HIV-1 Matrix Protein p17 Promotes Lymphangiogenesis and Activates the Endothelin-1/Endothelin B Receptor Axis

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Objective—AIDS-related lymphomas are high grade and aggressively metastatic with poor prognosis. Lymphangiogenesis is essential in supporting proliferation and survival of lymphoma, as well as tumor dissemination. Data suggest that aberrant lymphangiogenesis relies on action of HIV-1 proteins rather than on a direct effect of the virus itself. HIV-1 matrix protein p17 was found to accumulate and persist in lymph nodes of patients even under highly active antiretroviral therapy. Because p17 was recently found to exert a potent proangiogenic activity by interacting with chemokine (C-X-C motif) receptors 1 and 2, we tested the prolymphangiogenic activity of the viral protein.

Approach and Results—Human primary lymph node–derived lymphatic endothelial cells were used to perform capillary-like structure formation, wound healing, spheroids, and Western blot assays after stimulation with or without p17. Here, we show that p17 promotes lymphangiogenesis by binding to chemokine (C-X-C motif) receptor-1 and chemokine (C-X-C motif) receptor-2 expressed on lymph node–derived lymphatic endothelial cells and activating the Akt/extracellular signal–regulated kinase signaling pathway. In particular, it was found to induce capillary-like structure formation, sprout formation from spheroids, and increase lymph node–derived lymphatic endothelial cells motility. The p17 lymphangiogenic activity was, in part, sustained by activation of the endothelin-1/endothelin receptor B axis. A Matrigel plug assay showed that p17 was able to promote the outgrowth of lymphatic vessels in vivo, demonstrating that p17 directly regulates lymphatic vessel formation.

Conclusions—Our results suggest that p17 may generate a prolymphangiogenic microenvironment and plays a role in predisposing the lymph node to lymphoma growth and metastasis. This finding offers new opportunities to identify treatment strategies in combating AIDS-related lymphomas. (Arterioscler Thromb Vasc Biol. 2014;34:846-856.)

Key Words: angiogenesis factor ■ endothelin-1 ■ HIV-1 ■ lymphatic endothelial cells ■ MAP kinase signaling pathways ■ p17 matrix protein

AIDS-related lymphomas (ARLs) account for a large proportion of malignancies in HIV-1–infected individuals. ARLs comprise a narrow spectrum of histological types consisting almost exclusively of aggressive B-cell tumors derived from either germinal or postgerminal centers. Although the risk of AIDS-defining cancers has declined dramatically after the introduction of highly active antiretroviral therapy (HAART) and the consequent immune reconstitution, the risk of AIDS-related cancers has not declined to the same extent. The greatest difference between ARLs and lymphomas in HIV-1–seronegative individuals is that ARLs are usually high grade and aggressively metastatic.1 Indeed, ≈80% of ARLs arise systemically and have poor prognosis.4,5 Lymphangiogenesis is essential in supporting proliferation and survival of lymphomas and plays a key role in tumor dissemination.6 Several studies have provided direct evidence for the importance of an accelerated lymphangiogenesis in lymph nodes of malignant lymphomas, and this might be induced by the presence of lymphangiogenic molecules in the tumor microenvironment.7,8 Although several lymphangiogenic growth factors, including vascular endothelial growth factor (VEGF)-C, have been recognized in recent years,9,10 the molecular and cellular regulation of lymphangiogenesis is still
The HIV-1 matrix protein p17 (p17) is a 17-kDa myristoylated protein derived from the extreme N-terminal region of the Gag precursor protein p55. It is a key component of the HIV-1 preintegration complex and performs crucial functions of the Gag precursor protein p55. It is a key component of the HIV-1 protein did not noticeably differ in lymph nodes of patients either before initiation of HAART or during treatment where the virus burden was significantly decreased. This suggests that HIV-1 may contribute to the incidence of highly active antiretroviral therapy (HAART).11 Because both receptors are expressed on LN-LECs, we aimed to determine the effect of p17 on human LEC lymphangiogenesis and to elucidate the mechanism underlying the activity of the viral protein.

Herein, we report that primary human lymph node–derived LECs (LN-LECs) are positive for CXCR1 and CXCR2 expression. We demonstrate that both CXCR1 and CXCR2 are responsible for p17 lymphangiogenic activity on LN-LECs. Our results also reveal that p17-induced lymphangiogenesis is mediated, in part, by the endothelin-1 (ET-1)/ET-1 B receptor (ETBR) axis. Finally, we provide in vivo evidence on the capability of p17 to induce lymphangiogenesis directly.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
CXCR1 and CXCR2 Are Expressed on Human Primary LN-LECs
To explore the presence of CXCR1 and CXCR2 on the surface of LN-LECs, we examined the expression of both receptors in cultured LN-LECs under confocal imaging. CXCR1 and CXCR2 are expressed on the surface of LN-LECs as shown by immunofluorescence on fixed nonpermeabilized cells using mAbs to CXCR1 and CXCR2 as specific reagents (Figure 1A). Immunofluorescence showed a higher intensity for CXCR1 than for CXCR2, suggesting CXCR1 to be more highly expressed than CXCR2 on the surface of LN-LECs. Whereas CXCR2 signals on the cell surface defined a punctate pattern evenly distributed over the entire plasma membrane, the distribution of CXCR1 on the surface of LN-LEC appeared as larger circular patterns concentrated on distinct membrane areas or structures (Figure 1A). This distribution was not maintained when cells were permeabilized or when the receptor was engaged by CXCR1 mAb before cells were fixed but not permeabilized. This finding suggests a CXCR1 cell surface distribution at plasma membrane structures or domains that are not stable and hence, not preserved to be detected by antibodies unless conserved directly by fixation of live, nonpermeabilized cells. These structures do not seem to be supported by the actin cytoskeleton: immunofluorescence using actin-binding fluorescently conjugated phallolidin did not show any colocalization with CXCR1 (Figure 1B). The expression of CXCR1 and CXCR2 was then studied both at the protein and at the transcriptional level. As shown in Figure 1 in the online-only Data Supplement, both receptors were detected by Western blot in the total protein extract obtained from LN-LECs. Although CXCR1 was found to be more highly expressed than CXCR2, the difference was not statistically significant. Similar results were obtained by real-time polymerase chain reaction.

P17 Requires Both CXCR1 and CXCR2 to Induce Capillary-Like Structures
CXCR1 and CXCR2 are involved in p17-induced angiogenesis.23 Because both receptors are expressed on LN-LECs, we...
investigated the capability of p17 to promote capillary-like structure formation in vitro. To minimize the physiological lymphangiogenic activity of LN-LECs, low passage cells were nutrient starved for 24 hours before harvesting. Cells were then seeded on 48-well plates (5x10^4 per well) containing polymerized plugs of growth factor–reduced basement membrane extract (Cultrex). As shown in Figure II in the online-only Data Supplement, starved LN-LECs seeded on Cultrex and cultured ≤8 hours formed a cellular monolayer. In the presence of p17, LN-LECs formed a consistent network of capillary-like structures. Dose–response experiments showed that p17 exerted its lymphangiogenic activity at a concentration as low as 5 ng/mL, reaching a peak of lymphangiogenic potency at a protein concentration of 10 ng/mL. Parallel experiments showed that the lymphangiogenic activity of p17 was similar to that exerted by VEGF-C and IL-8, whereas the irrelevant protein glutathione S-transferase did not induce any capillary-like structure formation (Figure II in the online-only Data Supplement).

The activity of p17 was blocked by preincubating the medium containing p17 (10 ng/mL) with the p17 neutralizing mAb MBS-315 (1 μg/mL), whereas incubation with irrelevant control mAb (1 μg/mL) had no effect, thus confirming the specificity of the p17 effect (Figure 2A). Because the angiogenic response of ECs requires coordinated cytoskeletal rearrangement mediated by CXCR1 and CXCR2 and both receptors are required for angiogenic activity of p17 and IL-8,21 we determined the involvement of both receptors in p17-mediated capillary-like structure formation by examining the effect exerted by neutralizing mAbs to CXCR1 and CXCR2. As shown in Figure 2B, after 8 hours of culture, LN-LECs formed capillary-like structures in medium containing p17 or p17 plus the isotype-matched mAb. The neutralizing mAb to CXCR1 was strongly inhibitory (63.1±3.6%) toward p17-induced capillary-like structure formation, whereas mAb to CXCR2 accounted for a smaller 21.6±4.2% decrease. Blocking of both receptors by using a combination of the 2 mAbs resulted in an almost complete inhibition (83.8±2.7%) of p17-induced capillary-like structure formation. Our data demonstrate that both CXCR1 and CXCR2 are involved in p17-induced capillary-like structure formation and most likely cooperate in promoting lymphangiogenesis, with a major role for CXCR1 in mediating the p17 lymphangiogenic activity. This finding was confirmed by a knockdown approach based on small interfering RNA (siRNA). As shown in Figure III in the online-only Data Supplement, CXCR1 and CXCR2 siRNAs were equally capable of downmodulating the expression of the targeted mRNAs in LN-LECs (Figure IIIA in the online-only Data Supplement). As expected, the lymphangiogenic activity of LN-LECs was more reduced in the CXCR1 siRNA–treated cells (P<0.01) compared with CXCR2 siRNA–treated ones (P<0.05). No inhibition was observed in control cells treated with a scrambled siRNA (Figure IIIB in the online-only Data Supplement).
These data support a major role for CXCR1 in the p17-driven lymphangiogenic process.

**P17-Induced Capillary-Like Structure Formation Requires Activation of the Akt-Dependent ERK Signaling Pathway**

ERK signaling via Akt was defined as the pathway responsible for p17-induced angiogenesis. Phosphatidylinositol 3-kinase (PI3K) activity was identified as a key intermediate in coupling CXCR1 and CXCR2 to ERK1/2 and Akt phosphorylation, a key event for triggering EC angiogenic functions. These data prompted us to investigate the ability of p17 to induce Akt and ERK1/2 phosphorylation in LN-LECs. As shown in Figure 3A, p17 markedly stimulated the phosphorylation of both Akt and ERK1/2. This occurred through the interaction of p17 with CXCR1 and CXCR2 (Figure IV in the online-only Data Supplement). Phosphorylation was transient for pAkt because it reached a peak at 30 minutes and declined after 1 hour of p17 stimulation. As expected, the irrelevant protein glutathione S-transferase did not induce any Akt and ERK1/2 activation.

To investigate further whether these pathways play a role in p17-induced capillary-like structure formation and to characterize the intracellular signaling mechanisms, LN-LECs were incubated for 8 hours with p17 and optimal concentration of the inhibitors of PI3K (LY294002 [10 μmol/L] and wortmannin [100 nmol/L]), mitogen-activated protein kinase kinase [MEK]/ERK1/2 (PD98059 [10 μmol/L]), and Akt (Akt inhibitor VIII [1 μmol/L]). As shown in Figure 3B, capillary-like structure formation in p17-stimulated LN-LECs was significantly inhibited by LY294002, wortmannin, PD98059, and Akt inhibitor VIII. Our results suggest that similarly to what was observed in human umbilical vein ECs, the PI3K/Akt and MEK/ERK1/2 pathways are both required and critical for production and maintenance of p17-induced LN-LEC–derived capillary-like networks on growth factor–reduced basement membrane extract.

**P17 Induces Formation of Sprouts From LN-LEC Spheroids**

The entrapment of EC spheroids in biopolymeric gels represents a 3-dimensional (3D) cell model that was found to be...
an attractive potential method of forming a microvascular network that mimics in vivo sprouting angiogenesis. The 3D organotypic culture is based on the property of LECs to form spheroids under nonadherent conditions, and it has proven useful for studies on endothelial capillary sprouting. Modifying the recently established LEC spheroid differentiation model, we developed a spheroidal system of LN-LECs aimed at mimicking the correct 3D assembly of the normal lymphatic vessel wall. LN-LEC–derived spheroids were treated with p17, and a 3D organotypic culture was performed under nonadherent conditions. As shown in Figure V in the online-only Data Supplement, p17 induced a dramatic outgrowth of sprouts in 24 hours. The number of sprouts obtained in p17-treated LN-LEC spheroids was superimposable to that obtained in the same period of time with a molecule known to promote lymphangiogenesis (IL-8). On the contrary, minimal sprouting was observed in control LN-LECs and in LN-LECs treated for 24 hours with the irrelevant protein glutathione S-transferase. These data strongly suggest that also in the 3D environment p17 displays potent lymphangiogenic activity, which is comparable to that induced by IL-8.

P17 Stimulates LN-LEC Migration

Capillary-like formation and sprout formation from spheroids are in vitro assays that involve ECs present at different levels of confluence and are indicative of 2 different angiogenic functions, migration, and morphogenesis. To investigate the effect of p17 on LN-LECs migratory activity, further studies were performed using the wound healing assay. This method allows us to investigate the ability of viral protein to modulate cell migration by sealing a confluent cell monolayer after a mechanical injury. LN-LECs were grown on collagen-coated plates and starved for 24 hours. Confluent monolayers were scratched with a 200-μL tip, and the percentage of wound healing was observed during a period of 12 hours. As shown in Figure VIA in the online-only Data Supplement, control, as well as glutathione S-transferase–treated, LN-LECs reached ≈35% healing (range from 28% to 44%) only after 12 hours of culture, whereas at the same time LN-LECs treated with p17 reached 100% healing, showing a considerable improvement in scrape wound repair ability. To determine the involvement of CXCR1 and CXCR2 in p17-induced LN-LEC migration, immediately after scratching, cell monolayers were cultured for 12 hours.
with or without p17 (10 ng/mL) in the presence or absence of a neutralizing mAb to CXCR1, CXCR2 (2.5 μg/mL), or a control isotype-matched mAb (2.5 μg/mL). LN-LECs reached 100% of wound healing after 12 hours of culture in the presence of p17 or p17 plus the isotype-matched mAb. The neutralizing mAb to CXCR1 was strongly inhibitory (range from 77.2% to 88.8%), whereas the neutralizing mAb to CXCR2 accounted for a more modest decrease (range from 18.7% to 22.3%) of p17 activity (Figure VII in the online-only Data Supplement).

ET-1 Is Selectively Released by LN-LECs on p17 Stimulation

To understand whether the lymphangiogenic activity of p17 was direct or, at least in part, mediated by other lymphangiogenic molecules, we performed analysis of p17-stimulated LN-LEC secretome by using a human angiogenesis array. As shown in Figure 4A, after 6 hours of culture, LN-LECs did not spontaneously release any of the tested molecules, including VEGF and VEGF-C, with the only exceptions being Serpin E1 and low levels of ET-1. At the same time, p17 (10 ng/mL) was found to trigger the secretion of ET-1 powerfully, whereas it proved unable to induce secretion of any other proangiogenic/lymphangiogenic factors tested, with the exception of low levels of pentraxin 3, monocyte chemotactic protein-1, tissue inhibitor of metalloproteinase-1, and thrombospondin-1, which were detected in the supernatant of p17-treated LN-LECs.

To confirm the capability of p17 to promote ET-1 release by LN-LECs, a kinetic study was performed by collecting the supernatant of p17-treated or p17-untreated cells at 1, 3, and 6 hours after the beginning of stimulation. Culture supernatants were analyzed for the presence of ET-1 by a standard quantitative ELISA. Bars represent the mean±SD of triplicate samples. Statistical analysis was performed by 1-way ANOVA and by 1-way ANOVA performed separately for each concentration of p17 across the 3 groups. Bonferroni post test was used to compare data (*P<0.05, **P<0.01, ***P<0.001). ADAMTS-1 indicates a disintegrin and metalloproteinase with a thrombospondin type 1 motif; CTR, control; EGF, epidermal growth factor; FGF, fibroblast growth factor; IL-8, interleukin-8; LAP (TGF-β1), transforming growth factor beta 1; MCP, monocyte chemotactic protein; NT, not treated cells; PD-ECGF, platelet-derived endothelial cell growth factor; PDGF-AA, platelet-derived growth factor- AA; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; and VEGF, vascular endothelial growth factor.
hours after stimulation. As shown in Figure 4B, p17 induced a significant increase of ET-1 release at all tested times. The effect on ET-1 secretion by LN-LECs obtained using p17 at the concentration of 10 ng/mL did not differ from those obtained using a higher dose (100 ng/mL) of viral protein.

To investigate further whether PI3K/Akt and MEK/ERK1/2 signaling pathways play a role in p17-induced ET-1 secretion, LN-LECs were incubated for 6 hours with p17 and optimal concentrations of the inhibitors of PI3K (LY294002 [10 μmol/L] and wortmannin [100 nmol/L]), MEK/ERK1/2 (PD98059 [10 μmol/L]), and Akt (Akt inhibitor VIII [1 μmol/L]). As shown in Figure 4C, ET-1 secretion was significantly inhibited by LY294002, wortmannin, PD98059, and Akt inhibitor VIII. Our results suggest that the ET-1 secretion from LN-LECs is dependent on p17-induced activation of both PI3K/Akt and MEK/ERK1/2 pathways.

Increased level of ET-1 protein secretion induced by p17 treatment was not paralleled by an increase in specific ET-1 mRNA expression. When ET-1 messages were quantified by real-time polymerase chain reaction Taqman Assay in LN-LECs treated or not with p17 (10 ng/mL), we observed that ET-1 mRNA was expressed at constant levels, irrespective of p17 treatment (Figure VII in the online-only Data Supplement).

ET-1 is accumulated into Weibel–Palade bodies, the most recognizable endothelial storage granule,25 whose exocytosis is blocked by neutralization of the acidic intra-Weibel–Palade bodies pH by treatment with monensin.26 Therefore, to evaluate if the increased amount of ET-1 present in the supernatant of p17-treated LN-LEC was because of the capability of p17 to induce secretion of ET-1, monensin was added to LN-LEC cultures. As shown in Figure 4D, treatment with monensin inhibited the release of ET-1 by p17-treated LN-LECs within 1 hour of incubation, whereas the percentage of inhibition increased with duration of exposure.

**P17-Induced Capillary-Like Structure Formation Is Partially Mediated by the ET-1/ET_AR Axis**

We recently demonstrated that LECs express high levels of ET_AR both in vitro and in vivo, whereas the ET-1A receptor is only barely expressed on these cells.27 Moreover, we found that ET-1 exerts a potent lymphangiogenic activity by interacting with ET_AR.27 We investigated whether p17-induced capillary-like structure formation and migration were mediated by ET-1 by selectively blocking ET_AR with specific ET_AR antagonist BQ788. As shown in Figure 5A, p17-induced capillary-like structure formation was significantly inhibited in the presence of specific ET_AR antagonist BQ788. The presence of BQ788 in medium during p17 stimulation accounted for a 61.2±5.2% inhibition of LN-LEC tube formation. No significant effect on p17 angiogenic activity was observed when LN-LECs were stimulated with p17 in the presence of specific ET-1A receptor antagonist BQ123. These findings suggest that p17 lymphangiogenic activity is strongly, but not entirely, supported by activation of the ET-1/ET_AR axis. A strong involvement of ET-1/ET_AR axis also in p17-induced LN-LEC migratory activity was demonstrated using the wound healing assay. As shown in Figure 5B, a pronounced impairment in wound repair occurred in p17-treated LN-LECs when cells were cultured in the presence of BQ788, whereas the presence of BQ123 did not affect p17-dependent cell migratory activity. Our results clearly demonstrate that ET-1 through ET_AR cooperates with p17 in promoting and sustaining LN-LEC angiogenic and migratory activity.

**Figure 5.** Protein p17 (P17)–induced capillary-like structure formation and cell migration are partly mediated by activation of the endothelin-1 (ET-1)/ET-1 A receptor (ET_AR) axis. A, Lymph node–derived lymphatic endothelial cells (LN-LECs) were stimulated for 8 hours at 37°C with medium alone (not treated [NT]) or 10 ng/mL of p17. When indicated, LN-LECs were stimulated with p17 in combination with 0.65 μg/mL ET_AR antagonist BQ788 or with 0.65 μg/mL ET_AR antagonist BQ123. Percentages are representative of 3 independent experiments with similar results (magnification ×10). Values reported for LN-LEC capillary-like structure (tubes) formation are the mean±SD of 3 independent experiments. Statistical analysis was performed by 1-way ANOVA, and the Bonferroni post test was used to compare data (**P<0.001).
P17 Promotes Lymphangiogenesis In Vivo

Because p17 stimulated lymphangiogenesis of LECs in vitro by promoting the secretion of ET-1 and possibly of other lymphangiogenic molecules, we investigated whether p17 may induce per se lymphangiogenesis in vivo. To this end, we implanted Matrigel containing p17, ET-1 (positive control), or PBS (negative control) into the dorsal subcutaneous tissue of C57BL/6 mice. As shown in Figure 6, immunostaining of Matrigel plugs with polyclonal Ab to lymphatic vessel endothelial receptor-1 identified pronounced lymphatic vessel formation in those containing p17 and ET-1 compared with Matrigel controls. Staining was confined within discrete areas at the boundaries with the surrounding murine tissue. The number of LECs recruited into the Matrigel plugs was quantified by flow cytometry. As shown in the Figure 6 graph, flow cytometric analysis revealed a consistent increase in the number of LECs (CD31+/lymphatic vessel endothelial receptor-1+) recruited to Matrigel after p17 and ET-1 stimulation compared with the control cells. These results indicate that p17 acts as a lymphangiogenic factor via direct stimulatory effects on LN-LECs because its presence in Matrigel plugs is per se able to recruit LECs and promote the outgrowth of lymphatic vessels in vivo.

Discussion

IL-8 exerts a potent angiogenic and lymphangiogenic activity by interacting with CXCR1 and CXCR2. In previous studies, we showed that HIV-1 matrix protein p17 displays IL-8–like activities by binding to both IL-8 receptors. In particular, the viral protein was found to promote angiogenesis strongly in vitro and in vivo using the same IL-8 machinery. In this study, we demonstrate that LN-LECs do express CXCR1 and CXCR2 and that p17 interaction with CXCR1 and CXCR2 is responsible for a potent activation of the lymphangiogenic process.

We previously showed that exogenous p17 is present in the serum of HIV-infected patients at nanomolar concentrations. The viral protein was also detected in lymphoid organs, where it accumulates and persists for years even in patients undergoing successful HAART. This finding, together with the more recent evidence that latently HIV-1–infected resting T-cells are capable of producing HIV-1 Gag without supporting spreading infection, strengthens the possibility that p17 synthesis and release may occur even under HAART.

In this study, we show that p17 exerts a potent lymphangiogenic activity on LN-LECs at nanomolar concentrations (10 ng/mL), suggesting that the p17 biological activity we observe in vitro may likely occur in the HIV-1–infected host. It is worth noting that several authors demonstrated the binding of p17 to heparan sulfate proteoglycans. Such interaction, as already demonstrated for many heparin-binding chemokines, may allow a better presentation of p17 to CXCR1 and CXCR2 setting up synergistic and cooperative interactions leading to increased concentrations of the viral protein at the LN-LEC surface.

CXCR1 and CXCR2 use different signal transduction cascades that result in the activation of small G proteins and evoke pleiotropic responses in target cells. Our previous results

![Figure 6](http://atvb.ahajournals.org/)
obtained in human ECs have shown that both receptors are involved in promoting angiogenic activity, defining Akt and ERK as the signaling molecules responsible for p17 proangiogenic function, whereas PI3K activity was identified as a key intermediate in coupling CXCR1 and CXCR2 to Akt and ERK signaling.23 Here, we show that p17 requires both CXCR1 and CXCR2 to exert its lymphangiogenic activity and that signaling events similar to those observed in p17-treated ECs are evoked in LN-LECs after p17 stimulation. As in p17-stimulated ECs, p17 treatment of LN-LECs gave rise to the activation of PI3K/Akt and MEK/ERK1/2 signaling pathways, because specific inhibitors of both pathways completely blocked p17-induced capillary-like structure formation. All these results confirm that both pathways are required and may function cooperatively in driving p17-induced capillary-like structure formation.

ET-1 is a proangiogenic factor.32,33 Members of the ET-1 family and their expression has been correlated with increased lymphatic dissemination.22 In a recent article, we described the capability of ET-1 to promote lymphangiogenesis and migration of LECs through a mechanism mediated by ET\textsubscript{R}R-ET-1–driven effects being completely abrogated in the presence of the specific ET\textsubscript{R}R antagonist BQ788.27 Therefore, the finding that ET-1 was increased in the supernatant of LN-LECs after p17 stimulation led us to hypothesize a role of the ET-1/ET\textsubscript{R}R axis in p17-driven lymphangiogenic activity of LN-LECs. Indeed, the presence of the ET\textsubscript{R}R specific antagonist BQ788 was able to downmodulate p17-induced capillary-like structure formation. Furthermore, we demonstrated that in the Matrigel plug neovascularization assay, p17 promotes per se the outgrowth of lymphatic vessels in vivo. Data obtained support the hypothesis that p17 stimulates lymphangiogenesis directly and also indirectly via the ET-1/ET\textsubscript{R}R axis. It is worth noting that ET-1/ET\textsubscript{R}R interaction leading to lymphangiogenesis is dependent on activation of ERK1/2 and Akt.27 Thus, the signaling cascade involving activation of the MEK-dependent ERK signaling pathway triggered within LN-LECs by p17 interaction with CXCR1 and CXCR2 is likely to be enforced and sustained by a secondary ET-1/ET\textsubscript{R}R interaction, which may contribute to exert a stronger and more prolonged p17 lymphangiogenic activity.

Previous data have shown that ET-1–induced lymphangiogenesis is mediated by the release of other lymphangiogenic factors by LN-LECs and sustained by a secondary ET-1/ET\textsubscript{R}R interaction, which are potential candidates in the treatment of lymphangiogenesis associated with ARLs.

ARLs comprise a narrow spectrum of histological types consisting almost exclusively of aggressive B-cell tumors derived from either germinal centers or postgerminal centers. HIV-1 creates a milieu of combined immune suppression and chronic antigenic stimulation in lymph nodes.40 This environment with dysregulated cytokine/chemokine release and B-cell hyperstimulation41 along with the presence of concomitant infection (eg, Epstein–Barr virus, human herpesvirus type 8, cytomegalovirus) may promote a permissive environment for HIV-1–induced B-cell expansion and impaired immune surveillance, culminating in lympho-proliferative disorders.42 Up until now, the molecular mechanisms responsible for B-cell transformation associated with ARLs have not been elucidated. However, in this respect it is worth noting that recent data have highlighted a role for p17 variants in promoting B-cell growth and malignant transformation in vitro.18 This was related to a p17 variant–induced dysregulation of the PI3K/Akt signal transduction pathway, a hallmark of different types of malignancies known to play a central role in tumorigenesis and cancer progression.43,44 Collectively, all these evidences corroborate the hypothesis that p17 may play a key role in producing a microenvironment that fosters lymphoma development, progression, and metastasis. The significant importance of blocking the biological activity of p17 is becoming apparent.45 This may be achieved by using p17-specific drugs46 or by specific vaccines aimed to generate a neutralizing p17 antibody response.47 The knowledge that p17 triggers common pathways of lymphangiogenesis and angiogenesis, which represent important routes of metastasis, may offer new opportunities to identify novel treatment strategies in preventing and combating ARLs.

Chronic inflammation because of the presence of activated monocytes and macrophages in lymph nodes is known to trigger and sustain aberrant lymphangiogenesis37 and to induce the development of genomic abnormalities and tumor progression.38 Complexity of p17 activities includes also its capability to trigger the production of proinflammatory cytokines and chemokines from different immune cells, including monocytes/macrophages.15,17 This could have a particular relevance in lymphoid organs where it may recruit activated monocytes through CXCR1 engagement19 and induce the release of inflammatory and angiogenic molecules in situ, including ET-1.17,39

Although pharmacological inhibition of lymphangiogenesis may be clinically relevant in ARLs, the number of available drugs is limited.36 In this context, the present study indicates that molecules targeting the p17/CXCR1 and CXCR2 axis, as well as the ET-1/ET\textsubscript{R}R axis, are potential candidates in the treatment of lymphangiogenesis associated with ARLs.

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Disclosures

None.

References


**Significance**

Lymphangiogenesis is essential in supporting proliferation and survival of lymphoma, as well as tumor dissemination. The HIV-1 matrix protein p17 is secreted by HIV-1–infected cells and accumulates and persists in the lymph nodes of patients under highly active antiretroviral therapy in the absence of any HIV-1 replicative activity. This study identifies the HIV-1 matrix protein p17 as a positive regulator of lymphangiogenesis. Both chemokine (C-X-C motif) receptor-1 and chemokine (C-X-C motif) receptor-2, expressed on human lymph node–derived lymphatic endothelial cells, were required by the viral protein to exert its lymphangiogenic activity. This protein p17 activity was found to be, in part, sustained by activation of the endothelin-1/endothelin-1 B receptor axis. Our results suggest that protein p17 may generate a prolymphangiogenic microenvironment and play a role in predisposing the lymph node to lymphoma growth and metastasis. This finding offers new opportunities to identify treatment strategies in combating AIDS-related lymphomas.
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Materials and Methods

Recombinant HIV-1 p17 protein and mAb to p17

Purified endotoxin-free recombinant HIV-1 matrix protein p17 and GST were produced as previously described\(^1\). The p17 neutralizing mAb MBS-3\(^1\) was produced in our laboratory.

Human primary lymph node-derived endothelial cell (LN-LEC)

LN-LECs have been developed and characterized as previously described\(^2\). Cells were cultured in endothelial growth medium (EGM) (Lonza, Milan) containing 10% fetal bovine serum (FBS) and supplemented with VEGF-C (25 ng/mL) (Reliatech, Wolfenbuettel, Germany). All experiments were carried out with cells at passages 2–6.

Immunofluorescence

LN-LECs were grown on collagen-coated glass coverslips, starved overnight in endothelial basal medium (EBM), fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS) when indicated. Hoechst 33258, phalloidin and the following antibodies were applied in blocking buffer (2% BSA, 2% FBS in PBS): anti-human CXCR1 (R&D, Minneapolis, MN), anti-human CXCR2 (R&D), AlexaFlour 488-conjugated goat anti-mouse IgG (Invitrogen, Grand Island, NY) or rhodamine-conjugated phalloidin (Invitrogen). Immunofluorescence was analysed using the laser scanning fluorescence microscope Leica TCS SP5 and the imaging software Leica Application Suite (Buffalo Grove, IL).

In Vitro Tube Formation Assay

LN-LECs were nutrient starved for 24 h in endothelial basal medium with 0.5% FBS and then harvested and resuspended in EGM containing 10% FBS. Cells were seeded in growth factors-reduced Cultrex basement membrane extract (Trevigen, Gaithersburg, MD)-coated wells (5 x 10^4 per well) and treated or not with 5, 10, 100 ng/ml of p17, VEGF-C, IL-8, or GST. When reported, p17 was preincubated for 30 min at 37 °C with 2.5 \(\mu\)g/ml of unrelated control mAb (Ctrl mAb) or mAb MBS-3. Starved LN-LECs were cultured, when indicated, with an isotype-matched mAb, a neutralizing mAb to CXCR1 (mAb330; R&D), and/or a neutralizing mAb to CXCR2 (mAb331; R&D). When indicated, cells were nucleofected with siRNAs specific for CXCR1 or CXCR2 or irrelevant siRNAs used as negative control. LN-LECs were also pretreated with the inhibitors of phosphatidylinositol 3-kinase (PI3-K) LY294002 (10 \(\mu\)M) or wortmannin (100
nM) (Enzo, Farmingdale, NY, USA), with the inhibitor of MAPK kinase PD98059 (10 µM) (Calbiochem, Billerica, MA), or with the Akt inhibitor VIII (1 µM) (Sigma-Aldrich, St. Louis, MO). In some experiments, 24 h starved LN-LECs were cultured in EGM containing 10% FBS in the presence or absence of not with p17 (10 ng/ml), endothelin A receptor (ET_{A}R) antagonist BQ123 (0.65 µg/ml) or endothelin B receptor (ET_{B}R) antagonist BQ788 (0.65 µg/ml). Wells were then analyzed for the formation of tubule structure.

**siRNA technique**

Nucleoporation of LN-LECs was performed using the Amaxa Nucleofector Technology (Lonza). siRNAs (100 nM) were added to 1 x 10^6 cells resuspended in 100 µl of nucleofection buffer. CXCR1 and CXCR2 silencing was performed using 4 distinct siRNAs targeting 4 different regions of CXCR1 and CXCR2 receptors (Dharmacon, Fisher Scientific, Milan, Italy). Irrelevant (scr) siRNAs (Invitrogen, Milan) were used as negative control. The efficacy of CXCR1 and CXCR2 siRNAs was evaluated by real-time polymerase chain reaction (PCR) analysis. Quantification of CXCR1 and CXCR2 mRNAs was normalized in each reaction according to the internal β-actin control.

**Quantitative Nucleic Acid Analysis**

Total RNA was isolated from LN-LECs (1 x 10^6 cells) using the RNeasy Mini Kit (QIAGEN, Milan, Italy). After retrotranscription, 50 ng of cDNA was mixed with sterile water and the SYBR Green qPCR Master Mix (Promega, Milan, Italy) and amplified using the following PCR primers (0.2 µM each; Primm, Milan, Italy): CXCR1, 5'-TGG GAA ATG ACA CAG CAA AA-3' (forward) and 5'-AGT GTA CGC AGG GTG AAT CC-3' (reverse); CXCR2, 5'-TTGTTGGCTTTCTTCAAGG-3' (forward) and 5'-TGAGGCTTGGAAATGTGAAC-3' (reverse); human β-actin, 5'-GGCACCCAGCACAATGAAG-3' (forward), and 5'-GCTGATCCACATCTGCTGG-3' (reverse). PCR fragments were separated on a 3% agarose gel containing ethidium bromide. CXCR1 and CXCR2 gene expression was indicated as expression index calculated by comparing the levels of intensity for the specific gene to the housekeeping gene β-actin. The relative amounts of ET-1 and β-actin (endogenous control gene) transcripts were determined using a commercially available TaqMan Real-time PCR assay (Taqman gene expression assay, Applied Biosystems, Foster City, CA). Results are expressed as a percentage of control.

**Western Blot Analysis**

LN-LECs (1 x 10^6) were starved for 24 h, stimulated with p17 10 ng/ml at different time points (15, 30 and 60 min) and then processed as previously described. Starved cells, when indicated, were pretreated with a mAb to CXCR1 (R&D) or a
mAb to CXCR2 (R&D) (1 µg/ml). Cells were lysed in 200µL of lysis buffer [10 mM Hepes (pH7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM EDTA, 0.6% Nonidet P-40], containing a mixture of protease inhibitors (Complete Mini; Roche, Milan, Italy) and phosphatase inhibitors [1 mM sodium orthovanadate, 20 µM phenylarsine oxide (PAO) and 30 mM sodium fluoride; Sigma]. Equal amounts of total proteins were resolved on an 12% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. The blots were incubated overnight at 4 °C with mAb to pAkt and mAb to total Akt (Cell Signaling Technology, Boston, MA), mAb to pERK1/2 and polyclonal Ab to ERK1/2 (Santa Cruz Biotechnology, Dallas, TX), mAb to CXCR1 or mAb to CXCR2 (R&D) and mAb to GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (Santa Cruz Biotechnology). The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Thermo Scientific) and revealed using the ECL System (Santa Cruz Biotechnology). Images were acquired by ChemiDoc-it System (Bio-Rad, Segrate, Milan, Italy) and protein expression was determined using Gel-Pro Analyzer software (Media Cybernetics, Rockville, MD).

**Wound Healing Assay**

LN-LECs were plated into 24-well plates (1 x 10⁵ cells/well) in EGM containing 10% FCS and VEGF-C (25 ng/ml). Confluent monolayers were nutrient starved for 24 h and then scratched using a 200 µl pipette tip. After washing, cells were treated or not with p17, IL-8 or GST. In some experiments, 24 h starved LN-LECs were cultured in EGM containing 10% FCS and VEGF-C (25 ng/ml), and treated or not p17 (10 ng/ml), BQ123 (0.65 µg/ml) or BQ788 (0.65 µg/ml).

**Human Angiogenesis Array and Quantitative ELISA**

Sub-confluent LN-LECs were starved for 24 h and cultured for 1, 3, and 6 h in the presence or absence of p17 (10 ng/ml). The conditioned medium was then collected at 1, 3, and 6 h after the beginning of culture and stored in aliquots at -20 °C. An angiogenesis array (Proteome Profiler Array; R&D) was performed to evaluate the presence of angiogenic molecules in conditioned medium. ET-1 was measured in the conditioned medium by ELISA (R&D). In some experiments, 24 h starved LN-LECs were treated for 1 h with 10 µM monensin (Calbiochem) or, when indicated, with the inhibitors of phosphatidylinositol 3-kinase (PI3-K) LY294002 (10 µM) or wortmannin (100 nM), with the inhibitor of MAPK kinase PD98059 (10 µM), or with the Akt inhibitor VIII (1 µM). Cells were then washed and treated or not with p17.

**Preparation of spheroids**

LN-LECs spheroids were prepared according to the method of Laib et al.⁴ with minor modifications. Briefly, LN-LECs (1 x 10⁵) were cultured for 24 h in hanging
drops of 35 µl medium containing 80% EGM supplemented with 2% FCS and 20% methocel stock solution. Twelve spheroids were harvested all together and transferred in a 15 ml centrifuge falcon tube containing 2 ml of sterile PBS. Tubes were centrifuged (5 min at 150, no brake) and the pellet, resuspended in 300 µl of matrix solution [(10% PBS 10X, 2.3% NaOH 1N, 32.7% Sterile H₂O, 55% collagen type 1 (BD, Buccinasco, Milan, Italy)], was transferred in a well of a 48-well plate. 10 ng/ml of IL-8, p17 or GST were supplemented to the spheroid sediment before matrix solution was added. Lymphangiogenesis was quantified by counting the number of sprouts originating from spheroids.

**In vivo lymphangiogenesis assay**

Lymphangiogenesis was evaluated in vivo using a Matrigel plug assay as described previously. Female C57BL/6 mice (age 6-8 weeks, 5 per group) were handled according to the local government of Lower Saxony, Germany (No. 33.9-42502- 04-11/0368). Mice were subcutaneous injected with 500 µl growth factors reduced Cultrex. Gel implants were supplemented with 200 ng/ml p17, 200 ng/ml ET-1 (R&D), or PBS (control). Ten days post injection the gel implants were removed from the mice, fixed, and paraffin-embedded. De-paraffinized and re-hydrated sections were decorated with polyclonal anti-mouse LYVE-1 (Lymphatic Vessel Endothelial Receptor 1) antibody (AngioBio Co., Del Mar, CA) and indirect peroxidase staining was performed. Nuclei were stained using hematoxylin. The sections were analyzed by transmission light microscopy (BX51, Olympus Corp., Center Valley, PA).

**Flow cytometry and antibodies**

To perform flow cytometric analysis, cells were isolated from gel implants according to the method of Adini et al. (2008). The implants were incubated for 1 h at 37 °C in the following enzymatic digestive mixture: 25 µg/ml hyaluronidase (MP biomedical, Solon, OH), 25 µg/ml DNase (Sigma-Aldrich), 3 U/ml Dispase and 3 U/ml Liberase (Roche) dissolved in PBS. The liberated cells were filtered using a 40 µm nylon mesh (BD Falcon), washed three times with PBS containing 1% BSA and 0.01% sodium azide. APC-conjugated mAb to mouse CD31 (LifeSpan BioSciences, Seattle, WA), biotin-conjugated mAb to mouse LYVE-1 (eBioscience, San Diego, CA) and PerCp Cyanine5.5-conjugated Streptavidin (eBioscience) were used to distinguish the ECs within the whole cell population. Samples were analyzed using FACSCalibur and CellQuest software (BD, San Jose, CA).

**Statistical Analysis**

Data were analyzed for statistical significance using one-way ANOVA. Bonferroni’s post test was used to compare data. Differences were considered
significant at $P < 0.05$. Statistical tests were performed using GraphPad Prism 5 software (GraphPad).
References


Supplementary Figure SI. Western blot and RT-PCR analyses of CXCR1 and CXCR2 expression in LN-LECs. (A) LN-LEC lysates were evaluated for expression of CXCR1 and CXCR2 by Western blot using mAb to CXCR1 and CXCR2 as specific reagents. (Upper) Blots from one representative experiment of three with similar results are shown. Equal loading of cell extracts was evaluated by GAPDH protein staining. (Lower) Western blot signals were analyzed semiquantitatively using densitometric scanning. Expression levels are given in arbitrary units and the values reported are the mean ± SD of three independent experiments. (B) Analysis of CXCR1 and CXCR2 gene expression in human LN-LECs performed using quantitative real-time PCR. (Upper) The real time PCR products (CXCR1 115 bp, CXCR2 108 bp, β-actin 114 bp) were visualized by 3 % agarose gel electrophoresis. (Lower) The mRNA expression of CXCR1 and CXCR2 was indicated as Expression level calculated by comparing the levels of intensity for the specific gene to the housekeeping gene β-actin. Expression level is given in arbitrary units and the values reported are the mean ± SD of three independent experiments.
Supplementary Figure SII. Effect of different doses of p17, IL-8 and VEGF-C on organization of LN-LECs in capillary-like structures. Lymphangiogenic response of LN-LECs to the indicated treatments. Pictures were taken after 8 h incubation at 37 °C (magnification 10x). LN-LECs were stimulated with PBS (not treated cells, NT) or with different doses of VEGF-C, IL-8, p17 or of the irrelevant protein GST. Pictures are representative of three independent experiments with similar results. Values (number/well) reported for LN-LEC capillary-like structure (tubes) formation are the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA performed separately for each concentration of VEGF-C, IL-8 or p17 across the three groups. Bonferroni’s post test was used to compare data; ***P < 0.001.
Supplementary Figure SIII. P17-induced capillary–like structure formation is mediated by CXCR1 and CXCR2. (A) Analysis of CXCR1 and CXCR2 gene expression performed using quantitative real-time PCR. LN-LECs were nucleoporated with scr siRNAs used as negative control or with a pool of siRNAs specific for CXCR1 or CXCR2. Analysis of real-time PCR data were performed with the $2^{-\Delta\Delta Ct}$ method using relative quantitation study software. Quantification of CXCR1 and CXCR2 mRNAs was normalized in each reaction according to the internal β-actin control. Bars represent the means ± SD of 3 independent experiments performed in triplicate. (B) Capillary-like structure formation assay of LN-LECs nucleoporated with CXCR1 siRNAs, CXCR2 siRNAs or with scr siRNAs in response to the indicated treatments. Bars represent the means ± SD. This is representative of 3 independent experiments performed in triplicate. Statistical analysis was calculated using one-way ANOVA performed separately for each treatment. Bonferroni's post test was used to compare data: * P<0.05, *** P<0.001.
Supplementary Figure SIV. P17-activation of PI3K/Akt and MEK/ERK pathways requires both CXCR1 and CXCR2. LN-LECs were treated or not for 30 min with p17 (10 ng/ml). When indicated, cells were pretreated with 2.5 µg/ml of neutralizing mAb to CXCR1 or to CXCR2. Cell lysates were evaluated for the expression of pAkt, Akt, pERK1/2 and ERK1/2 by Western blot analysis using mAbs to pAkt, Akt, pERK1/2 or ERK1/2. Phosphorylation of ERK1/2 and Akt was quantified by densitometric analysis and plotting of the pAkt/Akt and pERK1/2/ERK1/2. (Left) Blots from one representative experiment of three with similar results are shown. (Right) Values reported for pERK1/2 and pAkt are the mean ± SD of three independent experiments. Statistical analysis was calculated using one-way ANOVA and Bonferroni’s post test was used to compare data. * P<0.05, *** P<0.001. NT, not treated.
**Supplementary Figure SV. P17 induces sprouting from LN-LEC spheroids.** Sprouting of LN-LEC spheroids treated with medium alone (NT), or 10 ng/ml of GST, IL-8 or p17 (magnification 10x). Values reported for LN-LEC sprouts (number/well) are the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA and the Bonferroni’s post test was used to compare data; *** P< 0.001.
Supplementary Figure SVI. P17 promotes LN-LEC migration. In the wound-healing assay, confluent LN-LEC monolayers were scratched using a 200 µl pipette tip and cultured in the presence of medium alone (not treated cells, NT) or 10 ng/ml of GST, IL-8, or p17 (A). Cell migration was recorded by light microscopy over a 12 h time course after wound scratch. (B) LN-LECs were stimulated for 12 h at 37 °C with medium alone (NT) or p17 (10 ng/ml) after preincubation of viral protein with 2.5 µg/ml of a neutralizing mAb to CXCR1, a neutralizing mAb to CXCR2, or a control isotype-matched mAb (Ctrl mAb). Images are representative of three independent experiments with similar results (magnification 10x). Statistical analysis was calculated using one-way ANOVA and Bonferroni’s post test was used to compare data: ** P<0.01, *** P<0.001.
Supplementary Figure SVII. Analysis of ET-1 gene expression by real-time PCR. LN-LECs were treated or not with p17 or with the irrelevant protein GST at the indicated concentrations. Cells were collected 6 h after the beginning of treatment and evaluated for ET-1 gene expression by TaqMan Real-time PCR. Analysis of real-time PCR data were performed with the $2^{-\Delta\Delta Ct}$ method using relative quantitation study software. Quantification of ET-1 mRNA was normalized in each reaction according to the internal β-actin control. Data are expressed as means ± SD. This is representative of 3 independent experiments performed in triplicate.