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HIV envelope protein gp120-induced apoptosis in lung microvascular endothelial cells by concerted upregulation of EMAP II and its receptor, CXCR3

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Green LA, Yi R, Petrusca D, Wang T, Elghouche A, Gupta SK, Petrache I, Clauss M. HIV envelope protein gp120-induced apoptosis in lung microvascular endothelial cells by concerted upregulation of EMAP II and its receptor, CXCR3. *Am J Physiol Lung Cell Mol Physiol* 306: L372–L382, 2014. First published December 6, 2013; doi:10.1152/ajplung.00193.2013.—Chronic lung diseases, such as pulmonary emphysema, are increasingly recognized complications of infection with the human immunodeficiency virus (HIV). Emphysema in HIV may occur independent of cigarette smoking, via mechanisms that are poorly understood but may involve lung endothelial cell apoptosis induced by the HIV envelope protein gp120. Recently, we have demonstrated that lung endothelial apoptosis is an important contributor to the development of experimental emphysema, via upregulation of the proinflammatory cytokine endothelial monocyte-activating polypeptide II (EMAP II) in the lung. Here we investigated the role of EMAP II and its receptor, CXCR3, in gp120-induced lung endothelial cell apoptosis. We could demonstrate that gp120 induces a rapid and robust increase in cell surface expression of EMAP II and its receptor CXCR3. This surface expression occurred via a mechanism involving gp120 signaling through its CXCR4 receptor and p38 MAPK activation. Both EMAP II and CXCR3 were essentially required for gp120-induced apoptosis and exposures to low gp120 concentrations enhanced the susceptibility of endothelial cells to undergo apoptosis when exposed to soluble cigarette smoke extract. These data indicate a novel mechanism by which HIV infection causes endothelial cell loss involved in lung emphysema formation, independent but potentially synergistic with smoking, and suggest therapeutic targets for emphysema prevention and/or treatment.

emphysema; gp120; HIV; lung; endothelium

INDIVIDUALS CHRONICALLY INFECTED with HIV have an increased incidence of developing several lung complications, including emphysema, pulmonary hypertension, and lung cancer (6, 9, 23, 26, 28, 33). Interestingly, development of emphysema often occurs at an earlier age in HIV patients (i.e., 20–40 yr) than in non-HIV positive smokers (i.e., 50–70 yr), independent of cigarette smoking status (6, 8, 9). Because emphysema carriers a high morbidity and mortality, identifying the determining factors involved in HIV-induced emphysema is of great importance.

The HIV envelope protein gp120 has been shown to have several effects on endothelial cells, most notably induction of apoptosis (10, 15, 18). Apoptosis has been shown to be caspase and p38 MAPK dependent and mediated by the gp120 receptor CXCR4 in various endothelial cell types (25, 41, 42). With regards to lung pathology, gp120 has been shown to increase ceramides, which can induce oxidative stress and apoptosis of endothelial cells leading to the development of emphysema (34). In addition to increased endothelial cell apoptosis, emphysema is characterized by variable levels of lung inflammation. In this context, we and others have shown that gp120, in synergy with cytokines, activates endothelial cells to release chemokines and to express vascular adhesion molecules (13, 16), further augmenting an inflammatory response.

Recently, we have demonstrated that a molecular link between excessive endothelial apoptosis and inflammation, which is significantly involved in the development of cigarette smoke-induced emphysema in mice, is the release of the proinflammatory cytokine endothelial monocyte-activating polypeptide II (EMAP II) (5). Interestingly, cigarette smoke exposure leads to the concerted upregulation of EMAP II and its chemokine receptor CXCR3 in mouse lungs and in primary human lung microvascular endothelial cells, a pathway leading to apoptosis (5, 14).

EMAP II is an intracellular protein, which is transported to the cell surface and released in response to various stresses including hypoxia, mechanical strain, apoptosis, TNF-α, and cigarette smoke (2, 24, 27, 32). Here we investigated if the HIV envelope protein gp120 induces apoptosis in lung microvascular cells via EMAP II and its receptor, CXCR3, independent of cigarette smoke exposure. We show that gp120 signaling through its coreceptor, CXCR4, and p38 MAPK signaling leads to a rapid surface expression of EMAP II and CXCR3. This concerted upregulation of EMAP II and CXCR3 is essential for gp120-induced apoptosis, suggesting a novel autocrine/paracrine mechanism of endothelial cell apoptosis in HIV infection.

MATERIALS AND METHODS

Reagents and cells. Anti-CXCR3 (MAB160) and CXCR4 (MAB170) were obtained from R&D Systems. AlexaFluor secondary antibodies and Tempol were from Invitrogen. p38 MAPK inhibitor (SB203580) was purchased from Sigma. Recombinant EMAP II and monoclonal neutralizing antibodies to EMAP II were produced and purified as recently described (34, 37). Adult human lung microvascular endothelial cells (HLMVEC; Clonetics, Lonza) were cultured in EGM2MV media. CXCR4-tropic recombinant HIV gp120 was

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purchased from Immunodiagnostics (Woburn, MA) and derived from CHO cells.

Determination of CXCR3 and EMAP II by flow-assisted cytometric analysis. Flow-assisted cytometric analysis (FACS) was performed as previously described (36). Briefly, HLMVEC (1×10^6) were fixed in 1% paraformaldehyde (PFA) and stained with anti-human CXCR3 or EMAP II antibodies and the appropriate secondary antibodies. In some cases, HLMVEC were permeabilized with 0.01% Triton X-100 in PBS and stained again using the same primary but different secondary antibodies to detect intracellular CXCR3 or EMAP II. Cells were then analyzed with a FACS-Calibur flow cytometer (BD), and fold induction was calculated based on isotype controls.

Determination of total and surface CXCR3 and EMAP II by immunofluorescence microscopy. HLMVEC were fixed in 1% PFA and stained with CXCR3 (Clone 220803; R&D Systems; 1:100) or EMAP II (1:200) followed by incubation with secondary antibody [Alexa Fluor(r) 546 goat anti-mouse IgG and Alexa Fluor(r) 546 goat anti-rabbit IgG; Invitrogen; 1:1,000]. In some case, HLMVEC were then permeabilized with 0.01% Triton X-100 in PBS to detect intracellular CXCR3 or EMAP II. Confocal microscopy (Olympus FV1000-MPE)-derived images were analyzed using MetaMorph software.

Detection of apoptosis by assessing DNA fragmentation (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) and annexin V/PI. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL Apoptosis Detection Kit; Millipore) in attached endothelial cells as previously described (37). Briefly, images were analyzed by MetaMorph image software, and results were expressed by computing the ratios between TUNEL-positive and total (DAPI positive) cells. Detection of apoptosis by measuring DNA fragmentation via FACS was also carried out using a fluorescein labeling system to detect dUTP end nicks according to the manufacturer's instructions (APO-BRDU kit; Becton Dickinson).

For detection of apoptosis by annexin V, cells were stained using the BD Pharmingen PE annexin V apoptosis detection kit according to the manufacturer's instructions.

p38 MAP kinase silencing in endothelial cells with small interfering RNA. For p38 gene knockdown by RNAi we used Ambion's Silencer Select Custom Designed small interfering (si)RNA against p38 using a protocol as previously described (38). Briefly, cells were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) and after incubation for 2 days at 37°C, total cell lysate was used to determine the knockdown of p38 by Western blotting.

Determination of p38 MAP kinase phosphorylation by Western blot. Cells were lysed in cell lysis buffer (no. 9803S; Cell Signaling;) containing protease inhibitor cocktail (no. 88663; ThermoScientific) with protein concentrations determined by BCA assay. Proteins (20 ng) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting using phospho-p38 or GAPDH antibodies. The chemiluminescent signals were quantified by densitometry using Adobe Photoshop.

Cigarette smoke extract preparation. Filtered research-grade cigarettes (1R3F) from the Kentucky Tobacco Research and Development Center (University of Kentucky, Lexington, KY) were used for preparing aqueous cigarette smoke extract (100%) as previously described (4). Briefly, smoke from two cigarettes or air (ambient air controls) was blown into 20 ml of PBS, followed by pH adjustment to 7.4- and 0.2-μm filtration.

Statistical analysis. Each experiment was performed in triplicate, with a minimum of three independent experiments. The differences between groups were compared using paired Student's *t*-test (for Fig. 1*D* and Fig. 2*E*) or ANOVA with Bonferroni corrections (for all others). Where applicable, means ± SE of multiple measurements are reported as indicated.

RESULTS

gp120 Induces CXCR3 and EMAP II surface expression. Based on our previous finding that stresses can increase CXCR3 receptor surface upregulation in endothelial cells (14), we wanted to know whether HIV envelope (gp120) would have the same ability. Therefore, we treated human microvascular endothelial cells (HMVEC) with recombinant gp120 for up to 18 h and then determined surface CXCR3 expression by flow cytometry (Fig. 1, *A* and *B*). We found that recombinant gp120 induced rapid upregulation of CXCR3 surface expression as early as 3 h after addition of gp120 and remained high for 18 h. To confirm the membrane upregulation of CXCR3, we examined CXCR3 expression in unpermeabilized and permeabilized HMVEC and found that the ratio of extracellular to total CXCR3 was increased dramatically (Fig. 1, *C* and *D*) while total expression of CXCR3 remained unchanged, indicating that increased surface CXCR3 expression is due to protein trafficking rather than overall increased protein synthesis.

To explore the possibility that CXCR3 upregulation was accompanied by induction of its ligand, EMAP II, we then examined the expression of EMAP II in HMVEC after exposure to gp120. We found a two- to threefold increase in surface EMAP II after only 3 h of exposure, followed by a gradual reduction to basal levels over 18 h (Fig. 2, *A* and *B*). The decrease of EMAP II surface expression was accompanied by a corresponding increase in EMAP II released in the supernatant, as determined by EMAP II ELISA (Fig. 2*C*), suggesting active EMAP II secretion from endothelial cells in response to gp120. We then confirmed upregulation by determining the ratio of extracellular to total EMAP II. We compared EMAP II expression in unpermeabilized and permeabilized HMVEC and found that the ratio of surface to total EMAP II increases significantly after gp120 treatment, suggesting that EMAP II surface upregulation is due to increased transport of EMAP II to the membrane surface (Fig. 2, *D* and *E*).

CXCR3 and EMAP II expression mediate gp120-induced endothelial apoptosis. Next, we determined whether the concerted upregulation of CXCR3 and EMAP II led to a corresponding increase of apoptosis. Using a FACS-based TUNEL assay, we analyzed apoptosis in HMVEC treated with either gp120 alone or with a combination of gp120 and EMAP II. Interestingly, while gp120-induced endothelial apoptosis alone was very robust and highly significant, we observed further increase in apoptosis when additional EMAP II (Fig. 3*A*) was added, suggesting that gp120-induced upregulation of the EMAP II CXCR3 receptor apoptosis is involved. Indeed, the addition of CXCR3 neutralizing antibodies abolished gp120-induced endothelial apoptosis (Fig. 3*B*). Importantly and in line with gp120-induced upregulation of EMAP II surface expression described above, neutralizing antibodies to EMAP II also abrogated gp120-induced endothelial apoptosis (Fig. 3*C*) suggesting that concerted induction of EMAP II and its receptor CXCR3 by gp120 is responsible for gp120-induced endothelial apoptosis. To confirm induction of apoptosis by gp120, we then determined apoptosis by annexin V staining and found a similar increase in apoptosis (Fig. 3*D*).

Signal transduction analysis of gp120-induced endothelial apoptosis. p38 MAPK has been previously implicated in gp120-induced apoptosis. In addition, both p38 and reactive

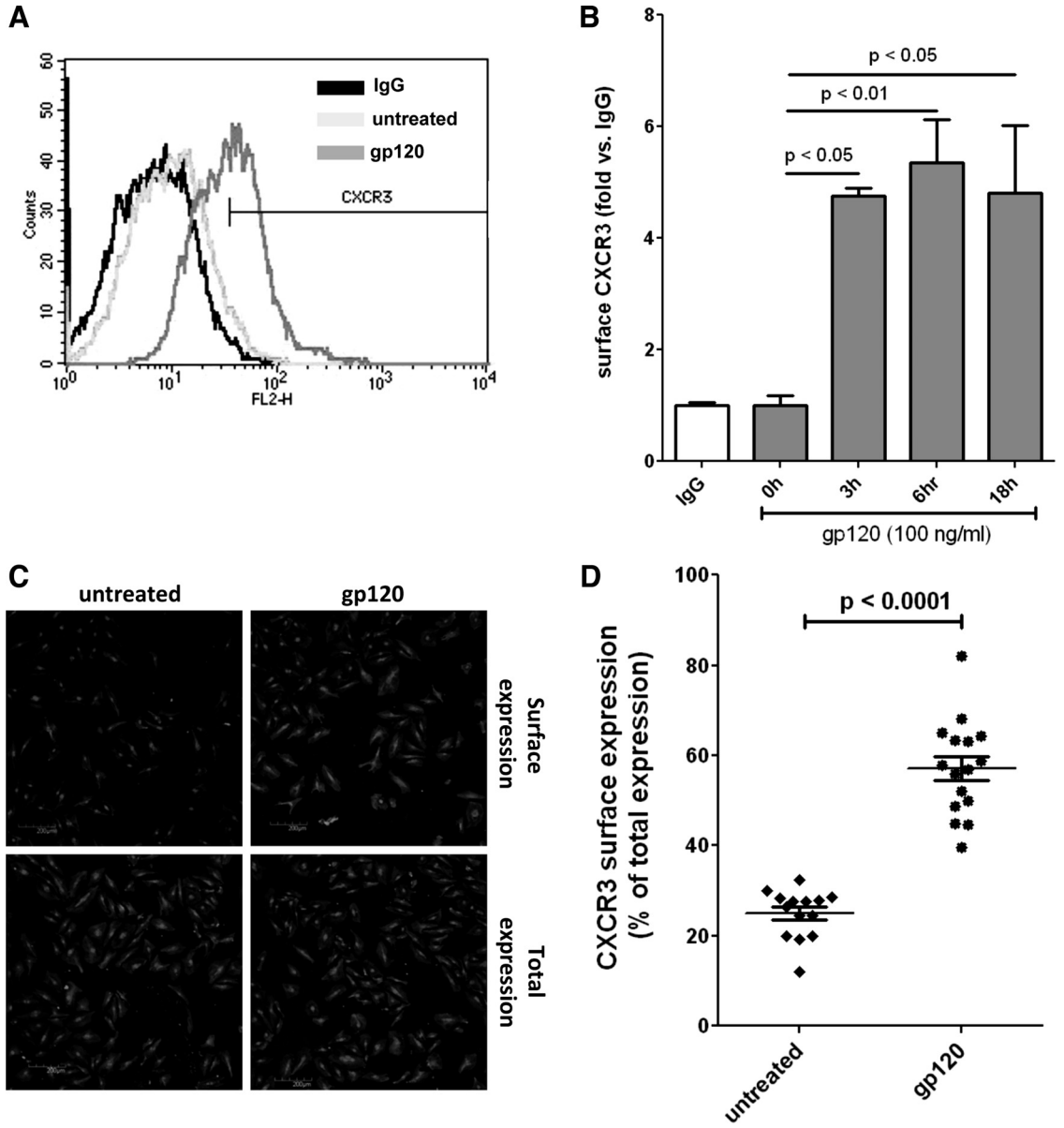

Fig. 1. Upregulation of CXCR3 by gp120. *A* and *B*: human lung microvascular endothelial cells (HMVEC) were treated with 100 ng/ml gp120 for the indicated time points, then stained for surface CXCR3, and analyzed by flow cytometry. Data are expressed as fold CXCR3 expression, normalized to IgG controls. *A*: representative histogram. *C*: extracellular (*top*) and total (*bottom*) CXCR3 was determined before and after 3-h gp120 treatment (100 ng/ml). *D*: surface expression of CXCR3 was determined before and after gp120 treatment and expressed as percent of total expression.

oxygen species are implicated in cigarette smoke-induced endothelial cell injury and apoptosis (14). Therefore, we focused on the role these signal transduction relays. We used a specific p38 MAPK inhibitor or the SOD mimetic Tempol, which we have previously demonstrated to inhibit cigarette smoke induced apoptosis and CXCR3 induction, respectively. Inhibition of p38 MAPK potently blocked gp120-induced apoptosis. However, Tempol failed to have an effect on gp120-induced apoptosis (Fig. 3*C*), suggesting that gp120 may have both shared and distinct signaling pathways required for apoptosis compared with cigarette smoking.

Signal transduction analysis of gp120-induced CXCR3 and EMAP II expression. To determine if p38 MAPK upregulation was upstream of gp120-induced EMAP II and CXCR3 surface upregulation, we incubated HMVEC with gp120 for 4 h in the presence of p38i or Tempol and then measured CXCR3 (Fig. 4*A*) or EMAP II (Fig. 4*B*) surface expression by FACS. While Tempol did not have an effect on CXCR3 upregulation, it did provide a slight inhibition of EMAP II upregulation. In contrast, p38 inhibition caused a marked decrease in the amount of both surface CXCR3 and EMAP II expression, suggesting that p38 is involved in both the upregulation of CXCR3 and EMAP II by gp120.

Differential analysis of p38 MAPK in gp120-induced CXCR3/EMAP II expression and apoptosis. To distinguish between the involvement of p38 MAPK in the gp120-induced upregulation of CXCR3/EMAP II from its role in CXCR3-mediated apoptosis induction (35), we added a p38 MAPK inhibitor (SB203580)

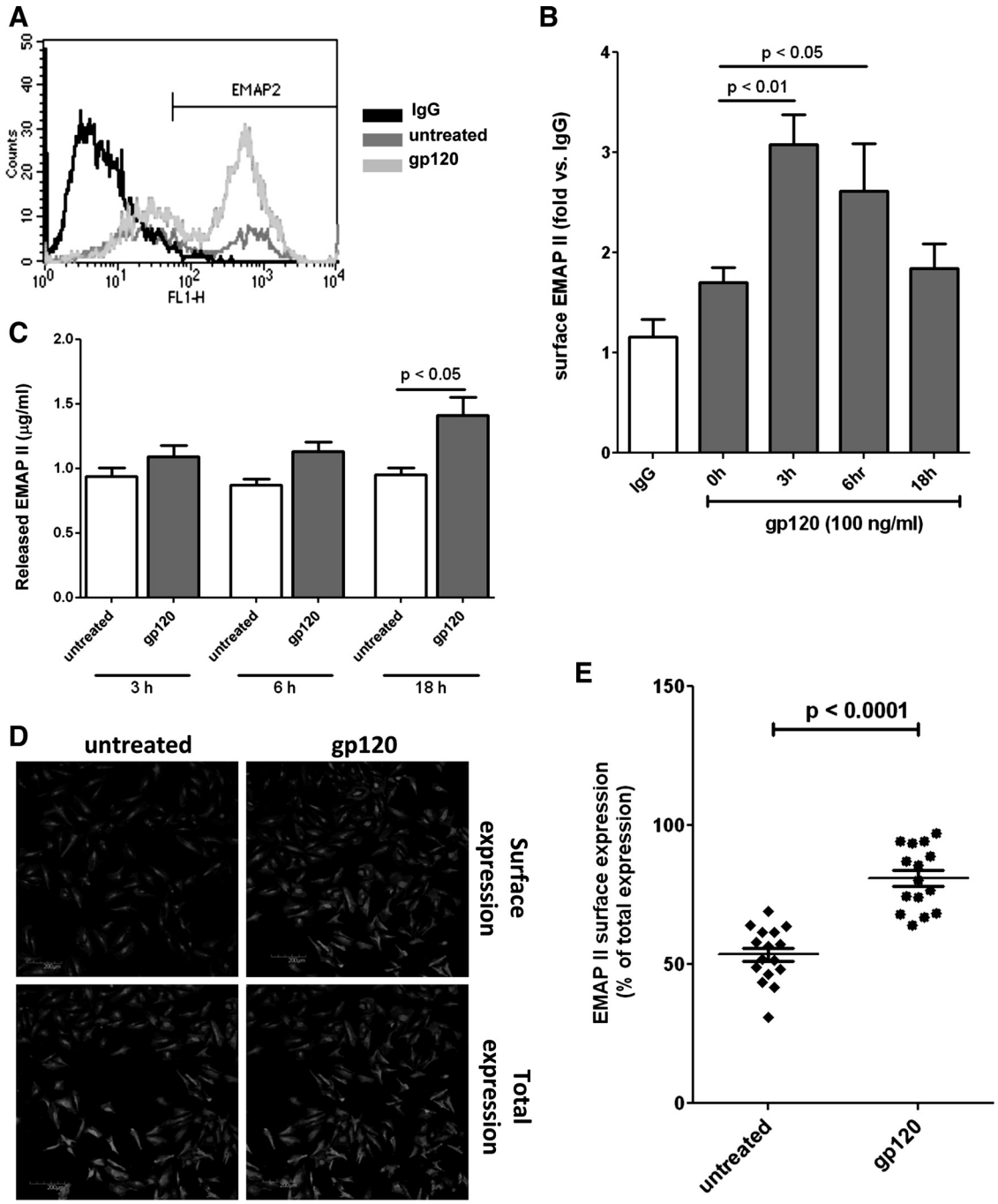

Fig. 2. Upregulation of endothelial monocyte-activating polypeptide II (EMAP II) by gp120. *A* and *B*: HMVEC were treated with 100 ng/ml gp120 for the indicated time points and then stained for surface EMAP II and analyzed by flow cytometry. Data are expressed as fold EMAP II expression, normalized to IgG controls. *A*: representative histogram. *C*: supernatants were collected for all time points and EMAP II concentration was determined by ELISA (Antigenix, Huntington Station, NY). *D*: extracellular (*top*) and total (*bottom*) EMAP II was determined before and after 3-h gp120 treatment (100 ng/ml). *E*: surface expression of EMAP II was determined before and after gp120 treatment and expressed as percentage of total expression.

immediately upon addition of gp120, or after 4 h of treatment with gp120, sufficient for CXCR3 and EMAP II upregulation, and then determined apoptosis. Consistent with the hypothesis that p38 is involved in both CXCR3 upregulation and CXCR3 apoptosis signaling, p38 inhibition abolished apoptosis at both early and late time points (Fig. 5*A*). To confirm the involvement of p38 in gp120-induced apoptosis, we transfected HMVEC with p38 siRNA or scrambled control siRNA and determined gp120-induced apoptosis after knockdown of p38. In fact, we found no induction of apoptosis after p38 knockdown, while cells transfected with scrambled control siRNA maintained strong apoptosis (Fig. 5*B*). Next we analyzed p38 MAPK activation after treatment with gp120 by Western blot analysis of HMVEC. This analysis indicated biphasic p38 phosphorylation and activation, with an initial peak after 30 min of gp120 treatment and a second peak around 16 h after addition of gp120 (Fig. 5, *C* and *D*).

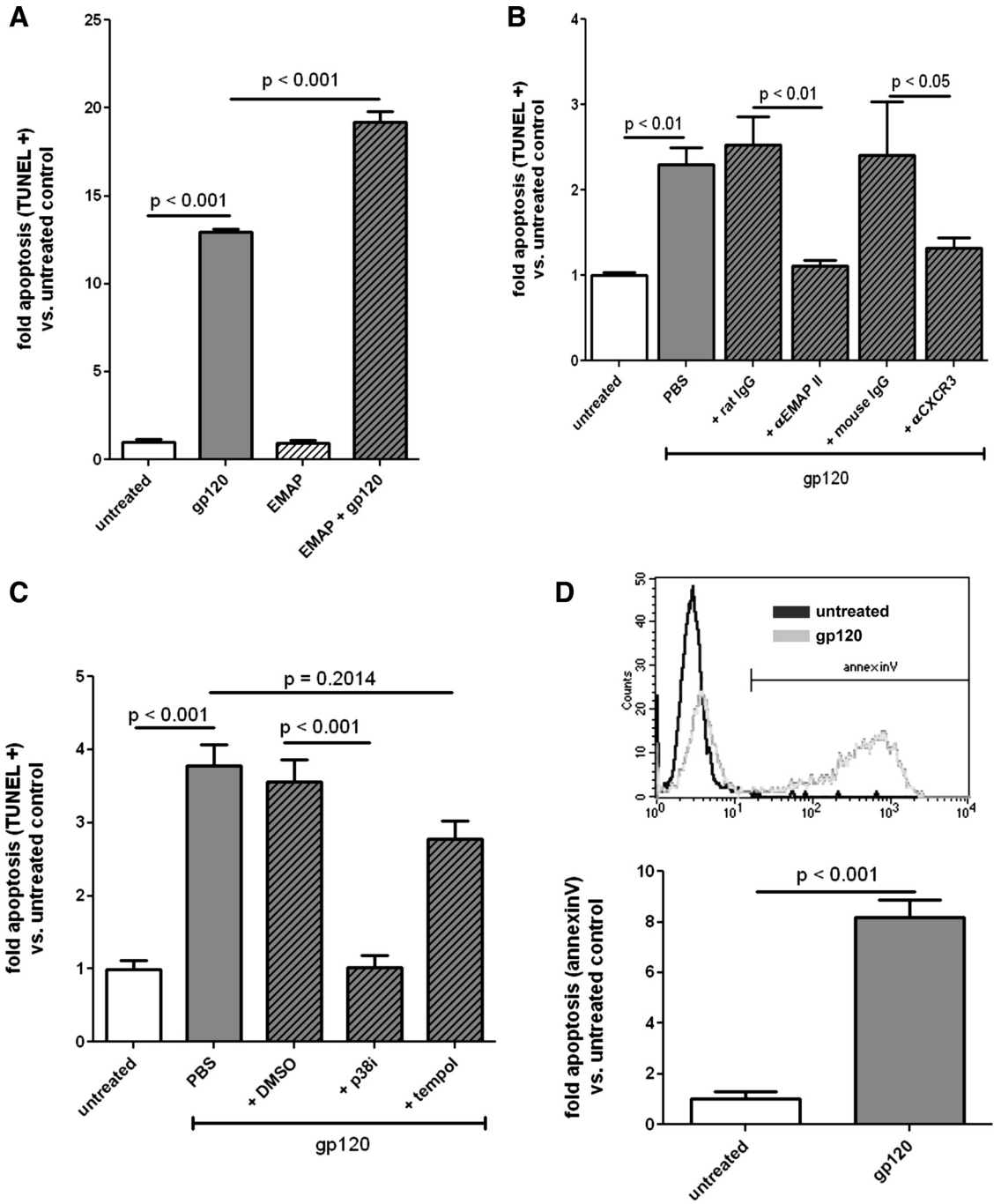

Fig. 3. EMAP II and CXCR3 are required for gp120-induced apoptosis. HMVEC were treated with 100 ng/ml gp120 alone or in combination with EMAP II (30 μg/ml; *A*), neutralizing antibodies to EMAP II (3.5 μg/ml) and CXCR3 (1 μg/ml; *B*), or p38 inhibitor SB203580 (10 μg/ml) and Tempol (6 mM; *C*) for 22 h. Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and normalized to untreated controls. *D*: detection of apoptosis was determined by annexin V staining after 22-h gp120 treatment and normalized to untreated controls (representative histogram top panel).

CXCR4 mediates gp120-induced CXCR3 and EMAP II expression but is not directly responsible for gp120-induced apoptosis induction. In immune cells gp120 signals via a main receptor, CD4, and two coreceptors, CXCR4 and CCR5. Endothelial cells do not express CD4; however, they do express CXCR4 in small amounts, a receptor that has been previously established to mediate gp120-induced endothelial apoptosis (25, 41, 42). We therefore treated HMVEC with gp120 in the presence of CXCR4 neutralizing antibodies and determined CXCR3 and EMAP II surface upregulation. The CXCR4 neutralizing antibody completely abolished gp120-induced both CXCR3 (Fig. 6*A*) and EMAP II (Fig. 6*B*) upregulation, whereas a control IgG had no effect. Next, we tested the effect of CXCR4 neutralizing antibody to gp120-induced apoptosis. In contrast to p38 inhibitors, the anti-CXCR4 antibody only inhibited apoptosis when included at the same time as gp120

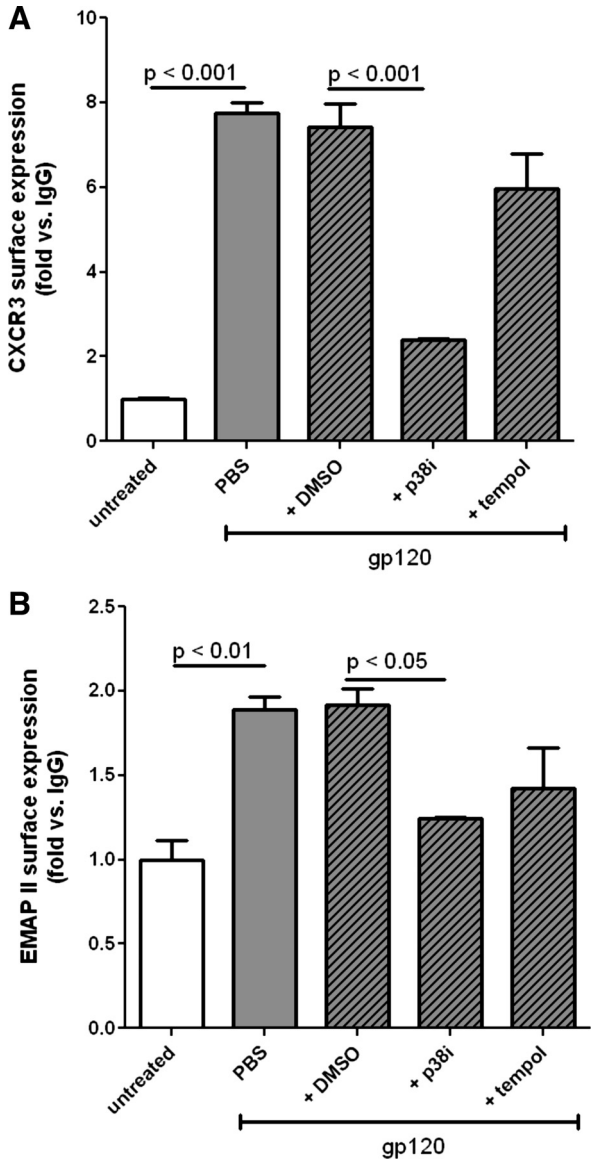

Fig. 4. Signal transduction of CXCR3 and EMAP II surface upregulation. HMVEC were treated with 100 ng/ml gp120 for 4 h in combination with p38 inhibitor SB203580 (10 μg/ml) and Tempol (6 mM) and then stained for surface CXCR3 (*A*) or EMAP II (*B*). Data are expressed as fold expression and normalized to IgG controls.

(Fig. 6*C*). Altogether, these data confirm our overall model of two stages, in which CXCR4 is required in the early first stage of gp120-induced endothelial apoptosis to upregulate CXCR3 and EMAP II. Once CXCR3 and EMAP II are upregulated they are sufficient to trigger apoptosis in a gp120 and CXCR4-independent manner.

Cigarette smoke extract enhances gp120-induced apoptosis induction. Because of the high prevalence of smoking in the HIV-positive population and the established role of cigarette smoke in lung emphysema development, we determined whether cigarette smoke exposure had a synergistic or additive effect with gp120 on endothelial cells. This is particularly relevant because of the earlier onset of emphysema in HIV-positive patients, suggesting the possibility that gp120 can provoke an increased susceptibility to tobacco damage. We treated HMVEC with concentrations of gp120 that were insufficient to induce apoptosis of endothelial cells alone, along with an established concentration of cigarette smoke extract (14), and found that gp120 enhanced cigarette smoke-dependent apoptosis in a dose-dependent manner (Fig. 7).

DISCUSSION

Our data suggest a mechanism for the observed ability of the HIV envelope to induce endothelial cell death (10, 15, 18, 25, 41, 42). According to our two-step model (see Fig. 8), gp120 activates first the CXCR4 receptor to induce concerted upregulation of proapoptotic EMAP II and its receptor CXCR3. In *step 2* EMAP II and its receptor CXCR3 induce p38 MAPK-dependent apoptosis. This is in line with our previous demonstration that cigarette smoke also induces upregulation of EMAP II and its CXCR3 receptor leading to endothelial apoptosis even in nonproliferating endothelial cells (5, 14). Intriguingly, endothelial cell death has been suggested as an important mechanism to induce lung emphysema in rodents (11, 21, 34, 39) and humans (20), and lung capillary endothelial cell injury and apoptosis may explain the increased incidence of emphysema in HIV-infected patients (33).

There are several lines of evidence in support of our hypothesis that the HIV envelope can evoke a series of events including EMAP II/CXCR3 upregulation to induce endothelial apoptosis and vascular injury during development of pulmonary emphysema. First, HIV-infected cells appeared adjacent to emphysematous areas of lung while normal appearing areas were devoid of infected cells, suggesting that infected cells could potentially directly cause lung damage (43). Secondly, injected HIV virus in small rodents suggested endothelial dysfunction in the cardiopulmonary system including oxidative stress and upregulation of proinflammatory cytokines, similar to what is observed in humans with HIV infections (1). Together with reports that gp120 can cause endothelial apoptosis (15, 41), we postulate that gp120 induction of endothelial apoptosis could be a mechanism for the increased incidence of emphysema observed in HIV infected patients. Our previous work has revealed EMAP II as an important mediator of emphysema development, which was able to link inflammation, a hallmark in human emphysema, to tissue destruction. Indeed, treatment with an EMAP II neutralizing antibody inhibited the development of cigarette smoke-induced emphysema in mice (5). Additionally, prolonged upregulation of secreted EMAP II in the lung of a transgenic model led to increased cell death in lung structural cells, including endothelial cells, an accumulation of monocytes, upregulation of matrix metalloproteinases, and an increase in airspace, which, altogether, correlate with features of pulmonary emphysema in humans (5). Interestingly, we could reduce excess upregulation of EMAP II in this model using broad range caspase inhibitors, a finding that is in line with the hypothesis that apoptosis further upregulates EMAP II release, resulting in a feed-forward loop and further destruction of alveolar compartments. Based on these results, we propose that gp120-induced endothelial apoptosis and EMAP II-

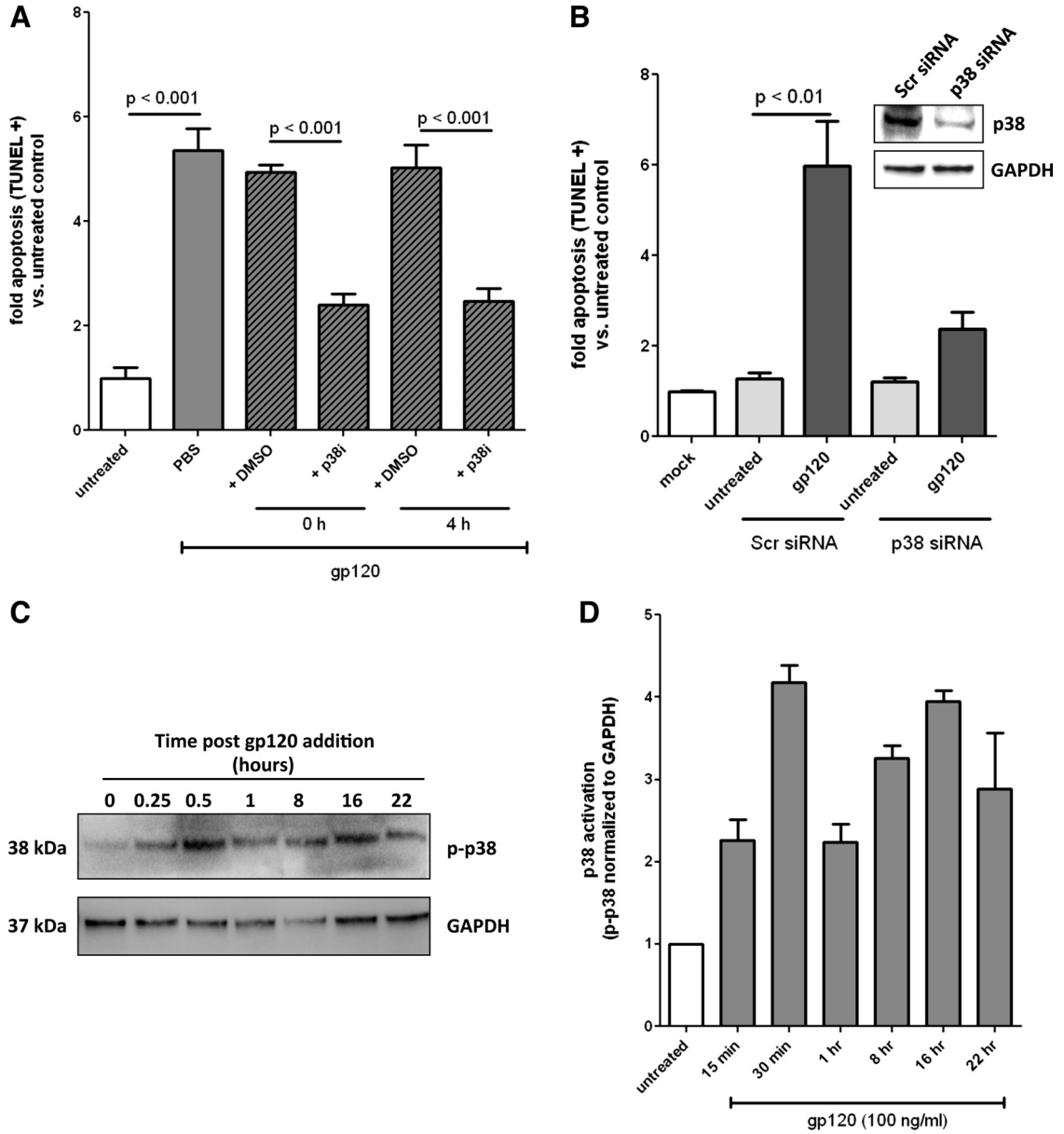

Fig. 5. Involvement of p38 in gp120-induced apoptosis. *A*: HMVEC were treated with 100 ng/ml gp120 for 22 h, with p38 inhibitor SB203580 (10 μg/ml) added either immediately upon addition of gp120 (0 h) or after 4 h (4 h) of treatment. Apoptosis was determined by TUNEL staining and normalized to untreated controls. *B*: HMVEC were transfected with p38 small interfering (si)RNA or control scrambled siRNA and tested for p38 expression at 2 days posttransfection by Western blot (*inset*). Cells were then treated with gp120 and apoptosis was determined by TUNEL staining and normalized to mock transfected control cells. *C*: HMVEC were treated with 100 ng/ml gp120 for the indicated time points and then cell lysates were subjected to phospho-p38 Western blot. *C*: densitometry analysis of phospho-p38 was normalized to GAPDH loading controls.

CXCR3 signaling could lead to inflammation by recruiting mononuclear phagocytes and eliciting positive feedback loops by inducing endothelial apoptosis, a mechanism known to release further amounts of intracellular EMAP II (24).

In this study, we used a gp120 concentration of 100 ng/ml, which has been previously established to induce endothelial cell death and to be relevant for modeling in vivo exposures of cells to HIV (25). Our finding that gp120-induced upregulation of CXCR3 is time dependent is in line with what we have observed previously with cigarette smoke-induced CXCR3 upregulation. On the other hand, gp120-induced upregulation of EMAP II surface expression is biphasic, with a late decrease, which was accompanied by a significant increase in free EMAP II released in the supernatant, consistent with a scenario wherein intracellular EMAP II is first externalized at the membrane surface and then released, causing an autocrine and potentially paracrine response. The mechanism of EMAP II membrane binding and release is unknown. It is possible this mechanism is similar to the alternative release of FGF1, whereby several proteins including S100A13 and synaptotagmin 1, form a membrane-associated protein complex that mediates the flip-flop from intracellular to extracellular membrane localization (12, 17, 22). The apparently delayed release of surface bound EMAP II may be a result of such a mechanism with yet unknown binding partners.

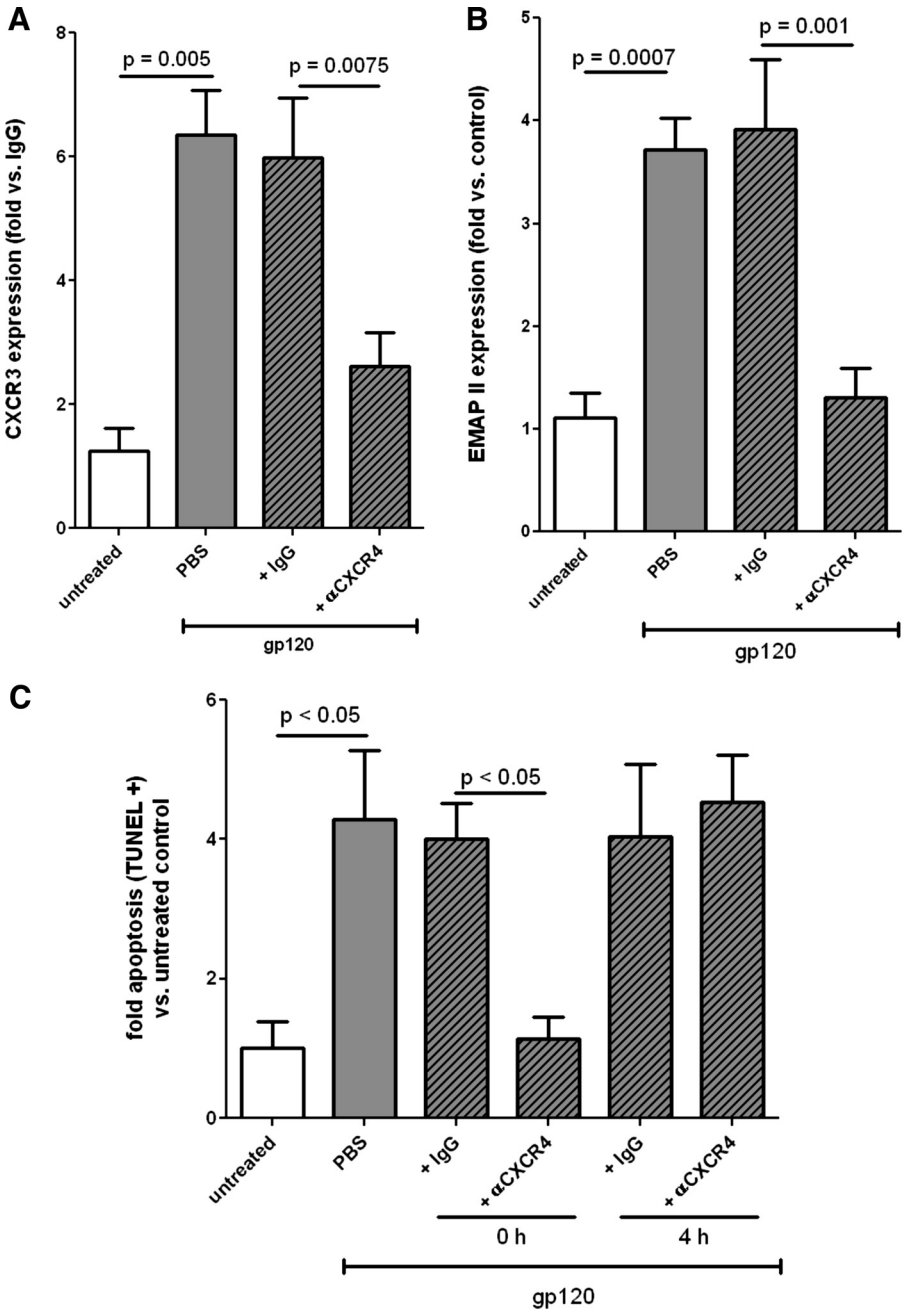

Fig. 6. Involvement of CXCR4 in gp120-induced apoptosis. HMVEC were treated with 100 ng/ml gp120 for 4 h alone or in combination with neutralizing anti-CXCR4 antibodies (1 μg/ml) then stained for either CXCR3 (*A*) or EMAP II (*B*). Data are expressed as fold CXCR3/EMAP II expression, normalized to IgG controls. *C*: HMVEC were treated with 100 ng/ml gp120 for 22 h, with neutralizing anti-CXCR4 antibodies (1 μg/ml) added either immediately upon addition of gp120 (0 h) or after 3 h (3 h) of treatment. Apoptosis was determined by TUNEL staining and normalized to untreated controls.

Notably, the degree of difference between the ratio of extracellular to total CXCR3 and EMAP II (Fig. 1, *C* and *D*, vs. Fig. 2, *D* and *E*) is most likely a result of the large intracellular pool of EMAP II (40). This also explains the high basal level of EMAP II observed in the supernatant even in unstimulated HMVEC (Fig. 2*C*). This large intracellular pool of EMAP II allows for significant release of EMAP II due to the minor stress conditions associated with tissue culture. In fact, it is typically not possible to detect an increase in supernatant EMAP II in vitro. Because of this, upregulation of CXCR3 is the rate-limiting step in in vitro EMAP II/CXCR3 apoptosis. However, the fact that we see an additional increase in released EMAP II over this initial high level is very telling and likely indicative of an even greater fold increase in vivo.

While it has been known that gp120 induces apoptosis of multiple types of endothelial cells, the mechanism for this observations has not been established other than a known requirement for CXCR4. Because of our experience with EMAP II-induced apoptosis of endothelial cells, we thought it is likely that gp120-dependent apoptosis was mediated through EMAP II/CXCR3 signaling, and indeed, blocking either EMAP II or CXCR3 completely abolished gp120-induced apoptosis. Our findings that gp120 upregulates EMAP II and CXCR3 and that gp120-dependent apoptosis requires EMAP II signaling through CXCR3 is indirectly supported by previous reports that gp120 apoptosis is caspase driven and involves mitochondrial dysfunction, since CXCR3-dependent apoptosis is also caspase dependent.

To further analyze the signaling mechanism for upregulation of CXCR3 and EMAP II compared with endothelial apoptosis, we had added candidate inhibitors previously shown to be involved in cigarette smoke-induced CXCR3 upregulation and apoptosis induction, at early and late time points. Intriguingly, independent of the time point of application, p38 MAP kinase inhibitors were able to both block CXCR3 upregulation and apoptosis. This finding is in line with previous reports that p38 activation is downstream of CXCR4 signaling and essential for CXCR3-mediated apoptosis (19, 30). Our Western blot analysis of p38 phosphorylation further supports this, as we observed an initial peak shortly after addition of gp120, consistent with the initial activation of the CXCR4 receptor, and a second peak after 16 h of gp120 treatment, consistent with apoptosis signaling through CXCR3 (see Fig. 8 for scheme) (7). In contrast, inhibition of reactive oxygen species using a SOD-mimetic inhibited neither CXCR3 upregulation nor apoptosis, which is distinct from signaling employed by cigarette smoke to upregulate CXCR3.

According to demographic information, there is a high prevalence of cigarette smoke exposure in the HIV-positive population (3, 29, 31). Although the risk of emphysema development in HIV patients appears higher than the risk posed by cigarette smoke alone, it is possible there is a synergistic effect between the two risk factors. Our results support the hypothesis that such a relationship may exist, since low concentrations of gp120 enhanced the induction of apoptosis in cigarette smoke exposed endothelial cells. Future studies will have to identify the molecular underpinnings of such a synergy; however, it is very likely that this synergy is a result of the differing pathways involved in CXCR3 upregulation. Because of the contrast between the available intracellular pools of EMAP II and CXCR3, the surface expression of CXCR3 is the key step in EMAP II/CXCR3-mediated endothelial apoptosis. Therefore, the use of two distinct pathways to mediate CXCR3 surface expression would allow for a faster response time and subsequently a greater amount of apoptosis.

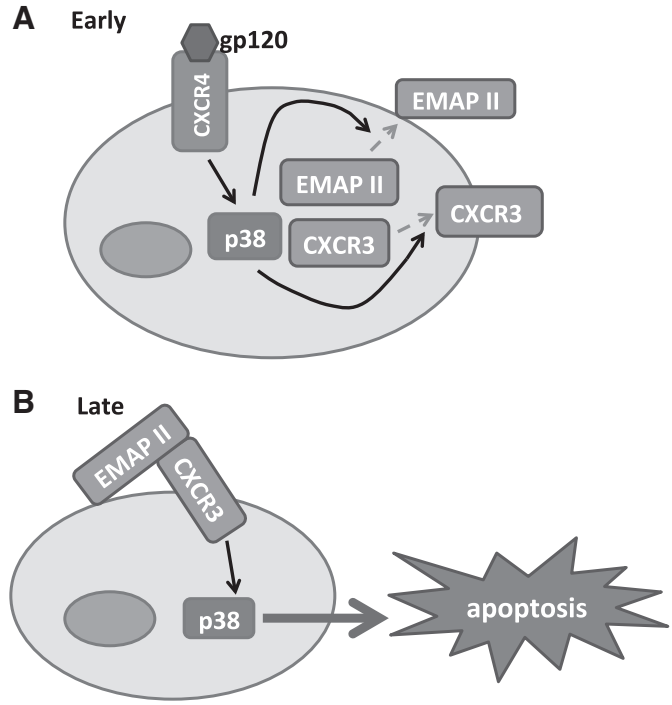

Fig. 8. Schematic for gp120-induced apoptosis. *A*: shortly following gp120 binding to its receptor, CXCR4, p38 MAP kinase is activated and induces the transport of EMAP II and CXCR3 to the cell surface (dashed lines). *B*: after surface upregulation, EMAP II signals through CXCR3 to induce apoptosis in a p38-dependent manner.

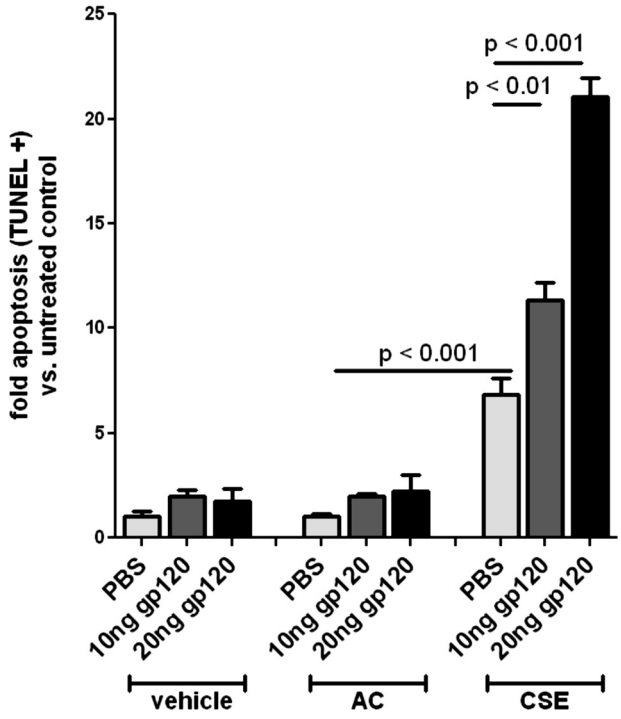

Fig. 7. Gp120 increases apoptosis in combination with cigarette smoke. HMVEC were treated with 10 or 20 ng/ml gp120 for 22 h alone or in combination with 1% cigarette smoke extract (CSE) or ambient air controls (AC). Apoptosis was determined by TUNEL staining.

In conclusion, these mechanistic insights into the gp120-induced autocrine and possibly paracrine proapoptotic and proinflammatory effects involving CXCR3 and EMAP II release may lead to the development of therapeutics targeting emphysema and other diseases caused by endothelial damage in the HIV-infected population.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.A.G., S.K.G., I.P., and M.C. conception and design of research; L.A.G., R.Y., D.P., T.W., and A.E. performed experiments; L.A.G., R.Y., D.P., and A.E. analyzed data; L.A.G., D.P., and M.C. interpreted results of experiments; L.A.G. prepared figures; L.A.G. drafted manuscript; I.P. and M.C. edited and revised manuscript; M.C. approved final version of manuscript.

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