjects in which 3 was in Group A were considered hypertriglyceridemic as TG levels were higher than normal (≤ 2 mmol/ml). There were no statistically significant differences in the basal values of the two groups of patients for any of the parameters studied between the from group A and group B (Table 1)

Induction of inflammatory cytokines

We then compared changes in the levels of inflammatory cytokines (CXCL8, IL-1β, IL-6 and TNF-α.) during the periods of interruption. The levels of IL-1β, TNF-α and IL-6 were undetectable in all except two patients, but we observed a sharp increase in CXCL8 in each period of interruption followed by a reduction in CXCL-8 during the periods of treatment (Table 2). That increment was higher in Group A than in Group B (13.19 ± 2.35 pg/mL; P= 0.001 vs. 2.51 ± 0.81 pg/mL; P = 0.015). Furthermore, no significant time effect was observed in any of the groups as the mean increments were similar over time. Interestingly during the 4 weeks of reintroduction of HAART, we observed a reduction in both viral load and in CXCL8 levels.

Effect of pVL rebound on other cardiovascular disease biomarkers

We then investigated whether the increase in viral load had any effect on the levels of CRP (a protein that has the ability to oxidize and opsonize LDL-C, facilitating its uptake by macrophages). In this study, we observed cyclical changes in CRP plasma concentration with

peaks in periods of ART interruption in subjects of Group A (Table 2). Nevertheless, a significant effect of time was observed as the mean increment in CRP tended to decrease over time (1.14 ± 0.07 μg/mL; P < 0.0001). In those subjects without rebound (Group B), although the mean increment in CRP was significant, it was 4-fold lower than that in the former group (0.32 ± 0.06 μg/mL; P = 0.0001). No statistically significant changes were observed over time.

In a similar way we observed a significant increase in the mean increment of plasma D-dimer (a fibrin degradation product present in plasma after a blood clot is degraded by fibrinolysis) and of MMP-9 (a protease that contributes to plaque instability and rupture) in patients from Group A and Group B (D-dimer: 62.9 ± 8.1 ng/mL; P<0.0001 vs. 16.7 ± 3.5 ng/mL; P = 0.016; MMP9: 122.3 ± 20.8 ng/mL; P < 0.0001 vs. 12.1 ± 2.1 ng/mL; P < 0.0001) (Table 2). Overall, the mean increment in D-dimer and MMP-9 levels closely paralleled viral load levels. The mean increments at each interruption were similar and did not vary over time.

In contrast there was no parallelism between changes in pVL and sCD40L (the sCD40 ligand can activate CD40 expressed on endothelial cells and, thereby, induce a proinflammatory cascade in the vessel wall [6]). There was a drop in sCD40L levels during treatment interruption in subjects with or without pVL rebound, although it did not reach statistical significance. Nevertheless the increase in the levels of sCD40L during interruption seemed to be associated with an increase in the CD4+ T cell count.

Plasma lipids

The group of subjects with pVL rebound (Group A) had abnormally high levels of TG when ART was interrupted (2.65 ± 0.71; P = 0.002) when compared with the group of subjects without viral rebound (Group B), in which no significant increment in TG levels was observed (-0.01 ± 0.12 mmol/mL; P = 0.999).

Moreover, the concentration of HDL-C remained unchanged in both groups while there was a reduction in LDL-C levels in all subjects. No significant time effect was observed for TG, HDL-C or LDL-C in either group. Interestingly, there was no parallelism between LDL-C levels and pVL, suggesting that other factors such as HAART administration may play a critical role in lipid disorders caused by LDL-C in HIV-1-infected subjects.

DISCUSSION

Cardiovascular complications have become one of the prominent causes of mortality and morbidity in HIV-1 subjects [1, 4, 11-16]. The risk of having a cardiovascular event increases with treatment interruptions [17, 18], and although STI are not currently recommended, they form a model for subjects that do not adhere to treatment. The SMART study [5] has shown that higher levels of IL-6 and D-dimer in plasma were strongly associated with an increase risk of death. In STACCATO study [3], soluble cell adhesion molecule-1 (s-VCAM-1) and monocyte chemoattractive protein-1 (MCP-1) increased after treatment interruption followed by a decrease after treatment reinitiation, but they did not found changes in IL-6.

The design of our study differs from the others in that, all 21 participants underwent six cycles of a “2 weeks off” / “4 weeks on” antiretroviral treatment interruption schedule and allowed us to compare those that had a rebound of viral load and those that did not at the early events of STI . It also allowed us to investigate the time effect of repeated cycles of interruptions on the different markers.

Furthermore, at early stages of interruption, we could not detect any IL-1, IL-6 or TNF except for two patients that were positive for IL-6. It is possible that the levels of circulating cytokines in plasma were too low to be detectable with our assays. In contrast, after only 2 weeks of interruption we already found high level of IL-8 in the patients in group A, a cytokine / chemokine that stimulates HIV-1 replication, attracts neutrophils and T cells, stimulates the adhesion of monocytes, and contributes to angiogenesis [8]. Furthermore it’s produced by macrophages and endothelial cells. Interestingly, once treatment was reinitiated, the level of these markers decreased to basal levels including viral load in only 4 weeks. the same ratio that viral load returned to negative levels, reinforcing the hypothesis that the activation induced by viral load might explain our observations.

At the same time the levels of CRP, D-dimer, MMP9 and TG mean also incremented rapidly in the subjects from that group A and all play an important role in the formation and instability of the atheroma plaque. [5, 6, 8, 9, 19-24]. It is unlikely that the changes observed in the levels of these markers are due to the local production of inflammatory cytokines or from the plaques themselves as they will get diluted in the plasma at very low levels. A plausible explanation for the increased risk of acute CAD after STIs is that the rebound of HIV replication following ART interruption could lead to a burst of inflammatory mediators that, in turn, could induce instability in a previously stable atherosclerotic plaque [3, 25, 26].

In group B we also observed statistically significant change in the levels of CRP, MMP9 during the interruption periods ($p<0.0001$) but these changes were not clinically relevant as their values were always between the normal plasmatic levels. These results also show that very low or undetectable levels of viral load rebound may induces baseline changes of some CAD markers. In this study, and as an exception, sCD40L expression was not only dependent on pVL during the "off" treatment periods but was correlated with the expansion and activation of CD4 lymphocytes during treatment reintroduction [27] .

Less is known about the lipid profile in HIV subjects; the positive correlation between viral load and triglycerides that we found in this study revealed that subjects without an adequate virological response often suffer from hypertriglyceridemia associated with hypoalphalipoproteinemia [1, 15, 16, 18]. Interestingly, hypertriglyceridemia may depend on, or mirror, the severity of the infection, as those subjects with high CD4+ T cell counts did not suffer from hypertriglyceridemia [1], whereas patients with low CD4+ T cell counts sometimes might [17, 28, 29]. No changes were observed in the lipidic profile of HDL in any of the two groups and no changes were observed between the two stages of the interruption and reinitiation of the treatment. In contrast, big changes in LDL cholesterol were observed in both groups supporting the idea that antiretroviral drugs might influence the levels of LDL. Furthermore in agreement with recent studies [18] , we found that STI contributes to the baseline reduction in high LDL-C levels.

In conclusion, although STI reduces the risk of toxicity associated with long-term treatment with ART, short and repeated interruptions do not prevent the increase in mediators of atherosclerosis. The up-regulation and down regulation of these factors are very quick as in only two weeks we can have major changes in these inflammatory markers. In a similar way in only 4 weeks the levels of these markers can return to the normal. Changes in biomarkers plasma levels are not related to the number of interruptions. Furthermore, we found that STI induces hypertriglyceridemia associated with a reduction in HDL-C levels in subjects without control of pVL rebound. Repeated interruptions change the lipid profile towards a less atherogenic profile due to the progressive reduction in LDL-C. Thus these data emphasize the importance of adhering to treatment to avoid the risk of cardiovascular complications.

ABBREVIATIONS

CAD = Coronary artery disease

CRP = C-reactive protein

HIV = Human immunodeficiency virus

pVL = plasma viral load

MMP-9 = Matrix metalloproteinase-9

sCD40L = soluble CD40 ligand

CXCL8, IL8 = Interleukin-8

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REFERENCES

[1] Rose H, Hoy J, Woolley I, *et al*. HIV infection and high density lipoprotein metabolism. Atherosclerosis 2008; 199:79-86.

[2] Neuhaus J, Angus B, Kowalska JD, *et al*. Risk of all-cause mortality associated with nonfatal AIDS and serious non-AIDS events among adults infected with HIV. AIDS 2010; 24:697-706.

[3] Calmy A, Gayet-Ageron A, Montecucco F, *et al*. HIV increases markers of cardiovascular risk: results from a randomized, treatment interruption trial. AIDS 2009; 23:929-39.

[4] Grinspoon S, Carr A. Cardiovascular risk and body-fat abnormalities in HIV-infected adults. N Engl J Med 2005; 352:48-62.

[5] Kuller LH, Tracy R, Belloso W, *et al*. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. PLoS Med 2008; 5:e203.

[6] Fichtlscherer S, Heeschen C, Zeiher AM. Inflammatory markers and coronary artery disease. Curr Opin Pharmacol 2004; 4:124-31.

[7] Bukrinsky M, Sviridov D. Human immunodeficiency virus infection and macrophage cholesterol metabolism. J Leukoc Biol 2006; 80:1044-51.

[8] Lane BR, Lore K, Bock PJ, *et al*. Interleukin-8 stimulates human immunodeficiency virus type 1 replication and is a potential new target for antiretroviral therapy. J Virol 2001; 75:8195-202.

[9] Gough PJ, Gomez IG, Wille PT, Raines EW. Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice. J Clin Invest 2006; 116:59-69.

[10] Singer J, Willet J. Applied Longitudinal Data Analysis. Oxford University Press, NY 2003.

[11] Sabin CA, Worm SW, Weber R, *et al*. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIV-infected patients enrolled in the D:A:D study: a multi-cohort collaboration. Lancet 2008; 371:1417-26.

[12] Friis-Moller N, Reiss P, Sabin CA, *et al*. Class of antiretroviral drugs and the risk of myocardial infarction. N Engl J Med 2007; 356:1723-35.

[13] SMART/INSIGHT and the D:A:D Study Groups. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIV-infected patients. AIDS 2008; 22: F17-24.

[14] Fox Z, Phillips A, Cohen C, *et al*. Viral resuppression and detection of drug resistance following interruption of a suppressive non-nucleoside reverse transcriptase inhibitor-based regimen. AIDS 2008; 22:2279-89.

[15] Lampe FC, Duprez DA, Kuller LH, *et al*. Changes in lipids and lipoprotein particle concentrations after interruption of antiretroviral therapy. J Acquir Immune Defic Syndr 2010; 54:275-84.

[16] Duprez DA, Kuller LH, Tracy R, *et al*. Lipoprotein particle subclasses, cardiovascular disease and HIV infection. Atherosclerosis 2009; 207:524-9.

[17] Ananworanich J, Gayet-Ageron A, Le Braz M, *et al*. CD4-guided scheduled treatment interruptions compared with continuous therapy for patients infected with HIV-1: results of the Staccato randomised trial. Lancet 2006; 368:459-65.

[18] Seoane E, Resino S, Micheloud D, *et al*. Lipid and apoprotein profile in HIV-1-infected patients after CD4-guided treatment interruption. J Acquir Immune Defic Syndr 2008; 48:455-9.

[19] Inoue T, Kato T, Uchida T, *et al*. Local release of C-reactive protein from vulnerable plaque or coronary arterial wall injured by stenting. J Am Coll Cardiol 2005; 46:239-45.

[20] Calabro P, Willerson JT, Yeh ET. Inflammatory cytokines stimulated C-reactive protein production by human coronary artery smooth muscle cells. Circulation 2003; 108:1930-2.

[21] Gunther K, Dhlamini B. D-dimer levels are markedly raised in HIV-related thrombotic thrombocytopenic purpura. AIDS 2007; 21:1063-4.

[22] Tanaka A, Shimada K, Sano T, *et al*. Multiple plaque rupture and C-reactive protein in acute myocardial infarction. J Am Coll Cardiol 2005; 45:1594-9.

[23] Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. N Engl J Med 2002; 347:1557-65.

[24] Koenig W, Rothenbacher D, Hoffmeister A, Griesshammer M, Brenner H. Plasma fibrin D-dimer levels and risk of stable coronary artery disease: results of a large case-control study. Arterioscler Thromb Vasc Biol 2001; 21:1701-5.

[25] Papasavvas E, Azzoni L, Pistilli M, *et al*. Acute viral rebound as a result of structured treatment interruptions in chronically HIV-1-infected subjects: a pathway to cardiovascular risk? Program and Abstracts of the 15th Conference on Retroviruses and Opportunistic Infections (CROI); Boston, Massachusetts, Abstract #964

2008.

[26] Nakajima K, Nakajima Y, Takeichi S, Fujita MQ. Plasma remnant-like lipoprotein particles or LDL-C as major pathologic factors in sudden cardiac death cases. Atherosclerosis 2008; 198:237-46.

[27] Sipsas NV, Sfikakis PP, Kontos A, Kordossis T. Levels of soluble CD40 ligand (CD154) in serum are increased in human immunodeficiency virus type 1-infected patients and correlate with CD4(+) T-cell counts. Clin Diagn Lab Immunol 2002; 9:558-61.

[28] El-Sadr WM, Lundgren JD, Neaton JD, *et al*. CD4+ count-guided interruption of antiretroviral treatment. N Engl J Med 2006; 355:2283-96.

[29] Lundgren JD, Babiker A, El-Sadr W, *et al*. Inferior clinical outcome of the CD4+ cell count-guided antiretroviral treatment interruption strategy in the SMART study: role of CD4+ Cell counts and HIV RNA levels during follow-up. J Infect Dis 2008; 197:1145-55.

Table 1. Characteristics of subjects at baseline.

Group A represent subjects with plasma viral load rebound after each interruption and Group B subjects without plasma viral rebound throughout the "2X4" Study.

	Group A (n = 13)	**Group B (n = 8)**
Women	3	3
Men	10	5
Mean age	37.5± 6.3	40± 4.5
CD4+ T cell counts (Median, IQR)	901(695-1150)	863(705-1050)
CD4+ cell nadir in cells/ml	≥400	≥400
Median Log_{10} HIV RNA (copies/ml)	<20	<20
CXCL8 in pg/ml (M, IQR)	6.75 (6.23-7.91)	7.71 (6.78-8.20)
CRP in µg/ml (M,IQR)	0.2 (0.13-0.27)	0.13 (0.04-0.23)
D-dimer in ng/ml (M, IQR)	39.33 (30.98-50.75)	44.08 (26.29-47.13)
MMP-9 in ng/ml (M, IQR)	66.11 (53.75-74.33)	66.95 (56.51-69.09)
sCD40L in ng/ml (M, IQR)	0.95 (0.55-1.28)	0.84 (0.31-1.13)
T. Cholesterol in mmol/ml (M, IQR)	5.7 (4.7-7.4)	6.9 (5.75-7.45)
LDL Cholesterol in mol/ml (M, IQR)	4.5 (3.42-4.91)	4.89 (4.01-5.1)
HDL Cholesterol mmol/ml (M, IQR)	1.17 (0.88-1.37)	1.1 (1.04-1.2)
Triglycerides in mmol/ml (M, IQR)	1.9 (1.4-2.35)	1.55 (1.03-2.01)

M= Median; IQR= Interquartile range; CRP= C-reactive protein; MMP= Matrix Metalloproteinases; sCD40L= soluble CD40 ligand; LDL= low density lipoprotein; HDL= High density lipoprotein

Table 2. Changes in CAD marker levels during six cycles of structured treatment interruption. CAD marker levels were measured during six cycles of a "2 weeks off" and "4 weeks on" ART interruption schedule in 21 chronically HIV-1-infected subjects. Thirteen patients had a VL rebound following treatment interruption.

Data are presented as Mean and Standard Error. TE: time effect

		VIRAL REBOUND				WITHOUT VIRAL REBOUND			
Markers	**units**	**MEAN**	**SE**	**P**	**T. E**	**MEAN**	**SE**	**P**	**T. E**
CXCL8	pg/ml	13.19	2.35	0.001	NO	2.51	0.81	0.015	NO
CRP	µg/ml	1.14	0.07	<0.0001	YES,↓	0.32	0.06	0.0001	NO
D-dimer	ng/ml	62.97	8.12	<0.0001	NO	16.76	3.54	0.016	NO
MMP9	ng/ml	122.36	20.83	<0.0001	NO	12.06	2.18	<0.0001	NO
sCD40	ng/ml	-0.27	0.17	0.142	NO	-0.58	0.24	0.076	NO
HDL	mmol/ml	0.026	0.15	0.859	NO	-0.11	0.03	0.100	NO
LDL	mmol/ml	-0.367	0.03	<0.0001	NO	-0.38	0.06	<0.0001	NO
TRIG	mmol/ml	2.65	0.71	0.002	NO	-0.01	0.12	0.999	NO

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