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REVIEW

HIV-1 infection, microenvironment and endothelial cell dysfunction

Pietro Mazzuca, Arnaldo Caruso, Francesca Caccuri

Department of Molecular and Translational Medicine, Section of Microbiology, University of Brescia Medical School, Brescia, Italy

SUMMARY

HIV-1 promotes a generalized immune activation that involves the main targets of HIV-1 infection but also cells that are not sensitive to viral infection. ECs display major dysfunctions in HIV$^+$ patients during long-standing viral infection that persist even in the current cART era, in which new-generation drugs have reduced dysmetabolic side effects and successfully impeded viral replication. *In vivo* studies have failed to demonstrate the presence of replicating virus in ECs suggesting that a direct role of the virus is unlikely, and implying that the mechanism accounting for vascular dysfunction may rely on the indirect action of molecules released in the microenvironment by HIV-1-infected cells. This article reviews the current understanding of how HIV-1 infection can contribute to vascular dysfunction. In particular, we discuss the emerging role played by different HIV-1 proteins in driving inflammation and EC dysregulation, and highlight the need to target them for therapeutic benefit.

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INTRODUCTION

Retroviruses are not common viruses. Their discovery has revolutionized our concept of genetic information, transmission, and evolution. Retroviruses are programmed to integrate within cells and to live together with the infected host. Following the discovery of the first human retrovirus (Poiesz *et al*., 1981), we have become aware of the capability of these viruses to invade our organism and integrate in our genome. From that crucial starting point, we have incredibly realized that approximately 8% of the human genome consists of endogenous retroviruses (Medstrand *et al.,* 2002). This accounts for the presence of peculiar and biologically active proteins in the genome of infectious ancestral retroviruses that they have become an integral part of the human genome, thus providing important new and apparently essential functions of dramatic importance in human evolution. Therefore, it is likely that the exogenous forebears of our endogenous retroviruses once behaved as highly contagious and aggressive infections (Ryan, 2004). In this respect, it is interesting to consider that pandemic retroviral infections are the initial and most dramatic manifestation of a virus/host coevolution aimed at their coexistence and ultimately at changes in the host gene pool.

HIV-1 is the first human retroviral pandemic plague experiences in our time. HIV-1 displays an unusually high mutability having an extremely diverse viral population both within an infected individual and across populations of hosts (Rambaut *et al.,* 2004). HIV-1 is already well adapted to the human host. In fact, it takes advantage of cellular machinery to promote replication and transmission, while possessing adequate equipment for immune evasion strategies (Douek *et al.,* 2002; Kwong *et al.,* 2002). Evidence of additional recent evolutionary adaptations of HIV-1 to the specific immune system comes from the finding that HIV-1 is clearly replacing cytotoxic T lymphocyte (CTL) epitopes to avoid detection by CTLs (da Silva, 2003) and modifying amino acid composition and/or masking critical epitopes to escape antibody neutralization (McGuire *et al.,* 2014; Makvandi-Nejad *et al.,* 2015). Therefore, we expect that its chronic persistence in the human host will necessarily evolve by selecting for changes in its gene products that preserve and strengthen the relationship with its human host.

However, because of its young existence in the human host, HIV-1 is still highly pathogenic in our organism by promoting a generalized immune activation that involves the main targets of HIV-1 infection, such as CD4$^+$ T cells and monocytes/macrophages, but also cells that are not sensitive to viral infection. This is particularly true for ECs: although *in vivo* studies have failed to demonstrate the presence of replicating virus, these cells suffer major dysfunctions in HIV$^+$ patients during long-standing viral infection.

This article reviews the current understanding of how HIV-1 can directly and/or indirectly contribute to vascular dysfunction. In particular, we emphasize the emerging role played by different HIV-1 gene products released in the microenvironment in driving inflammation and EC dysregulation, and highlight the need to target them for therapeutic benefit.

Key words:
HIV-1, Viral proteins, Endothelial cells, Vascular dysfunction, Cytokines, Inflammation.

Corresponding author:
Francesca Caccuri
E-mail: francesca.caccuri@unibs.it

EC DYSFUNCTION IN HIV+ PATIENTS

Besides causing immunodeficiency, HIV-1 infection is characterized by hyperactivation of the immune system and chronic inflammation. In this regard, HIV-1 infec-

tion might potentiate other chronic inflammatory diseases such as atherosclerosis. Recent studies have demonstrated that HIV-1 infection induces a pro-thrombotic state and pro-inflammatory phenomena in the vascular endothelium, leading to increased cardiovascular disease risk (Hsue *et al.,* 2009). Moreover, high plasma HIV-1 RNA levels have been associated with endothelial dysfunction, a well-established predictor of atherosclerosis (Funderburg *et al.,* 2010). More recently, an increased rate of endothelial dysfunction and subclinical signs of atherosclerosis have been found in asymptomatic HIV$^+$ young men with long-standing HIV-1 disease, compared with uninfected subjects (Lo *et al.,* 2010). Since cART has reduced the risk of potentially fatal opportunistic infections and has significantly prolonged the average lifetime of HIV-1-infected people, long-term complications, and in particular cardiovascular events, have emerged as a clinically significant issue and have become the topic of several studies. HIV-1 and cART play crucial roles *per se* in the pathogenesis of endothelial dysfunction and atherosclerotic disease in HIV$^+$ patients (Piconi *et al.,* 2013). HIV-1 infection induces chronic inflammation that produces vascular changes (de Larrañaga *et al.,* 2003; Borderi *et al.,* 2001) and alterations of plasma lipoprotein metabolism (Rose *et al.,* 2008). Moreover, a few antiretroviral drugs, and in particular HIV-1 protease inhibitors, can cause dyslipidemia (Gibellini *et al.,* 2012), thus contributing to the increased risk for endothelial dysfunction. The high risk of endothelial dysfunction persists even in the current treatment era, in which new-generation cART has significantly reduced dysmetabolic side-effects (e.g., insulin resistance, dyslipidemia, and hypertension) (Cao *et al.,* 2010).

HIV-1-induced EC injury

EC damage/dysfunction is an accepted component of the pathophysiology of cardiovascular disease. It can be assessed by changes in EC responses to altered blood flow (e.g. flow-mediated dilatation) and differences in levels of EC-specific molecules released in the blood (e.g. von Willebrand factor).

Vascular integrity results from the equilibrium between the mechanism of vascular damage and repair. Injury to blood vessels is associated with high levels of circulating ECs (cECs) and microvesicles from the endothelium. Under normal conditions, restoration of vascular integrity mainly implies the activity of endothelial progenitor cells (EPCs), plaque neovascularization, and reverse cholesterol transport (Moreno *et al.,* 2009). EPCs are key determinants of endothelial dysfunction and show a high predictive value of early vascular disease. Interestingly, all mechanisms involved in vascular repair seem to be impaired in HIV$^+$ individuals.

These physiological processes are altered in HIV$^+$ individuals who have lower EPC levels than HIV-1-seronegative subjects (da Silva *et al.,* 2011). HIV-1 decreases the number of EPCs by a direct infection of these cells, which are characterized by the expression of the chemokine receptors CCR5 and CXCR4 on cell surface, and antiretroviral therapy restores EPC levels (Teofili *et al.,* 2010). Microvesicles released from the endothelium are discarded in response to vascular damage and have been proposed as biomarkers of endothelial dysfunction (Makin *et al.,* 2004; Blann *et al.,* 2005). Indeed, patients with uncontrolled HIV-1 infection present high levels of cECs and EC-derived microvesicles (Boulanger *et al.,* 2006; Kuller *et al.,* 2008). A combination of high levels of cECs and microvesicles from the endothelium along with reduced EPC levels impairs the necessary compensatory responses involved in the restoration of normal condition and supporting the development and progression of endothelial dysfunction.

HIV$^+$ patients also have high plasma concentrations of high sensitivity C-reactive protein (hsCRP), IL-6, TNF-α, D-dimer, fibrinogen, soluble intercellular adhesion molecule (sICAM), and soluble vascular cell adhesion molecule (sVCAM) suggesting endothelial activation and damage. These molecules are also responsible for an increased interaction of infected monocytes with ECs, thereby disrupting the integrity of the EC monolayer (Dhawan *et al.,* 1995; Gilles *et al.,* 1995) and promoting extravasation of HIV$^+$ cells into peripheral tissues and, consequently, viral dissemination. It is noteworthy that these molecules have higher plasma concentrations in cART-naïve patients than in cART-treated patients (Piconi *et al.,* 2013; Kuller *et al.,* 2008).

HIV-1 infection of ECs

HIV-1 is not an endothelium-tropic virus. It displays a narrow tropism predominantly determined by the cell surface receptors required for HIV-1 infection. CD4 and co-receptors are usually essential for HIV-1 to infect cells efficiently. The chemokine receptor CCR5 is the main co-receptor used *in vivo* but variants that use another co-receptor, CXCR4, evolve during disease. *In vitro*, more than a dozen different co-receptors have been shown to support the infection of cell lines by different HIV-1 strains (Clapham *et al.,* 2001). It is also known that HIV-1 viral particles interact with a range of other cell surface receptors via interactions that involve gp120 binding to the glycolipid galactocerebroside (gal)-C and its sulphated derivative (Clapham *et al.,* 2001).

The endothelium plays an important role in the pathogenesis of viral infection, and its potential involvement in HIV-1 infection is suggested by several *in vitro* observations. HIV-1 is likely to infect ECs depending on the tissue source of ECs and on their functional status. In fact, microvascular ECs from brain, kidney glomeruli, hepatic sinusoid and bone marrow may be infected by HIV-1 in the absence of cytolysis (Moses *et al.,* 1993; Lafon *et al.,* 1993). The infection of brain ECs has been extensively studied for its relevance in neurological diseases associated with HIV-1 infection.

T-cell tropic but not brain-derived macrophage tropic HIV-1 strains selectively infect *in vitro* brain endothelium suggesting that T-cell tropism is important for HIV-1 entry through the blood-brain barrier (Moses *et al.,* 1993) and for *in vivo* infection of ECs in the central nervous system (Bagasra *et al.,* 1996; Del Valle *et al.,* 2000).

Macrovascular ECs are generally found to be resistant to HIV-1 infection, unless treated with a combination of pro-inflammatory cytokines promoting cell activation and/or proliferation. The inflammatory process is often accompanied by conditions favoring EC proliferation, such as vessel repair. Therefore, the presence of HIV-1-induced stimulatory cytokines and the development of an inflammatory microenvironment may determine the switch from abortive to productive HIV-1 infection. Indeed, RANTES- and IFN-γ-mediated enhancement of ICAM-1 exposed on the surface of ECs increases the adhesion of HIV-1$^+$ T-cells

to ECs, promoting EC infection and leading to increased viral transmission (Conaldi *et al.,* 1995; Dianzani *et al.,* 1996). Similarly, treatment of ECs from large vessels with inflammatory cytokine, i.e. TNF-α or IL-1, allows virus replication (Conaldi *et al.,* 1995).

It is worth noting that *in vitro* data are not supported by *in vivo* studies, that have persuasively failed to demonstrate the presence of replicating virus in ECs, suggesting that a direct role of the virus is unlikely, and implying that the mechanism accounting for vascular dysfunction may therefore rely on the indirect action of molecules released in the microenvironment by HIV$^+$ cells.

In the light of these considerations, our aim in the following paragraphs is to assess two essential factors in the development of HIV-1-driven endothelial dysfunction:

a) the virus ability to promote pro-inflammatory cytokines secretion from infected cells;
b) the release of HIV-1-encoded proteins from infected cells in the microenvironment able to affect EC function.

CYTOKINES/CHEMOKINES IN THE HIV-1-CONDITIONED MICROENVIRONMENT

In addition to the progressive loss of CD4$^+$ T-cells, HIV-1 infection is characterized by hyperimmune activation, persistent inflammation, and elevated levels of pro-inflammatory cytokines/chemokines released from immune cells everywhere in the organism.

Cytokine and chemokine production during HIV-1 infection

Following transmission, HIV-1 initially replicates at the transmission site and in local lymphoid tissues and then disseminates (Gasper-smith *et al.,* 2008). Virus expansion is associated with a dramatic depletion of memory CD4$^+$ T cells, particularly from gut-associated lymphoid tissues (Haase, 2005) and the levels of cytokines and chemokines in the plasma are increased. Several groups have reported an upregulation of cytokines and chemokines in HIV$^+$ patients since acute viral replication, including IFN-α, TNF-α, INF-γ, IL-1β, IL-10, IP-10, IL-15, IL-8, IL-6, IL-18 and monocyte chemoattractant protein (MCP)-1 (Zauli *et al.,* 1993a; von Sydow *et al.,* 1991; Graziosi *et al.,* 1996; Zauli *et al.,* 1993b; Sinnico *et al.,* 1993; Stacey *et al.,* 2009). The cellular sources of cytokine and chemokine release during early HIV-1 infection include infected T cells, and activated dendritic cells (DCs), monocytes, macrophages, natural killer (NK) cells and NKT cells (McMichael *et al.,* 2010). The cytokine storm observed during early HIV-1 infection may contribute to the control of viral replication but also to the early immunopathology of the infection and to the associated long-term consequences. cART usually leads to prolonged control of HIV-1 replication but the blocking of viral replication is not linked to a complete recovery from immune dysfunction. A persistent level of immune activation and cytokines production are common features of HIV$^+$ patients even under cART (Vandergeeten *et al.,* 2012). This may be due to an incomplete restoration of the CD4$^+$ T cells and to persistent HIV-1 reservoirs. It is noteworthy that chronic HIV-1 infection and chronic immune activation are considered the leading causes of dramatic lymphoid tissue damage, lymph node cytokine profile changes, and naïve T cell depletion (Vandergeeten *et al.,* 2012; Bioncotto *et al.,* 2007).

Role of cytokines and chemokines in EC dysfunction

HIV-1 is able to generate a systemic chronic inflammatory disorder as a result of continuous stimulation of the immune response. TNF-α, IFN-γ, IL-1β, IL-6, IL-10, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), macrophage colony-stimulating factor (M-CSF) and IL-18 are upregulated by HIV-1 in T cells and monocytes cells (Zeng *et al.,* 2012; Ford *et al.,* 2009; Iannello *et al.,* 2009; Herbeuval *et al.,* 2005).

Systemic chronic inflammatory disorders enhance the development of endothelial dysfunction atherosclerosis, stenosis, arterial damage and cardiovascular injury (Hansson *et al.,* 2011; Gibellini *et al.,* 2013). In particular, increased levels of IL-6 have been associated with carotid atherosclerosis and progressive stenosis, thereby upregulating the lipid uptake in macrophages and inhibiting the activity of lipoprotein lipase (Thakore *et al.,* 2007). IL-18 is increased in the plasma of patients with increased carotid intima-media thickness (IMT) and hypertension (Rabkin, 2009), whereas TNF-α acts on macrophages, ECs and vascular smooth muscle cells via several mechanisms: increased cholesterol uptake and foam cell formation in macrophages; augmented leukocyte transmigration in sub-endothelial structures; and increased proliferation and migration of vascular smooth muscle cells (Kleinbongard *et al.,* 2010).

Liver-synthesized C-reactive protein (CRP) is a member of the pentraxin family factors and is considered a marker for coronary vascular disease and endothelial damage (Corrado *et al.,* 2010). CRP plasma levels are significantly upregulated in HIV$^+$ patients and inversely correlated with CD4$^+$ T lymphocyte count (De Lorenzo *et al.,* 2009), and elevated CRP levels have been associated with an increased risk of myocardial infarction in HIV$^+$ patients (Triant *et al.,* 2009). It is noteworthy that increased levels of IL-6, IL-1 and TNF-α induce CRP, which in turn is able to activate proinflammatory cytokines such as IL-6 and M-CSF in a positive feedback loop (Ballou *et al.,* 1992).

The HIV-1-mediated inflammatory microenvironment causes a continuous recruitment of monocytes that migrate across activated ECs in blood vessels, differentiate into macrophages and produce proinflammatory cytokines, thus determining the progressive damage to vessel structures. Furthermore, HIV-1 replicates in macrophages and induces activation and synthesis of several proinflammatory cytokines that in turn induce EC activation and leukocyte adhesion (Westhorpe *et al.,* 2009). VCAM-1 and ICAM-1 levels are raised during HIV-1 infection (Nordoy *et al.,* 1996) promoting trans-endothelial migration of immune cells and inhibiting their reverse migration (Westhorpe *et al.,* 2009), thus determining the localization of monocytes inside the vessel wall and promoting the formation of foam cells.

Monocytes that have infiltrated the arterial intima may differentiate into different kinds of macrophages: the M1 subset which promotes inflammation and the M2 subset which are inflammatory resolving cells. Polarization of monocytes into the M1 or M2 subset depends on the cytokine storm. Indeed, the T-helper 1 (Th1) cytokines, namely IFNγ and IL-1β, elicit an M1 profile, whereas the Th2 cytokines, as IL-4 and IL-13, drive monocytes to acquire an M2 profile (Mantovani *et al.,* 2004). HIV-1 is able to induce an imbalance in the M1/M2 ratio that may contribute to endothelial dysfunction through cytokine perturbation. Indeed *in vitro* infection of macrophages by HIV-1 polarizes

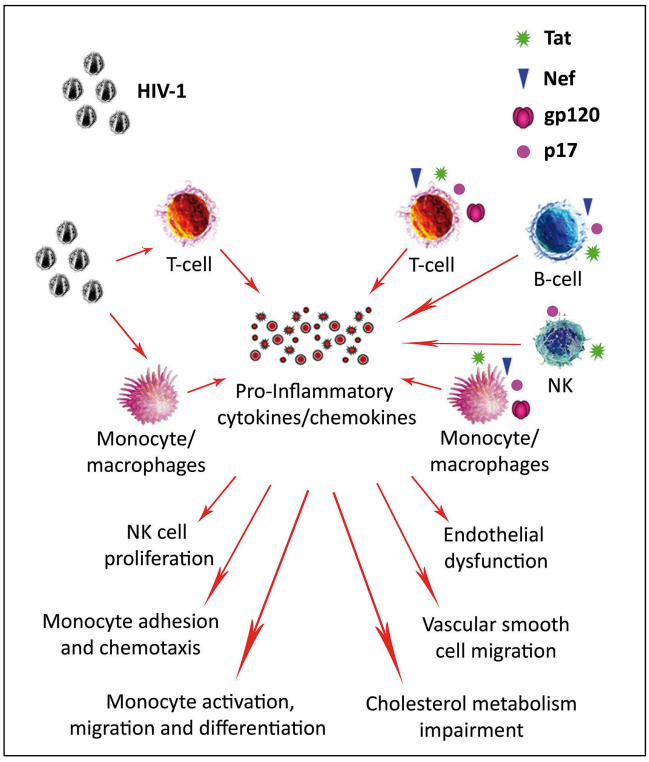

Figure 1 - *Role of HIV-1 and HIV-1 proteins in promoting inflammation, dyslipidemia and EC dysfunction.*

these cells toward the M1 phenotype (Cassol *et al.,* 2010), thus promoting the secretion of those proinflammatory cytokines that contribute to the persistence of inflammation (Tabas, 2010). HIV-1 infection of macrophages also triggers the release of multifunctional molecules, including endothelin-1 (ET-1) (Ehrenreich *et al.,* 1993). ET-1 is a potent vasoconstrictor that promotes the intracellular expression of mRNA for the growth-promoting proto-oncogenes c-fos and c-myc and, subsequently, the migration and proliferation of smooth muscle cells (Komuro *et al.,* 1988). As reviewed by Pellicelli *et al.* (2001), HIV-1-triggered secretion of ET-1, IL-1β, and IL-6 promotes a reduction of vascular nitric oxide production by ECs with the consequent proliferation and migration of smooth muscle cells leading to arterial vasoconstriction.

Interestingly, low high density lipoprotein (HDL) levels and increased triglyceride levels were detected in over 50% of naive HIV$^+$ patients (Grunfeld *et al.,* 1992). The increased levels of triglycerides and the decreased levels of HDL in HIV$^+$ patients are mediated by inflammatory cytokines (IFNγ, TNFα, and IL-1β) that upregulate hepatic fatty acid synthesis and very low density lipoprotein (VLDL) production, impairing cholesterol metabolism (Khovidhunkit *et al.,* 2000).

All these findings suggest that by generating an inflammatory microenvironment HIV-1 contributes to dyslipidemia, EC dysfunction, vascular smooth muscle cells proliferation and migration and, ultimately, to atherosclerotic plaque formation (*Figure 1*).

HIV-1-ENCODED PROTEINS AND EC DYSFUNCTION

HIV-1 has designed its structural and regulatory/accessory proteins to better adapt to the human host thereby promoting virus replication and transmission. Among the many functions in the virus life cycle, a major role played by different HIV-1 proteins in directly driving inflammation (*Figure 1*) and EC dysregulation (*Figure 2*) is taking shape, thus highlighting the need to target these proteins for therapeutic benefit.

The Tat protein

The HIV-1 Tat protein is a regulatory protein that drastically enhances viral transcription (Caputo *et al.,* 1995). Tat is also secreted in the extracellular microenvironment by HIV-1-infected T cells and monocyte/macrophages, where it may act as a proto-cytokine promoting several disease conditions ranging from pulmonary hypertension to sleep disorder (Ensoli *et al.,* 1993; Fiala *et al.,* 2004) by modulating the function of immune cells, mesenchymal cells and ECs (Barillari *et al.,* 1993).

One of the most striking effects of Tat is the induction of a functional program in vascular cells related to angiogenesis. Tat is a direct angiogenic factor (Barillari *et al.,* 1993) and is rich in arginine and lysine in its sequence similarly to potent angiogenic growth factors, such as vascular endothelial growth factor-A (VEGF-A). Tat specifically binds and activates the Flk-1/kinase insert domain receptor (Flk-1/KDR), a VEFG-A tyrosine kinase receptor, and promotes

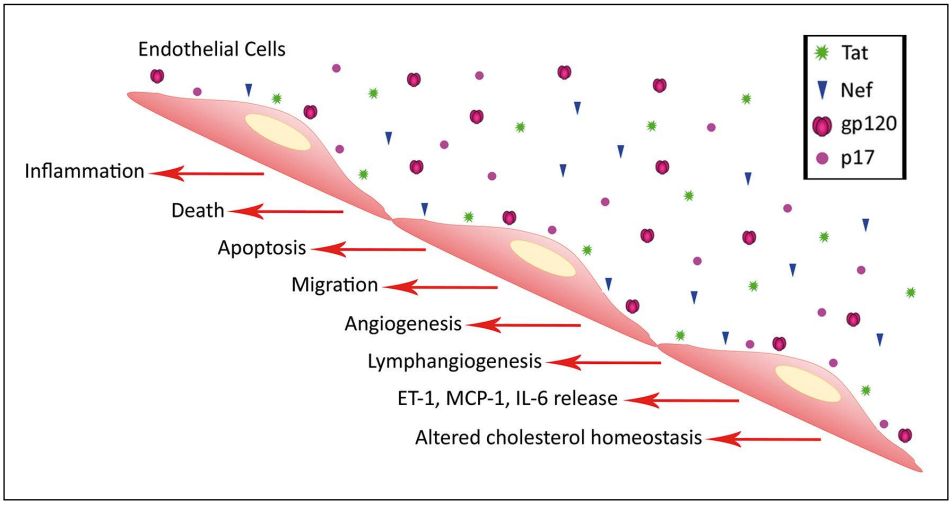

Figure 2 - *Role of HIV-1 proteins in directly driving inflammation and EC dysregulation. In particular, Tat was found to promote: angiogenesis, migration, inflammation, MCP-1 and IL-6 release, and apoptosis. Nef promoted: cell death, apoptosis, MCP-1 release, inflammation and altered cholesterol homeostasis. Gp120 was found to foster: cell death, apoptosis and ET-1 secretion, whereas p17 triggered: inflammation, migration, angiogenesis, lymphangiogenesis, ET-1 and MCP-1 release.*

angiogenesis (Albini *et al.,* 1996 b). Tat angiogenic activity, as well as EC growth, migration and invasion, are promoted by Tat after binding to EC-expressed heparan sulfate proteoglycans (Albini *et al.,* 1996 a).

Tat can be divided into five distinct domains termed N-terminal, cysteine rich, core, basic, and C-terminal. The C-terminal domain of Tat contains an Arg-Gly-Asp (RGD) sequence, which represents the major cell attachment motif recognized by integrin receptors. This Tat domain can bind with high affinity to the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ receptors for fibronectin and vitronectin (Ganju *et al.,* 1998). On the other hand, the basic domain of Tat is able to bind to the integrin $\alpha_v\beta_5$ receptor (Vogel *et al.,* 1993). Tat interaction with cellular receptors leads to the activation of several protein kinases (Albini *et al.,* 1996a; Herrmann *et al.,* 1993; Zidovetzki *et al.,* 1998), including Flk-1/KDR, FLt-1, MAP kinase and of other signaling pathways associated with EC growth, migration and angiogenesis.

Tat was also found to activate ECs thus promoting endothelial transmigration of monocytes and inflammation (Hofman *et al.,* 1993). EC activation includes Tat-mediated induction of E-selectin adhesion molecule expression, IL-6 production (Hofman *et al.,* 1993), and leukocyte adhesion molecules expression as ICAM-1, VCAM-1, and endothelial-leukocyte adhesion molecule 1 (ELAM-1). Tat-induced EC activation is likely to facilitate interaction of inflammatory cells with endothelium (Dhawan *et al.,* 1997) and to promote MCP-1 secretion by activation of the protein kinase C (PKC) signaling pathway (Park *et al.,* 2001b).

In contrast to its roles in angiogenesis, Tat can also impair EC proliferation (Gibellini *et al.,* 2012b) and cause apoptosis of primary microvascular ECs via either TNF-α secretion or through activation of the Fas-dependent pathway (Park *et al.,* 2001 a). Fiala et al. (2004) analyzed the pathogenesis of HIV-1-related cardiomyopathy, examining heart tissue from HIV$^+$ patients and demonstrated the presence of HIV-1 DNA and RNA in inflammatory cells, but not in ECs or cardiomyocytes, although these cells suffered apoptosis. These authors also showed the *in vitro* ability of HIV-1 to invade - but not to replicate - in neonatal rat ventricular myocytes (NRVMs) and coronary artery endothelial cells (CAECs). However, they found that exogenous Tat protein was capable of activating Erk 1/2 phosphorylation, caspase-3 and apoptosis of both cells types, thus concluding that cardiomyopathy pathogenesis may involve HIV-1 replication in immune cells, release of pro-inflammatory molecules, apoptosis of ECs and cardiomyocytes through activation of Tat signaling.

Tat is released by infected cells even during cART (Mediouni *et al.,* 2012). This evidence suggests that in the absence of HIV-1 detectable viremia, persistence of EC dysfunction in HIV$^+$ patients may be, at least in part, mediated by Tat.

The Nef protein

The HIV-1 viral protein Nef is a 27-kD, n-myristoylated protein devoid of enzymatic activity. Nef interaction with membrane and host cell proteins is crucial to sustain its biological activity (Kestler *et al.,* 1991; Kirchhoff *et al.,* 1995). The specific intracellular functions of Nef include alteration of protein trafficking and cell signaling cascades, inhibition of antibody maturation in B cells (Qiao *et al.,* 2006), and enhancement of HIV-1 infectivity (Qi *et al.,* 2008).

It is an adaptor protein with multiple domains important for the interaction with the cellular signaling machinery. Its proline-rich $(PxxP)_3$ domain interacts with Src homology3-containing signaling proteins (Saksela *et al.,* 1995), while other Nef domains interact with the endocytotic cellular machinery responsible for the down-regulation of CD4 (Stoddart *et al.,* 2003).

Nef has been found to induce the formation of conduit-like nanotubes, which connect HIV$^+$ cells to bystander cells (Sowinski *et al.,* 2008). Nef is also delivered to bystander cells through exosomes (Lenassi *et al.,* 2010). Wang *et al*. (2014) showed that Nef induces its own transfer from HIV-1-infected cells to ECs by nanotube-like conduits thus promoting EC activation, dysfunction and death (Wang *et al.,* 2014; Vilhardt *et al.,* 2002; Duffy *et al.,* 2009).

Many studies demonstrate that Nef is able to activate and induce NADPH oxidase with its SH3 binding site and this activity is most important in Nef-induced EC apoptosis. Indeed, this viral protein significantly decreases nitric oxide production and, in contrast, increases superoxide anion production, thus contributing to ROS production, cell oxide stress and cell death.

Acheampong *et al.* (2005) showed that both extracellular and endogenously expressed HIV-1 Nef potently induces apoptosis in primary human brain microvascular ECs (HB-MVECs) by activation of caspases. Treatment of HB-MVECs with extracellular Nef at low concentrations (i.e., 10 ng/ml) resulted in a moderate amount of programmed cell death, whereas at higher concentrations (i.e., 50 and 100 ng/ml) Nef caused a substantial induction of apoptosis. Similarly, Nef-transduced HB-MVECs exhibited dose-dependent programmed cell death. Human apoptosis gene microarray analysis of Nef-transduced HB-MVECs demonstrated that the up-regulated genes involved in Nef-dependent apoptosis belong to both mitochondrial and Fas/FasL apoptotic pathways, suggesting that HIV-1 Nef may utilize more than one pathway to induce apoptosis in HB-MVECs.

Furthermore, Nef protein can increase endothelial MCP-1 production that concurs with endothelial inflammation in HIV$^+$ patients through activation of the NF-kB signaling pathway (Wang *et al.,* 2014).

Recently, it has been shown that Nef is also involved in the alteration of EC cholesterol homeostasis by phosphorylation of Caveolin-1 (Cav-1) at Tyr14, that promotes Cav-1 redistribution and impairment of HDL-mediated cholesterol efflux in ECs (Lin *et al.,* 2015).

The envelope gp120

The HIV-1 envelope gp120 is essential for viral entry by binding to CD4 and to the co-receptor CCR5 or CXCR4. Co-receptor activation by gp120 affects the biological activity of T cells, macrophages, cardiomyocytes, ECs, and central nervous system cells (Ahr *et al.,* 2004). Interestingly, free gp120 was expressed on the surface of apoptotic CD4$^-$ cells (Twu *et al.,* 2002). This observation leads to the hypothesis that cell dysfunction and cell death occurring in HIV$^+$ patients may result not only from the direct cytopathic effects of HIV-1, but also from the effect of soluble gp120 on uninfected bystander cells. Gp120-induced cell death acts through chemokine receptors CXCR4 and CCR5, but interestingly natural ligands of these receptors like stroma cell-derived factor (SDF)-1α or RANTES cause cell growth and activate transcription (Huang *et al.,* 2000). This discrepancy may be explained by the different modality of gp120 interaction with these co-receptors compared to their natural ligands.

Many studies demonstrated that gp120 in the context of viral particles, on the surface of infected cells, or as free soluble protein is also able to damage the endothelium by direct interaction with CXCR4 and, to a lesser extent CCR5, thus promoting CXCR4-dependent caspase and p38 MAPK activation. Huang *et al*. (2000) demonstrated the involvement of PKC in gp120-mediated apoptosis, characterizing a direct relationship between gp120 exposure, PKC activity and apoptosis.

Ullrich *et al.* (2000) demonstrated that gp120-induced cell apoptosis is time- and concentration-dependent and occurred at a concentration very close to that reported in the circulation of patients (Schneider *et al.,* 1986; Oh *et al.,* 1992). The viral glycoptotein is also able to increase ET-1 secretion in primary human lung EC (Kanmogne *et al.,* 2002) in a time-dependent and dose-dependent manner. Induction of ET-1 secretion by gp120 was also observed in brain ECs (Finkel *et al.,* 1995) and human monocytes (Ehrenreich *et al.,* 1993; Didier *et al.,* 2002). Several *in vivo* and *in vitro* studies showed increased expression and secretion of ET-1 following vascular endothelium injury, in particular in lung (Giaid *et al.,* 1993; Jiang *et al.,* 2010). These findings suggest a double role of gp120 in EC dysfunction: the first is a direct effect on apoptosis and the second occurs indirectly, through secretion of ET-1 that may further contribute to cell disruption.

In the context of an inflammatory microenvironment, gp120 may also contribute to reduce the EC-derived nitric oxide (NO) synthesized by the NO synthase, that is a major mediator of endothelium-dependent vasorelaxation and EC dysfunction (Jiang *et al.,* 2010).

Endothelial monocyte activating polypeptide II (EMAPII) is an intracellular protein. It is transported to the cell surface and released in response to various stresses including hypoxia, mechanical strain and apoptosis (Knies *et al.,* 1998; Matschurat *et al.,* 2003; Barnett *et al.,* 2000), and acts as a pro-apoptotic factor. Recently, Green *et al.* (2014) showed that gp120 interaction with CXCR4 and activation of the p38 MAPK signaling pathway promotes a rapid surface expression of EMAPII and CXCR3. This concerted upregulation of EMAPII and CXCR3 is essential for gp120-induced apoptosis, suggesting a novel autocrine/paracrine mechanism of EC apoptosis during HIV-1 infection.

The fact that HIV-1 and/or gp120 can directly induce EC injury has serious implications for the pathogenesis of HIV-1 endothelial dysfunction. Indeed, secreted gp120 is found in the body fluids of HIV^+ patients (Schneider *et al.,* 1986; Oh *et al.,* 1992) and associated with cell membranes (Twu *et al.,* 2002) even in cART-treated patients. It is noteworthy that a long-term persistence of structural proteins, including gp120 has been reported in germinal centers of lymph nodes in the absence of detectable virus replication under cART (Popovic *et al.,* 2005).

The matrix protein

The HIV-1 matrix protein p17 is encoded by 396 nucleotides inside the *Gag* gene. It is a 17-kDa myristoylated protein involved in the HIV-1 pre-integration complex and is sufficient for the assembly of immature particles. Indeed this viral protein plays a key role in the HIV-1 life cycle by contributing to nuclear localization of the pre-integration complex after HIV-1 entry and to RNA binding and by promoting virus maturation and assembly (Fiorentini *et al.,* 2006).

In terms of structural features, p17 is similar to IFN-γ (Matthews *et al.,* 1994). A specific interaction between p17 and IFN-γ has been shown (Caruso *et al.,* 1989). However, p17 is not able to mimic IFN-γ immunomodulatory activity or to interact with IFN-γ receptors (Flamminio *et al.,* 1995). In particular, p17 can form heterodimers with IFN-γ, leaving the ability of the cytokine to perform its biological activities unchanged (Flamminio *et al.,* 1995).

All p17 functional activities occur after the interaction between a functional epitope identified at the N-terminus of the viral protein, with receptors expressed on different target cells (Iaria *et al.,* 2014). Extracellularly, p17 has been found to interact and deregulate the biological activity of many different immune cells as activated $CD4^+$ T-cells (De Francesco *et al.,* 2014), $CD8^+$ T-cells (Avolio *et al.,* 2008), NK cells (Vitale *et al.,* 2003), plasmacytoid dendritic cells (Fiorentini *et al.,* 2008), monocytes (Marini *et al.,* 2008) and B-cells (Caccuri *et al.,* 2014 a; Giagulli *et al.,* 2011).

P17 is able to induce the production and release of proinflammatory cytokines, such as IFN-γ and TNF-α, by pre-activated T-cells (De Francesco *et al.,* 2014). Vitale *et al.* (2003), showed that p17 increases the release of IL-2, IL-12, IL-15, TNF-α and IFN-γ by NK cells and induces NK cell proliferation. Treatment of human primary monocytes with p17 treatment was followed by a selective production of MCP-1, whose expression was regulated at the transcriptional level and was dependent on activation of the AP-1 transcriptional factor (Marini *et al.,* 2008). Similarly to monocytes, treatment of B cells with p17 was also able to generate a transient activation of AP-1 (Giagulli *et al.,* 2011). These findings show that p17 contributes to the production and release of pro-inflammatory molecules and to the development of an inflammatory microenvironment.

P17 exerts a chemokine-like activity by binding to chemokine receptors CXCR1 and CXCR2, and mimics some of the biological activities of the CXCR1 and CXCR2 natural ligand IL-8, such as the rapid adhesion and chemotaxis of monocytes through activation of the Rho/ROCK signaling pathway (Giagulli *et al.,* 2012). The chemokine-like activity of p17 was also revealed on human primary B-cells and mainly driven by CXCR2 engagement (Caccuri *et al.,* 2014 a). These findings suggest that p17 may recruit activated monocytes and B cells in different tissues and organs to participate and/or sustain inflammatory processes.

P17 is a potent angiogenic and lymphoangiogenic factor, and its pro-vasculogenic activity was found to be as potent as that exerted by VEGF-A (Caccuri *et al.,* 2012; Basta *et al.,* 2015). Angiogenesis and lymphangiogenesis triggered by p17 are dependent on the viral protein interaction with CXCR1 and CXCR2, both expressed on the EC surface (Caccuri *et al.,* 2012). This is not surprising since both receptors are known to cooperate in coordinating the cytoskeletal rearrangement required for angiogenesis (Brat *et al.,* 2005). The interaction of p17 with CXCR1 and CXCR2 activates the MAPK/ERK and PI3K/Akt signaling pathways, both responsible for p17-induced angiogenesis and lymphoangiogenesis. PI3K was shown to play a key role in coupling CXCR1 and CXCR2 to Akt and ERK signaling (Caccuri *et al.,* 2012).

Lymphangiogenesis induced by p17 was found to be partly mediated by the selective release of the pro-angiogenic/lymphangiogenic factor ET-1 (Caccuri *et al.,* 2014b), which acts through its B receptor (ETBR) expressed on lymph node-derived ECs (LECs) by activating PI3K/Akt

and MAPK/ERK1/2 signaling pathways (Spinella *et al.,* 2009). Activation of ERK1/2 and Akt pathways by ET-1/ETBR interaction is likely to enforce and sustain the p17 lymphangiogenic activity.

After exogenous p17 administration to ECs, the viral protein was first detected in the cytoplasm and then in the nucleus, despite the continuous presence of p17 protein in the culture medium (Caccuri *et al.,* 2014 a), highlighting the nuclear targeting capability of exogenously delivered p17. The presence of p17 stored in the nucleus of ECs, in the absence of other *Gag*-derived proteins, was also observed in liver biopsies of a patient under cART (Caccuri *et al.,* 2012). All together, these findings suggest that exogenous p17 can bind to ECs *in vivo* and drive EC activation and aberrant angiogenesis/lymphangiogenesis leading to vascular disease. The ability of p17 to stimulate monocyte migration (Marini *et al.,* 2008) and promote an activation status of monocytes with consequent release of MCP-1 (Fiorentini *et al.,* 2008), together with its ability to induce the release of inflammatory and angiogenic molecules *in situ* sustaining aberrant angiogenesis, indicate additional and indirect roles of p17 in causing endothelial dysfunction.

P17 is continuously released in the extracellular space from HIV-1-infected cells, and it is detected at nanomolar concentrations in the blood of HIV-1$^+$ patients even in the presence of anti-p17 antibodies (Fiorentini *et al.,* 2006; Vitale *et al.,* 2003). The viral protein is easily detected by immunohistochemistry in different organs and tissues of patients either before or during cART, even in the absence of viremia. In particular, p17 was found to accumulate and persist in germinal centers of lymph nodes of patients under cART in the absence of any *in situ* viral replication (Popovic *et al.,* 2005). Recently, many groups demonstrated that different stimuli are able to increase HIV-1 transcription (Reuse *et al.,* 2009) even in the presence of protease inhibitors (Vandergeeten *et al.,* 2007) and that latently HIV$^+$ resting T-cells are capable of producing HIV-1 *Gag* without supporting spreading infection (Pace *et al.,* 2012). All these findings suggest that p17 may be chronically present in the tissues of patients even during cART and may be very important for the progression of endothelial dysfunction and for the insurgence of vascular diseases.

Production of molecules with biological activity on ECs is triggered by HIV-1-encoded proteins

As described above, HIV-1-encoded proteins contribute to the synthesis and release of different molecules that may impact on EC function. HIV-1 matrix protein p17 is itself a viral chemokine that binds to the chemokine receptors CXCR1 and CXCR2 (Caccuri *et al.,* 2014 a; Giagulli *et al.,* 2012). Cell activation following p17/receptor interaction leads to an enhanced production of molecules with pro-inflammatory and pro-apoptotic activity on ECs, such as TNF-α, IFN-γ and MCP-1 (Flamminio *et al.,* 1995; Iaria *et al.,* 2014; Avolio *et al.,* 2008). Stimulation of LECs by p17 triggers the release of ET-1, that contributes together with p17 to promote angiogenesis and lymphangiogenesis (Caccuri *et al.,* 2014b). Tat is *per se* an exogenous cytokine promoting EC activation and angiogenesis (Hofman *et al.,* 1993). It is also able to induce IL-6 expression in human brain ECs (Zidovetzki *et al.,* 1998) and to promote MCP-1 secretion by human lung microvascular ECs (Park *et al.,* 2001b). The HIV-1 viral protein Nef triggers MCP-1 production and secretion from ECs (Wang *et al.,* 2014), whereas gp120 has a dual role in promoting either EC activation/angiogenesis or EC apoptosis/death by promoting ET-1 production and release (Ehrenreich *et al.,* 1993; Finkel *et al.,* 1995). All these findings attest the key role played by exogenously released HIV-1 proteins in conditioning a microenvironment capable of promoting and sustaining chronic inflammation and, consequently, vascular dysfunction.

ANIMAL MODELS FOR THE STUDY OF HIV-1 ENDOTHELIAL DYSFUNCTION

Several animal models have been used to simulate the arterial wall pathology to enhance insight into the mechanisms of HIV-1-associated endothelial dysfunction.

Tinkle *et al.* (1997) developed a model of transgenic mice infected by the HIV-1 provirus and demonstrated the development of an adventitial mixed inflammatory cell infiltrate, medial hypocellularity and intimal hyperplasia following smooth muscle migration, with sparing of the ECs. The intimal thickening produces intraluminal narrowing of some vessels causing distal tissue ischemia. In addition, viral components have been observed in smooth muscle cells, which in some instances have proliferated in the absence of inflammation (Tinkle *et al.,* 1997; Pipitone *et al.,* 2008). This model partially reproduces the characteristics of human HIV-1-associated endothelial dysfunction and highlights the conceptual principles of viral invasion, even if the pathophysiological features of endothelial dysfunction in human remain challenging. Nonhuman primate models of HIV-1 infection with either simian immunodeficiency virus (SIV) or genetically manipulated pathogenic viruses containing HIV-1 genes reproduce many of the end-organ pathologies and immune deficiencies seen in humans (Van Rompay, 2005).

Infection of macaque species with SIV results in a disease (simian AIDS) that shares many similarities with HIV-1 infection and AIDS in humans. Interestingly, Marecki *et al.* (2006), demonstrated complex vascular lesions in macaques infected with a chimeric viral construct containing the HIV-1 Nef gene in a SIV backbone (SHIV-1-nef) but not in SIV-infected animals. These authors also showed the presence of Nef in the vascular cells of patients with HIV-1-related pulmonary hypertension. They found complex plexiform-like lesions characterized by luminal obliteration, intimal disruption, medial hypertrophy and thrombosis exclusively in SHIV-1-nef-infected animals but not in SIV-infected animals. These findings seem to highlight a possible role of Nef at least in endothelial dysfunction leading to severe pulmonary arterial disease.

More recently, Panigrahi *et al.* (2016) showed that SIV and SHIV-1-infection of Rhesus macaques alters the vascular endothelium, triggering inflammatory changes characterized by sub-endothelial infiltration of immune cells. eNOS levels were significantly reduced in aortic ECs of infected animals, reflecting vascular dysfunction.

The vasculogenic activity of p17 was recently demonstrated using *ex vivo* and *in vivo* models Caccuri *et al.,* 2012; Basta *et al.,* 2015; Caccuri *et al.,* 2014b). The *ex vivo* rat aortic ring assay showed that p17 was able to promote vasculogenesis as potent as that observed using the pro-angiogenic factor VEGF-A (Caccuri *et al.,* 2012). Similar results were obtained in the *in vivo* chick chorioallantoic membrane (CAM) assay. At day 12 of incubation, a significant angiogenic response was induced by p17 in the form

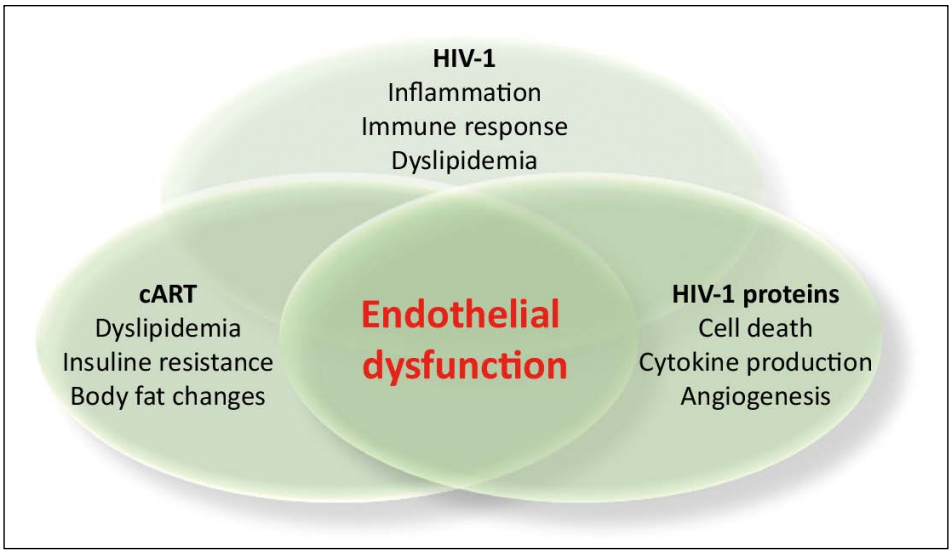

Figure 3 - *Multiple events concur to promote EC dysfunction in HIV+ patients.*

of numerous allantoic neovessels (Caccuri *et al.,* 2012). Matrigel plug assay was also used to test the lymphangiogenic activity of p17 in mice. Matrigel plugs containing the protein were implanted into the dorsal subcutaneous tissue of C57BL/6 mice and 10 days after injection the plugs were immunostained with polyclonal antibody to lymphatic vessel endothelial receptor-1 (LYVE-1) identifying pronounced lymphatic vessel formation in p17-treated mice, compared to controls (Caccuri *et al.,* 2012). Interestingly, matrigel plugs containing a p17 variant derived from an Ugandan clade A1, named S75X, disclosed adipocyte infiltration observed at histological level, thus suggesting that at least some p17 variants may trigger an interplay between angiogenesis, lymphangiogenesis and adipogenesis (Basta *et al.,* 2015).

DISCUSSION

As described above, HIV-1 proteins are able to induce strong changes in EC physiology and morphology by altering their homeostasis and function. Interestingly, HIV$^+$ patients have a high risk of endothelial dysfunction in the absence and presence of suppressive cART (Currier *et al.,* 2008; Obel *et al.,* 2007; Triant *et al.,* 2007; Grunfeld *et al.,* 2009; Freiberg *et al.,* 2013), although a low-level transcription of HIV-1 genes continues even after years of cART (Furtado *et al.,* 1999; Fischer *et al.,* 2000; Günthard *et al.,* 2001). Many studies have demonstrated the persistence of HIV-1-encoded proteins also during viral load suppression (Mediouni *et al.,* 2012; Lenassi *et al.,* 2010; Schneider *et al.,* 1986; Oh *et al.,* 1992; Popovic *et al.,* 2005; Fiorentini *et al.,* 2006; Vitale *et al.,* 2003). Since these proteins are able to induce direct EC damage and to develop an inflammatory microenvironment, we hypothesize that viral proteins and their circulation in HIV-1 patients are the most important factors involved in the development of endothelial dysfunction. Although animal models have limitations and can never completely mimic the HIV-1 infection of humans or the physiological relevance of a single protein product in the human microenvironment, they start to provide proof-of-concept for a general vascular dysregulation operated by HIV-1 and its products. All together, these data show that a microenvironment prone to endothelial dysfunction is a common feature in HIV$^+$ individuals (*Figure 3*). Recognizing the interaction of some HIV-1 protein products with their receptors as the key events in sustaining EC aberrant functioning could help us identify new therapeutic strategies in combating and/or preventing HIV-1-related vascular diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Abbreviations

HIV-1, human immunodeficiency virus type 1; HIV$^+$, HIV-1-seropositive/infected; cART, combined antiretroviral therapy; EC, endothelial cell; Tat, HIV-1 transactivator of transcription; Nef, HIV-1 negative regulatory factor; p17, HIV-1 matrix protein p17; gp120, HIV-1 envelope glycoprotein 120; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin.

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