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HIV-1 gp120 signaling through TLR4 modulates innate immune activation in human macrophages and the biology of hepatic stellate cells

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RECEIVED DECEMBER 1, 2015; REVISED FEBRUARY 2, 2016; ACCEPTED FEBRUARY 16, 2016. DOI: 10.1189/jlb.4A1215-534R

ABSTRACT

Highly active antiretroviral therapy has significantly improved the prognosis of HIV-infected subjects. However, patients treated long term still manifest increased mortality and, even with undetectable plasma viremia, often experience persistent immune activation. Furthermore, liver-related mortality is now the most common cause of non-AIDS-related death in HIV-infected individuals on highly active antiretroviral therapy through accelerated fibrosis progression. TLRs are the first line of the host response to pathogens and play an important role in human host defense against viruses through sensing of viral structural proteins. Growing evidence points to TLR4 as a key player in chronic immune activation, HIV recognition/replication, and liver fibrosis progression, suggesting that HIV triggering of TLR4 may dictate some aspects of the multifaceted AIDS pathogenesis. In this study, we provide evidence for an interplay between host TLR4 and HIV-1 gp120 in human monocyte-derived macrophages and hepatic stellate cells, leading to intracellular pathways and biologic activities that mediate proinflammatory and profibrogenic signals. Finally, we hypothesize that CCR5 and TLR4 are likely part of a common receptor cluster, as the blocking of CCR5 by specific antagonists impairs the macrophage capacity to produce chemokines in response to LPS. Chronic immune activation and liver fibrosis remain important obstacles for highly active antiretroviral therapy success. Thus, the identification of gp120-TLR4 axis as a novel determinant of immune system and hepatic stellate cell biology opens new perspectives to the management of HIV infection and disease. *J. Leukoc. Biol.* 100: 599–606; 2016.

Introduction

Chronic immune activation and systemic inflammation, hallmarks of HIV infection, are now considered a main driving force leading to progressive immune failure. Furthermore, accumulating evidence points to chronic immune activation as one of the multifactorial pathogenic mechanisms associated with HIV disease and HIV-associated non-AIDS disease [1]. In recent years, a better management of HIV infection with the use of HAART has led to a much longer survival of these patients and to the emergence of different causes of death, including end-stage liver disease [2]. However, HAART does not completely eliminate chronic immune activation and inflammation. As a consequence, patients may experience several non-AIDS-related complications, in spite of a successful antiviral treatment, which can be considered a direct or indirect consequence of a chronic inflammatory status. Liver disease in HIV-infected individuals, in the absence of coinfection with hepatotropic viruses, is an emerging issue in the management of these patients [3]. Whereas HAART-related toxicities are a main cause for liver damage, growing evidence suggests that HIV infection may have a direct impact on the pathogenesis of liver fibrosis and subsequent progression to advanced liver disease [3]. In addition, patients with HIV coinfected with hepatotropic viruses, particularly HCV, have a much faster progression of hepatic fibrosis, leading to the earlier appearance of liver-related complications [4].

As first responders to pathogens, TLRs, together with other pathogen recognition receptors, play a key role in antiviral host response [5]. In particular, TLR4 expression/function in innate immunity cells is modulated in vitro and in vivo during HIV-1 infection [6], and HIV-1 gp120 signaling through TLR4 and TLR2 has been reported recently in genital epithelial cells [7] and cancer cell lines [8], although scarce evidence has been achieved on HIV-1 recognition through surface TLR so far [9]. In macrophages, TLR4 triggering regulates HIV-1 replication

Abbreviations: AT-2 = aldrithiol-2, HAART = highly active antiretroviral therapy, HCV = hepatitis C virus, h.i. = heat inactivated, HSC = hepatic stellate cell, IRF3 = IFN regulatory factor 3, MDM = monocyte-derived macrophage, Pam3CSK4 = synthetic triacylated lipopeptide

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and contributes to AIDS-associated immune activation [10]. Moreover, a physical interaction between Tat protein and TLR4 was described recently in these cells [11]. TLR4 is also a key player in liver fibrosis, as its engagement may directly activate HSCs [12]. It is noteworthy that the HIV gp120 envelope protein interacts with MDMs and HSCs and exerts proinflammatory and profibrogenic activities independently of productive infection [13]. Although growing evidence suggests a role of TLR4 in the mechanisms promoting HIV-1 recognition/replication, liver fibrosis progression, and pathogenesis, the functional consequences of HIV-TLR4 cross-talk have not yet been defined. Here, we show that the engagement of host TLR4 by HIV-1 gp120, previously undescribed in MDMs and HSCs, leads to intracellular signaling pathways and functional activities. Additionally, we hypothesize that a common receptor cluster, including CCR5 and TLR4, is required to activate the innate proinflammatory cascade in MDMs, as the blocking of CCR5 by specific antagonists impairs the macrophage capacity to produce chemokines in response to LPS.

MATERIALS AND METHODS

Ethics statements

Healthy donor buffy coats were obtained from Centro Trasfusionale University of Rome "Sapienza." Buffy coats were not obtained specifically for this study. Informed consent is not required, as data were analyzed anonymously. Data from healthy donors have been treated by Centro Trasfusionale, according to the Italian law on personal data management—"Codice in materia di protezione dei dati personali" (Testo unico D.L., June 30, 2003, n. 196).

Cell separation and culture

Monocytes were isolated from PBMCs, obtained from healthy donor buffy coats by immunomagnetic selection using CD14 microbeads (MACS Monocyte Isolation kit; Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions. This procedure yields a ≥98% pure population of monocytes, as assessed by FACS analysis of lineage-specific surface markers (CD1a, CD14, CD3, CD19, CD56). MDMs were obtained from monocytes (1×10^6 cells/ml) after 5 d of in vitro culture in endotoxin-free Iscove's medium (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA) containing 10% FBS. HSCs were isolated from wedge sections of liver tissue unsuitable for transplantation by collagenase/pronase digestion and centrifugation on Stractan gradients, as described previously [14]. All of the experiments were conducted on cells cultured in IMDM, supplemented with 20% FBS on uncoated plastic dishes (passages 3–9), showing an "activated" or "myofibroblast-like" phenotype [15]. Cells isolated from at least 3 different donors were used in all experiments.

Reagents

All culture reagents were purchased from BioWhittaker (Lonza, Basel, Switzerland) as endotoxin-free lots. Ultrapure LPS from *Escherichia coli* (serotype EH100, Ra TLR grade) was purchased from Alexis Biochemicals (Nottingham, United Kingdom). Recombinant gp120 from CN54 HIV-1 strain, JRFL virus, and the inhibitors Tak779, Maraviroc, and AMD3100 were obtained from the NIH AIDS Research and Reference Reagent Program (Bethesda, MD, USA). Recombinant gp120 Ada and JRFL strains were kindly provided by Drs. Gao and Doms, respectively. The HIV-1 Ada (Advanced Biotechnologies, Eldersburg, MD, USA) and JRFL virus preparation were inactivated as described previously [16]. LPS contamination of reagents was excluded by checking their endotoxin activity by the limulus amebocyte assay (Endosafe; Charles River Laboratories, Charleston, SC, USA; detection limit: 0.125 endotoxin units/ml). The endotoxin content determined in gp120 preparations was <0.125 endotoxin unit/ml [17]. Polymixin B was purchased from Calbiochem (EMD Millipore, Billerica, MA, USA). Pam3CSK4 was purchased from InvivoGen (San Diego, CA, USA). mAb against TLR4 [18] (5 μg/ml; clone 15C1; 1 h pretreatment) and TLR2 (5 μg/ml; clone T2.5) were kindly provided by Greg Elson (Novimmune; current affiliation Glenmark Pharmaceuticals, La Chaux-de-Fonds, Switzerland). IgG isotype control was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used at the concentration of 5 μg/ml. To test the effect of specific inhibitors, cells were treated before and during gp120 exposure with 5 μM CLI-095 (EMD Millipore).

Cytokine and chemokine determination

Cytokine and chemokine levels in culture supernatants were measured by ELISA kits, purchased from R&D Systems (Minneapolis, MN, USA) for CCL2 and from BioLegend (San Diego, CA, USA) for CCL4, for IL-1β, CXCL8, and IL-6, according to the manufacturers' instructions.

Immunoblot Analysis

MDMs were exposed to gp120 in the presence or in the absence of anti-TLR4 mAb for the indicated time periods. Cell were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), containing a cocktail of protease and phosphatase inhibitors, and protein extracts were resolved by 8–12% SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis with antibodies specific for the total or phosphorylated forms of p65 NF-κB, p38 MAPK, ERK1/2, and IRF3 (Cell Signaling Technology, Danvers, MA, USA) and β-actin (BD Biosciences, San Jose, CA, USA). Levels of phosphorylated and nonphosphorylated proteins were quantified using ImageJ software (software developed by Wayne Rasband, NIH).

Chemotactic assay

Subconfluent HSCs were serum starved for 24 h, washed, trypsinized, and resuspended in serum-free medium at a concentration of 3×10^5 cells/ml. Cell migration was measured in modified Boyden chambers, equipped with 8 μm porosity polyvinylpyrrolidone-free polycarbonate filters (EMD Millipore), and precoated with collagen (20 mg/ml human type I collagen for 30 min at 37°C; Collaborative Biomedical Products, Bedford, MA, USA), as described previously [19]. At least 10 high-power fields per filter were counted in each experiment.

Statistical analysis

Statistical comparison among various groups was performed by 1-way ANOVA with Bonferroni post hoc tests, using GraphPad Prism 5 software for statistical analysis. Comparisons were made between means from independent experiments. Differences were considered significant when $P < 0.05$. Statistical significance is indicated in figure legends.

RESULTS

HIV-1 gp120 induces TLR4-mediated proinflammatory factors in MDMs

To determine the role of TLR4 as a potential sensor of viral structural proteins, such as the envelope protein, we performed experiments with blocking mAb to TLR4 in primary human macrophages exposed to recombinant R5 HIV-1 gp120 (full-length glycosylated, monomeric CN54 strain), as well as to ultrapure LPS from *E. coli* as positive control. We chose mAb neutralization, as this technique is commonly used to elucidate the physiology of virus–receptor interactions. MDMs generated from peripheral blood monocytes were pretreated with anti-TLR4

mAb [18] before stimulation with R5 gp120 (2 μg/ml) or LPS (10 ng/ml). We found that the blocking of TLR4 significantly reduced the secretion of CCL2 (A), CCL4 (B), CXCL8 (C), and IL-1β (D) induced by CN54 gp120 (**Fig. 1**) and as expected, totally inhibited the production of these cytokines upon LPS stimulation. Interestingly, treatment with anti-TLR4 mAb did not affect gp120-induced production of IL-6 (**Fig. 2A**), whereas it suppressed that induced by LPS, demonstrating that the impact of blocking TLR4 on HIV-1 gp120-induced cytokine production is not only cytokine specific but also independent of LPS contamination. To exclude further significant bacterial endotoxin contamination, MDMs were exposed to unmodified CN54 gp120 or CN54 gp120 h.i. by boiling. This procedure causes protein denaturation without affecting LPS activity, as demonstrated previously [20]. As shown in Fig. 2B, no CCL2 secretion was observed upon MDM treatment with h.i. gp120, whereas h.i. LPS retained its activity. Moreover, the LPS sequestering agent polymixin B did not attenuate gp120-induced CCL2 secretion, whereas it markedly inhibited LPS activity, firmly excluding LPS contamination (Fig. 2C). Antibody cross-reactivity and nonspecific effects were also excluded by testing the capacity of anti-TLR4 and -TLR2 to interfere with Pam3CSK4 and LPS, respectively (Fig. 2D).

The ability of anti-TLR4 to inhibit gp120-induced CCL2 secretion was not restricted to the CN54 strain, as inhibition was also observed after treatment with other gp120, as well as with AT-2-inactivated HIV-1 virions from different strains (R5 Ada and JRFL; 50 ng/ml p24).

In contrast to conventional methods of inactivation (i.e., heat or formalin treatment), the AT-2 procedure allows viruses to retain conformational and functional integrity of viral surface proteins [16]. As shown in **Table 1**, reduced CCL2 secretion, compared with gp120 or AT-2 virus alone, was observed in all donors tested in the presence of anti-TLR4 mAb, although at a lesser extent compared with CN54 gp120. Although statistical significance was not reached in the case of AT-2 Ada virus, a clear-cut trend toward CCL2 reduction was observed in the presence of anti-TLR4 for this condition. CN54 gp120 proved to be the most potent inducer of CCL2 compared with other stimuli. Therefore, all subsequent experiments aimed at defining the involvement of TLR4 in gp120 signaling in MDMs were performed with CN54 gp120. The involvement of TLR4 in gp120-mediated induction of CCL2, CCL4, CXCL8, and IL-1β was further confirmed by pretreating MDMs with the small molecule inhibitor CLI-095 (5 μM; **Fig. 3**) [21] that blocks signaling mediated by the intracellular domain of TLR4 but not the extracellular one. Altogether, these results indicate that TLR4 participates in the mechanisms by which HIV-1 gp120 stimulates the expression of proinflammatory cytokines/chemokines in human MDMs.

HIV-1 gp120 triggers TLR4 signaling pathways in MDMs

To provide additional evidence for the role of TLR4 in the modulation of cytokine/chemokine production by gp120, we investigated the effects of blockade of virus interaction with TLR4 on gp120-associated signaling. We evaluated the activation of the NF-κB pathway, the MAPK families, and finally, IRF3. These represent 3 major pathways involved in TLR4 signaling and at the same time, known to be induced by gp120 [17, 22, 23]. Thus, the phosphorylation status of p65, IRF3, p38MAPK, and ERK1/2 was assessed by Western blotting. As shown in **Fig. 4**, activation of NF-κB/MAPK through stimulation of MDM by gp120 was dependent on TLR4. Conversely, IRF3 did not appear to have a role in TLR4-mediated gp120 signaling, as its phosphorylation extent did not change in the presence of anti-TLR4 mAb (Fig. 4A and E), suggesting the involvement of a MyD88-dependent pathway. The levels of total p65 and IRF3 expression upon gp120 treatment were not modulated (data not shown). Of note, the gp120-induced level of phosphorylation of p65 (Fig. 4A and D), p38 (Fig. 4B and F), and ERK1/2 (Fig. 4C and G) MAPKs was reduced at a similar extent—94, 78.5, and 83%, respectively, by treatment with anti-TLR4 mAb, as determined by densitometry. These results highlight the role of TLR4 in the induction of signals triggered by HIV gp120, which are essential for the production of cytokines/chemokines in macrophages.

HIV-1 gp120 triggers TLR4-mediated profibrogenic action in HSCs

We have previously reported the ability of gp120 to induce a profibrogenic activation of HSCs [13]. These cells are considered the main players in the pathogenesis of liver fibrosis, and their infection by HIV-1 has been reported [24]. Thus, we sought to determine whether neutralizing mAb to TLR4 abrogated gp120-mediated activation of these cells. To this end, HSCs isolated from normal liver tissue and activated in culture [14] were

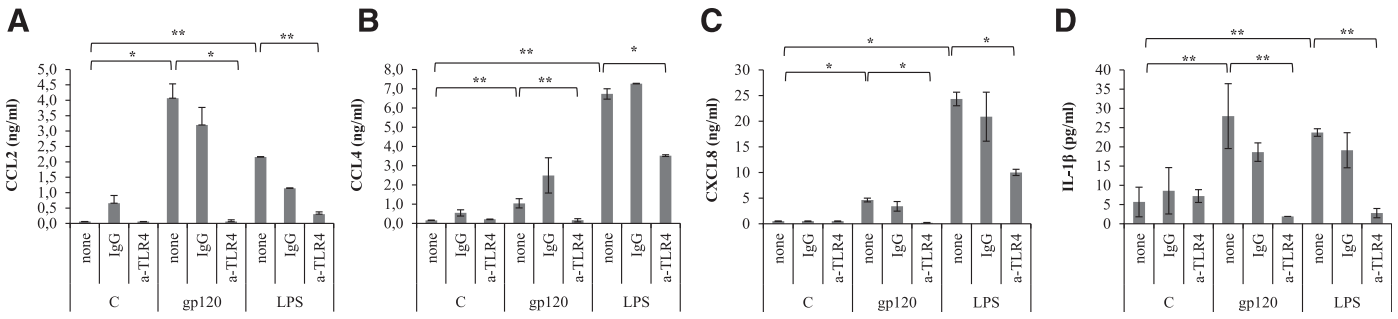

Figure 1. Effect of the TLR4-blocking antibody on gp120-induced production of cytokines/chemokines in primary MDMs. MDMs were preincubated for 1 h, with or without anti-TLR4 (a-TLR4; clone 15C1)-blocking mAb or isotype control (IgG), and then exposed to gp120 CN54 or LPS or left untreated [control (C)]. After 24 h of culture, supernatants were harvested and frozen before CCL2 (A), CCL4 (B), CXCL8 (C), and IL-1β (D) determination by ELISA. Data are represented as means ± SE of 8 (A and C) and 7 (B and D) independent experiments. $*P < 0.05$, $**P < 0.005$, calculated by ANOVA.

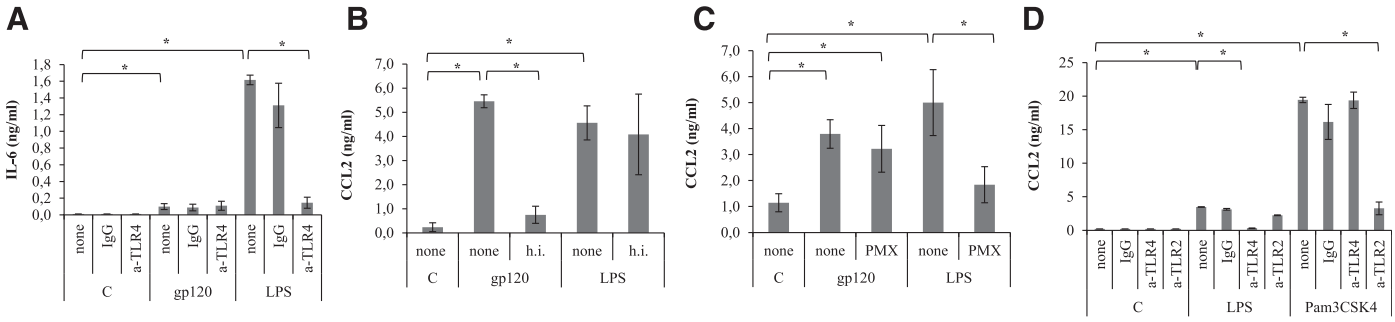

Figure 2. Specificity control. MDMs were preincubated for 1 h, with or without anti-TLR-blocking mAb or isotype control (IgG), and then exposed to gp120 CN54, LPS, or Pam3CSK4 or left untreated (control). After 24 h of culture, supernatants were harvested and frozen before IL-6 (A) determination by ELISA. To exclude bacterial endotoxin contamination of reagents, MDMs were exposed to CN54 gp120 or LPS, either active or h.i., by boiling (B) or pretreated with polymixin B (PMX) for 1 h (C). To test the specificity of anti-TLR4 mAb, MDMs were preincubated for 1 h, with or without anti-TLR4 (clone 15C1), anti-TLR2 (clone T2.5)-blocking mAb, or isotype control (IgG1; 5 μg/ml), and then exposed to the TLR ligands. The capacity of TLR2 and TLR4 mAb to block interaction with the specific ligand but not with others excludes antibody cross-reactivity and nonspecific effects (D). After 24 h of culture, supernatants were harvested and frozen before CCL2 determination by ELISA. Data are represented as means ± SE of 3 (A, C, and D), and 2 (B) independent experiments. $*P < 0.05$, calculated by ANOVA.

exposed to 500 ng/ml recombinant gp120 (CN54) for 24 h in the presence or absence of anti-TLR4 or TLR4 inhibitor CLI-095. As shown in **Fig. 5**, gp120-induced production of CCL2 (Fig. 5A) and CXCL8 (Fig. 5B), as well as migration (Fig. 5C) of HSCs, was reduced significantly when HSCs were pretreated with anti-TLR4 mAb or with the inhibitor CLI-095, strongly indicating a role for TLR4 in mediating profibrogenic signals in response to gp120, in addition to CCR5, as described previously [25]. Similar to what observed in MDMs, CCL2 and CXCL8 induction by gp120 was not restricted to CN54 gp120 but was also observed upon HSC exposure to JRFL and Ada gp120 strains. Induction of both chemokines by Ada gp120 was significantly inhibited by TLR4 blocking. Likewise, CCL2 secretion promoted by JRFL gp120 was reduced significantly in the presence of the anti-TLR4 antibody, whereas a clear-cut trend toward inhibition was observed for CXCL8 under the same experimental condition (Fig. 5D).

Effect of CCR5 blocking on TLR ligand-induced chemokine production

Finally, we tested the hypothesis that TLR4 may cocluster with CCR5, similarly to the TLR4/CXCR4 complex [26–28], by evaluating the inhibitor effect of the CCR5 receptor antagonists Tak779 (5 μM) and Maraviroc (0.1 μM) on LPS-induced

TABLE 1. Involvement of TLR4 in CCL2 secretion induced by different strains of HIV-1 gp120 and AT-2-inactivated virions in MDMs

		CCL2 production (pg/ml)					
Treatment	Anti-TLR4	Donor 1	Donor 2	Donor 3	Donor 4	Means ± SE	*P*
C	−	11	582	509	516	405 ± 132	–
	+	50	966	761	466	561 ± 199	ns
gp120							
CN54	−	11359	8030	3114	6735	7310 ± 1704	[a]
	+	3589	630	421	15.2	1164 ± 818	[b]
JRFL	−	1185	1870	1847	2001	1906 ± 42	[a]
	+	25	989	1291	716	755 ± 144	[c]
Ada	−	ND	1695	2370	2153	1797 ± 199	[d]
	+	ND	710	1398	1282	967 ± 213	[c]
AT-2 HIV							
JRFL	−	1107	1578	1552	1083	1330 ± 139	[a]
	+	511	704	682	487	596 ± 60	[c]
Ada	−	908	1001	2221	1532	1416 ± 302	[e]
	+	569	357	770	833	632 ± 108	ns

Involvement of TLR4 in CCL2 secretion induced by different strains of HIV-1 gp120 and AT-2-inactivated virions in MDMs, which were treated with gp120 (2 μg/ml) or AT-2-inactivated HIV-1 strains (50 pg/ml p24) in the presence (+) or absence (−) of anti-TLR4 mAb (clone 15C1; 5 μg/ml) or left untreated [control (C)]. After 24 h of culture, supernatants were harvested and tested for CCL2 content. Each value represents the mean of duplicate culture samples, and the mean values ± SE is also indicated. ns, not significant; ND, not determined. *P* values were calculated by ANOVA, and statistical significance is indicated. [e]$P < 0.05$, [a]$P < 0.005$, or [d]$P < 0.0005$ vs. control; [c]$P < 0.05$ or [b]$P < 0.005$ gp120/virus + anti-TLR4 versus gp120/virus alone.

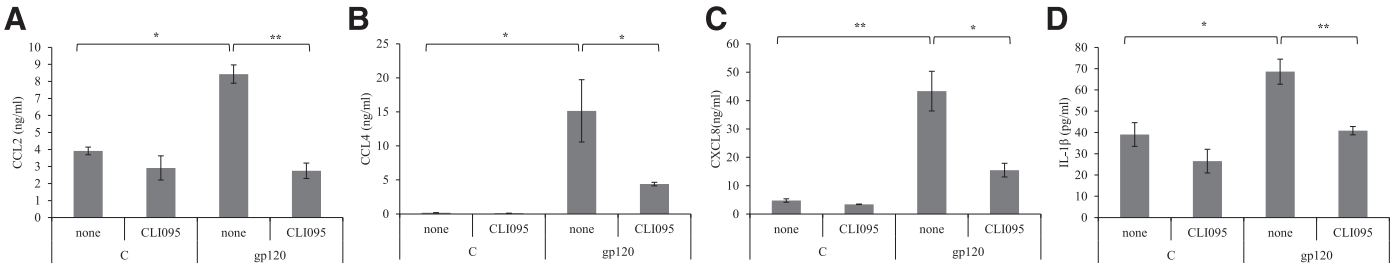

Figure 3. Effect of CLI-095 inhibitor on gp120-induced production of cytokines/chemokines in primary MDMs. MDMs were preincubated for 1 h, with or without CLI-095 inhibitor (5 μM), and then exposed to gp120 CN54 or not (control). After 24 h of culture, supernatants were harvested and frozen before CCL2 (A), CCL4 (B), CXCL8 (C), and IL-1β (D) determination by ELISA. Data are represented as means ± SE of 4 (A and B) or 5 (C and D) independent experiments. $*P < 0.05$, $**P < 0.005$, calculated by ANOVA.

cytokine production in MDMs. As shown in **Fig. 6**, preincubation with Maraviroc or Tak779 inhibited LPS-induced CCL2 (Fig. 6A and C) and CXCL8 (Fig. 6B and D) secretion and as expected, prevented CN54 gp120 but not ×4 (IIIB strain) gp120-mediated secretion of these chemokines. Conversely, addition of the CXCR4 antagonist AMD3100 (1 μg/ml) did not exert any effect on the production of chemokines induced by LPS, whereas it blocked the effects of IIIB gp120, as expected. Taken together, these results suggest that CCR5, but not CXCR4, and TLR4 are likely part of a common receptor cluster participating in the propagation of intracellular signals generated by gp120 in MDMs.

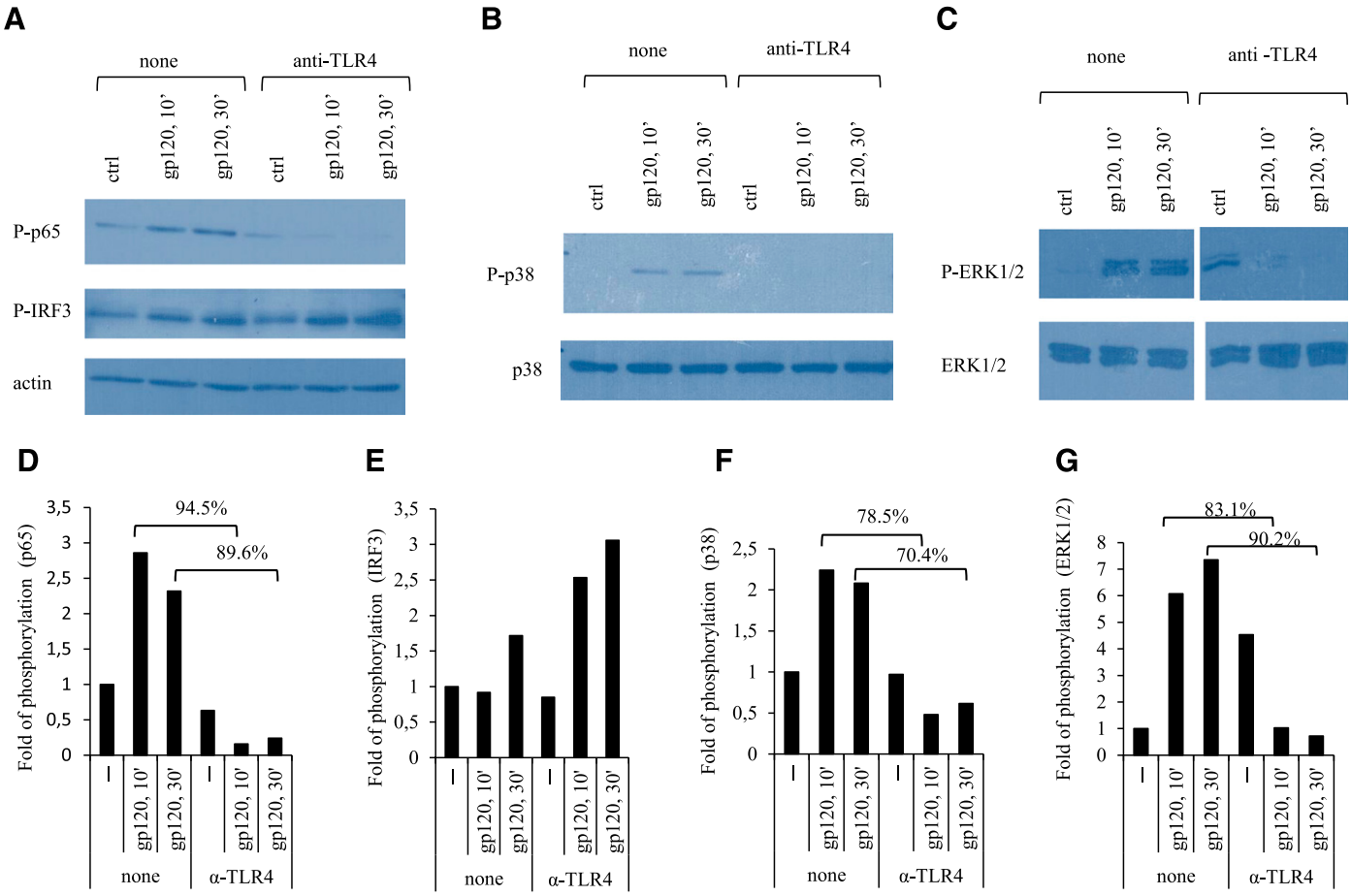

Figure 4. Effects of TLR4 blocking on gp120-mediated signals essential for the production of cytokines/chemokines in MDMs. MDMs, untreated [control (ctrl)] or stimulated with gp120 CN54 or LPS for the indicated time periods in the presence or absence of anti-TLR4 (α-TLR4)-blocking mAb, were used. Cell lysates were resolved by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis with antibody specific for the total or phosphorylated (P) forms of the following: (A) p65 NF-κB, IRF3, and actin; (B) p38 MAPK; and (C) ERK1/2. Blots shown are representative of 3 independent experiments using different donors. (D–G) Graphs show the ratio of phosphorylated to nonphosphorylated protein, where each sample was normalized to total proteins. Fold phosphorylation and percent of reduction were calculated relatively to untreated control.

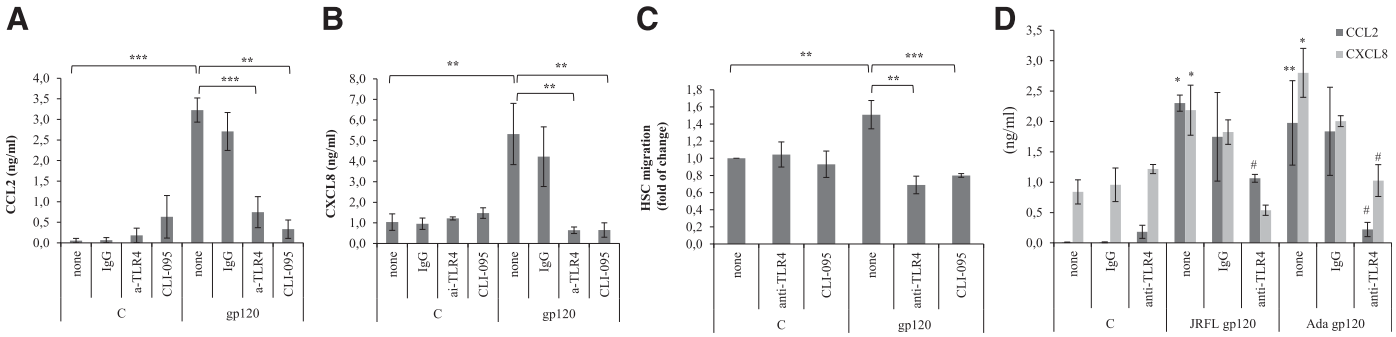

Figure 5. Involvement of TLR4 in the gp120-induced chemokine secretion and migratory response of HSCs. Serum-deprived primary HSCs were incubated for 1 h in the presence or absence of anti-TLR4-blocking mAb or isotype control or with the TLR4 inhibitor CLI-095 and then left untreated (control) or incubated with gp120 CN54 (500 ng/ml; A–C) or gp120 JRFL or Ada (D) for 24 h. At the end of the incubation, conditioned medium was assayed for CCL2 (A and D) and CXCL8 (B and D) by ELISA assay. Migration was measured in response to gp120 for 6 h in a Boyden chamber assay (C). Data are represented as means ± SE of 5 (A and B) or 3 (C and D) independent experiments. (A–C) $^{**}P < 0.005$, $^{***}P < 0.0005$, calculated by ANOVA. (D) $^{*}P < 0.05$ or $^{**}P < 0.005$ versus control; $\#P < 0.05$ gp120 + anti-TLR4 versus gp120 alone.

DISCUSSION

TLR4 has been reported, in addition to LPS, to interact with several other ligands, including structural viral proteins, and has been shown to recognize surface glycoproteins belonging to a diverse range of viruses [29]. In the present study, we described that TLR4 interaction with gp120 results in the activation of the NF-κB and MAPK pathways, leading to downstream up-regulation of proinflammatory cytokines and chemokines in MDMs and to cell migration and secretion of CCL2 and CXCL8 in HSCs.

HSCs are key cell types in the pathogenesis of fibrosis and have been shown previously to be a target of the action of gp120. Data from our group and from other laboratories have shown that gp120 stimulates migration of HSCs, expression of α-smooth muscle actin, and secretion of procollagen type I and of several cytokines involved in the fibrogenic process [13, 30]. In particular, gp120 induces CCL2 secretion by human macrophages [31] and by HSCs, which contributes to local amplification and maintenance of chronic inflammation [32]. Furthermore, chemokines, including CCL2 and CCL4, are chemoattractants for HSCs [33], suggesting that gp120 may lead to HSC accumulation via direct chemotaxis [13] and secretion of chemokines by HSCs themselves and by activated macrophages. Furthermore, induction of CXCL8 is linked to chronic liver disease progression, and monocytes/macrophages are the main responders to CXCL8 in liver fibrosis [34]. Other proinflammatory factors derived from macrophages, such as IL-1β, promote the survival of activated HSCs [35]. According to our results and previously published data, we proposed the schematic model depicted in **Fig. 7**. We speculated that the initial innate recognition of HIV-1 by TLR4 could be the first step for chronic

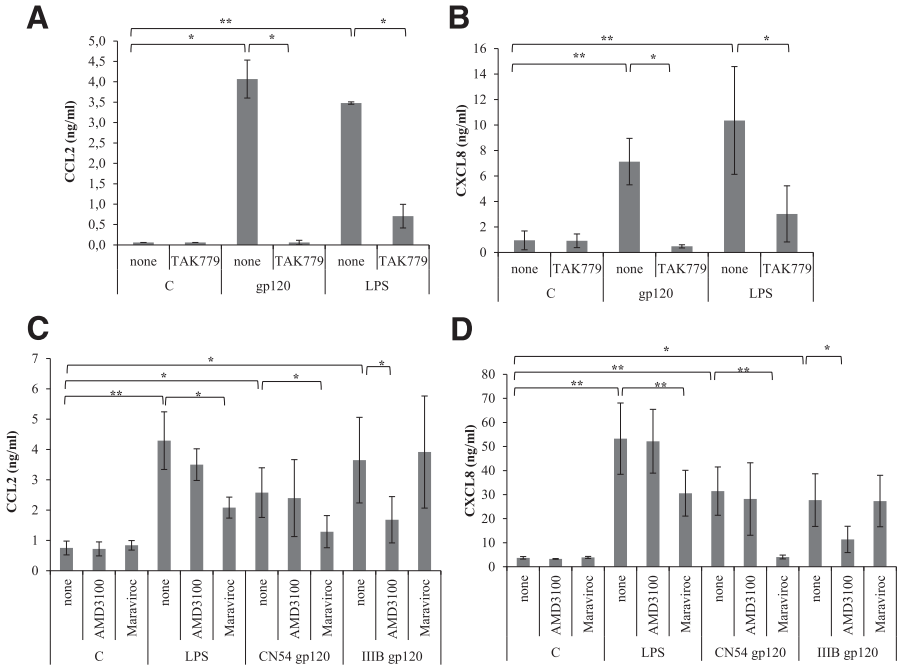

Figure 6. Effect of CCR5 blocking on the TLR ligand-induced chemokine production. MDMs were preincubated for 1 h, with or without CCR5 inhibitors Tak779 (A and B) and Maraviroc or CXCR4 inhibitor AMD3100 (C and D), and then exposed to CN54 gp120, IIIB gp120, or LPS or left untreated (control). After 24 h of culture, supernatants were harvested and frozen before CCL2 (A and C) and CXCL8 (B and D), determination by ELISA. Data are represented as means ± SE of 5 (A and B), 6 (C), or 4 (D) independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$, calculated by ANOVA.

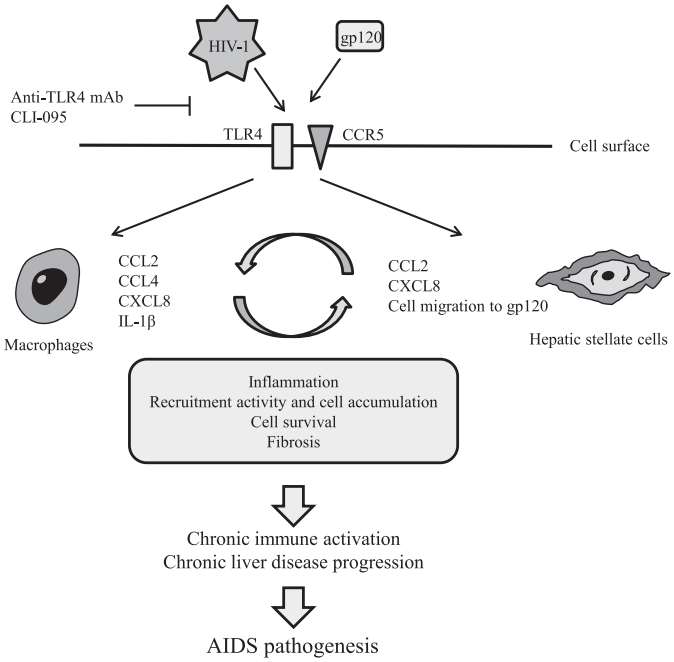

Figure 7. Schematic model of functional outcomes of gp120-TLR4 interplay. The cascade of signals triggered upon TLR4 interaction with R5 HIV-1 gp120 in MDMs and HSCs and its hypothetical contribute to the functional alterations of these cells and to AIDS pathogenesis.

immune activation, a hallmark of HIV-1 pathogenesis. On the other hand, this recognition could represent a direct pathway, possibly linking HIV infection with liver inflammation and fibrogenesis via envelope proteins. In this context, macrophage/HSC interactions could represent a key component of AIDS-associated liver disease progression.

In this study, we also examined the existence of a functional cross-talk between TLR4 and the HIV-1 coreceptor CCR5, demonstrating inhibitory effects of CCR5 antagonists Tak779 and Maraviroc, but not CXCR4 antagonist AMD3100, on LPS-induced chemokine production. Receptor clustering between TLRs and chemokine receptors was first demonstrated by Triantafilou et al. [26, 27], who identified CXCR4 as a component of TLR4-based receptor complexes involved in LPS recognition. A cross-talk between CXCR4 and TLR2 was also described in human monocytes [36]. However, in our experimental setting, we failed to demonstrate CXCR4/TLR4 coclustering, suggesting a cell type-specific receptor assembly. Although a limitation of our study is that we did not investigate the molecular mechanisms of the CCR5/TLR4 interaction, we believe that our results may represent a further example of the remarkable differences frequently observed between primary monocytes/macrophages and established cell lines in their response to exogenous stimuli [31]. We propose that TLR4 coclustering with HIV-1 coreceptors may be, at least in MDMs, a peculiarity of CCR5, thus expanding the repertoire of cellular proteins participating in the propagation of gp120 signals. Accordingly, Marchant et al. [37] demonstrated in the context of RSV infection that the virus may bind pathogen recognition receptors to enhance entry, in conjunction with the entry-fusion receptor. Moreover, the authors hypothesized that the entry and signaling receptors may comprise an entry complex that is unique to each virus.

Although the molecular details of the HIV-1/TLR4 interaction in target cells remain to be fully elucidated, the understanding of molecular and cellular mechanisms by which HIV gp120 hijacks the TLR4 pathway is of crucial interest for better knowledge of HIV-related immune system dysfunction and fibrosis and for the development of new therapeutic strategies.

AUTHORSHIP

M.D.C. designed and coordinated the research and wrote the manuscript. A.C. performed experiments on HSCs and participated in the interpretation and discussion of the results. G.D. performed experiments on MDMs and participated in the interpretation and discussion of the results. B.V. performed cell preparation. F.M. coordinated the research on HSCs. S.G. designed and coordinated the research and the manuscript preparation. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

This work was supported by grants from the Italian Ministry of Health, Bando Nazionale AIDS 2009–2010, 3H/31 (to S.G.) and FIRB project (RBAP10TPXK, 2010; to F.M.). Moreover, F.M. has received grant support and consultancy fees from ViiV Healthcare. The authors are indebted to the AIDS Reagents and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, for providing the HIV-1 CN54 gp120, JRFL virus, Tak779, Maraviroc, and AMD3100; to Drs. Doms and Gao for providing recombinant JRFL and Ada gp120, respectively; and to Dr. Elson (Novimmune; current affiliation Glenmark Pharmaceuticals, Switzerland) for providing TLR4-blocking mAb clone 15C1. The authors thank Fabiola Diamanti and Daniela Diamanti for excellent technical assistance.

DISCLOSURES

F.M. is supported, in part, by funds from ViiV Healthcare. The remaining authors have no funding and declare no conflicts of interest.

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KEY WORDS:
AIDS · receptor · cytokine · chemokine · migration