Disruption of the cholinergic anti-inflammatory response by R5-tropic HIV-1 protein gp120JRFL

Katherine Quiroz Figueroa, José A. Lasalde Dominicci, Sonnieliz Cotto Ríos, Orestes Quesada, and José O. Colón Sáez

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Despite current pharmacological intervention strategies, patients with HIV still suffer from chronic inflammation. The nicotinic acetylcholine receptors (nAChRs) are widely distributed throughout the nervous and immune systems. In macrophages, activation of α7-nAChR (α7-nAChR) controls inflammatory processes through the cholinergic anti-inflammatory response (CAR). Given that this innate immune response controls inflammation and α7-nAChR plays a critical role in the regulation of systemic inflammation, we investigated the effects of an R5-tropic HIV soluble component, gp120RFL, on the CAR functioning. We previously demonstrated that X4-tropic HIV-1 gp120HIV disrupts the CAR as well as inducing upregulation of the α7-nAChR in vitro in monocyte-derived macrophages (MDMs), which correlates with the upregulation observed in monocytes, T-lymphocytes, and MDMs recovered from HIV-infected people. We demonstrate here using imaging and molecular assays that the R5-tropic HIV-1 glycoprotein gp120RFL upregulates the α7-nAChR in MDMs dependent on CD4 and/or CCR5 activation. This upregulation was also dependent on MEK1 since its inhibition attenuates the upregulation of α7-nAChR induced by gp120RFL and was concomitant with an increase in basal calcium levels, which did not result in apoptosis. Moreover, the CAR was determined to be disrupted, since α7-nAChR activation in MDMs did not reduce the production of the proinflammatory cytokines IL-6, GRO-α, or I-309. Furthermore, a partial antagonist of α7-nAChR, bupropion, rescued IL-6 but not GRO-α or I-309 production. Together, these results demonstrate that gp120RFL disrupts the CAR in MDMs. Other medications targeting the α7-nAChR need to be tested to reactivate the CAR to ameliorate inflammation in HIV-infected subjects.

Worldwide, approximately 36.7 million people are HIV-positive, with 1.8 million new cases reported and 1.0 million AIDS-related deaths in 2016 alone (http://www.unaids.org/en/). The human immune deficiency virus type 1 (HIV-1) accomplishes cell entry by direct interaction between the viral envelope glycoproteins gp120 and gp41 with the CD4 receptor present in the host’s target cells. This interaction results in conformational changes on the gp120-CD4 receptor complex, enabling the virus to interact with either the CXCR4 coreceptor or CCR5 and culminating in the gp41-mediated fusion between the viral envelope to finally infect the target cell (1).

Macrophages are one of the key HIV-1-targeted cells that can be infected by M-tropic strains through the CCR5 (R5-tropic strain) coreceptor or by dual-tropic (X4/R5) strains that use both coreceptors for cell infection (2, 3). Importantly, because HIV-1 M-tropic strains (R5 strains) appear to play an essential role in HIV spread (4), it is crucial to understand how signaling triggered by soluble viral constituents (e.g., gp120), acting through the CCR5 receptor, affects the inflammatory phenotype of macrophages, and how these cells could be targeted pharmacologically to activate an innate anti-inflammatory mechanism, called the cholinergic anti-inflammatory response (CAR), to treat the chronic inflammation suffered by HIV-infected subjects.

Since 2000, special attention has been paid to the CAR as an innate neuroimmune mechanism that regulates and controls excessive inflammation. This is a prototypical neural circuit activated peripherally by cytokines and inflammatory products (5, 6). This anti-inflammatory mechanism is dependent on the signal transduction via macrophages α7 nicotinic acetylcholine receptor (α7-nAChR), which binds acetylcholine (ACh) to inhibit the production of inflammatory cytokines without altering IL-10 levels (7). Although there is robust evidence on the benefits of activating this anti-inflammatory mechanism to counteract inflammation, in the field of HIV there are practically no studies focused on this topic except for evidence recently reported by our group (8).

Indeed, studies performed in our laboratory using the X4-derived viral envelope gp120HIV show that activation of the CXCR4 by this viral glycoprotein increases the expression of α7-nAChR in human monocyte-derived macrophages (MDMs) (8, 9). Interestingly, this upregulation was also observed in T-lymphocytes, monocytes, and macrophages from HIV-infected patients (8). However, in this upregulated setting, α7-nAChR activation failed to inhibit the production...
Cholinergic anti-inflammatory response altered by gp120JRFL

of the majority of proinflammatory cytokines in MDMs deliberately pre-exposed to gp120IIIB followed by lipopolysaccharide (LPS) challenges. These results suggest that gp120IIIB disrupts an innate immune response mechanism that controls inflammation in HIV-infected subjects, the CAR. Moreover, these results underscore the importance of better understanding the mechanism behind the gp120-induced \( \alpha \)-nAChR upregulation, which may be associated with an unresolved problem in the HIV field: chronic inflammation. Although important advances have been made concerning the consequences of the X4-tropic gp120IIIB on macrophage's \( \alpha \)-nAChR expression and the CAR (8), the repercussions of the R5-tropic gp120JRFL on the \( \alpha \)-nAChR expression and the CAR operation in MDMs are still unknown.

In the current work, we found that gp120JRFL induced the upregulation of \( \alpha \)-nAChR in MDMs results that are consistent with those reported previously using gp120IIIB (8). Also, \( \alpha \)-nAChR's baseline levels correlated with the magnitude of the observed upregulation. Moreover, the \( \alpha \)-nAChR upregulation was significantly reduced after blocking CCR5 and CD4 receptors, suggesting that stimulation of these receptors could lead to \( \alpha \)-nAChR upregulation in these cells. Furthermore, CCR5 endogenous agonists RANTES and MIP-1\( \beta \) also abrogated gp120JRFL-induced upregulation of \( \alpha \)-nAChR, suggesting that activation of CCR5 or CD4 could trigger \( \alpha \)-nAChR upregulation in human macrophages. Also, \( \alpha \)-nAChR upregulation was attenuated by a MEK1 inhibitor and did not depend on de novo protein synthesis. Moreover, we found that the addition of gp120JRFL to MDMs promoted higher basal calcium levels, but not apoptosis. These cellular and molecular results underscore the molecular complexity behind the gp120-induced \( \alpha \)-nAChR upregulation in MDMs. Of note, from the inflammation standpoint, we found a CAR disruption for cytokines IL-6, GRO-\( \alpha \), and I-309, but not for TNF-\( \alpha \). Additionally, treatment with buprocain, a partial antagonist of \( \alpha \)-nAChR, reestablished the CAR for only IL-6, not the other cytokines.

Results

R5-tropic HIV-1 gp120JRFL upregulates the \( \alpha \)-nAChR in MDMs

To study the effects of the R5-tropic gp120JRFL on \( \alpha \)-nAChR's expression, MDMs were treated with different concentrations of gp120JRFL including those within the pathophysiological range (0.002–2.5 nM) found in HIV-infected patients (10–13). Binding assays were performed using Alexa-488-\( \alpha \)-bungarotoxin (\( \alpha \)-BuTX) to detect surface expression of \( \alpha \)-nAChR in MDMs upon gp120JRFL exposure (Fig. S1A). As shown in Figure 1A, gp120JRFL at different concentrations (0.15 and 150 nM) significantly increased binding of \( \alpha \)-BuTX in MDMs, suggesting higher levels of \( \alpha \)-nAChR. Moreover, Figure 1B shows that following exposure to a pathophysiological concentration (0.15 nM) of gp120JRFL, there was a significant increase in \( \alpha \)-BuTX binding in nine of the total 14 donors \( (p = 0.0134, 64\%) \). Not all donors exhibited elevated levels of \( \alpha \)-nAChR, possibly owing to genetic variations (14–16) between individuals, which are common in macrophages (8). Also, the upregulation detected for \( \alpha \)-nAChR in MDMs was directly proportional to the baseline levels that each donor had (Fig. S2), similar to what has been previously reported for gp120IIIB (8). Upregulation results were confirmed by immunoblot assays that also showed increased levels of \( \alpha \)-nAChR in gp120JRFL-treated MDMs (Fig. 1F). Since this study focused on the mechanism and effects of gp120JRFL-induced \( \alpha \)-nAChR upregulation in the inflammatory response of MDMs, from this point forward (Fig. 1) all analyses were done using donors who demonstrated \( \alpha \)-nAChR upregulation in their respective MDMs upon gp120JRFL exposure. Consistent with previous studies we found that, as shown in Figure 1C, treatment with X4-tropic gp120IIIB increased the levels of \( \alpha \)-BuTX binding in MDMs (8). Notably, similar results were observed after treatment with a different R5-tropic glycoprotein, gp120JRFL (Fig. 1C) and gp120ADA (Fig. 1D). Furthermore, to demonstrate the selective binding of \( \alpha \)-BuTX to the \( \alpha \)-nAChR, nicotine competitive assays were performed in upregulated MDMs. As expected, results showed that nicotine pretreatment significantly reduced \( \alpha \)-BuTX binding (Fig. 1E). Lastly, the increase in \( \alpha \)-nAChR levels occurred preceded by a nonsignificant increase in Egr1 after 6 h of incubation with gp120JRFL (Fig. S3).

CCR5 mediates gp120JRFL-induced upregulation of \( \alpha \)-nAChR in MDMs

Chemokine coreceptors, CXCR4 and CCR5, expressed on immune cells are important for HIV-1 virus entry into CD4 expressing cells. CCR5 is the principal coreceptor used by M-tropic strains (e.g., gp120RFL) to infect macrophages while CXCR4 (e.g., gp120IIIB) is used by T-tropic virus strains to infect and replicate activated CD4\(^{+}\) T-lymphocytes and macrophages (17, 18). Also, as mentioned, it has been shown that gp120IIIB, through CXCR4, induces \( \alpha \)-nAChR upregulation in MDMs while HIV-infected individuals exhibit elevated levels of \( \alpha \)-nAChR in their monocytes, T-lymphocytes, and MDMs (8). Given these findings, we hypothesized that the CCR5 coreceptor could also be involved in the mechanism of gp120JRFL-mediated upregulation of \( \alpha \)-nAChR in MDMs. Details of the treatments are presented in Figure S1B. As shown in Figure 2, A and B, the therapeutic CCR5 antagonist maraviroc prevented the increase of \( \alpha \)-BuTX binding induced by gp120RFL. Similar results were obtained in MDMs pretreated with the CD4 receptor antagonist, 4,4’-disothiocyantostilbene-2,2’-disulfonic acid (DIDS) (Fig. 2, A and B). Interestingly, the CCR5 natural agonists MIP-1\( \beta \) and RANTES also prevented the increase in \( \alpha \)-BuTX binding (Fig. 2, A and B), suggesting a reduction in the expression of surface \( \alpha \)-nAChR in MDMs. These suggest that gp120RFL could potentially activate other mechanisms that also result in \( \alpha \)-nAChR upregulation. Together, these results highlight the importance of CD4 and CCR5 stimulation for the upregulation of \( \alpha \)-nAChR in MDMs.

gp120JRFL-CCR5 interaction involves MAPK signaling in MDMs

Based on these findings, we next sought to investigate whether the signaling cascades resulting from gp120JRFL addition, shown to be dependent on activation of CD4 and...
CCR5, activate MAPK signaling in MDMs. Previous findings have shown that the CXCR4 receptor agonist SDF-1α and gp120JRFL upregulate the Egr1 gene, a transcription factor of α7-nAChR (18–20). To investigate further, we pretreated MDMs with PD98059, a highly selective inhibitor of MEK1, to prevent its activation (21). Details of the treatments are presented in Figure S1C. Our results show that α7-nAChR upregulation was attenuated by PD98059 pretreatment (Fig. 3, A and B), suggesting that gp120JRFL requires MAPK activation to upregulate α7-nAChR in MDMs. Unexpectedly, the addition of PD98059 alone did not reduce α7-nAChR to control levels. Taken together, these results suggest that gp120JRFL interaction with the CCR5 receptor activates MAPK signaling, which may promote the transcription of α7-nAChRs. However, protein synthesis inhibition results showed that the increase in α7-nAChR surface expression did not depend on de novo protein synthesis, suggesting that gp120JRFL could be affecting the trafficking of previously synthesized α7-nAChRs pool to the plasma membrane.

The CCR5 antagonist maraviroc did not rescue the CAR

Based on our findings, it seems that gp120JRFL activates a signaling cascade that depends on both CD4 and CCR5 stimulation, which is critical for gp120JRFL-mediated upregulation of α7-nAChRs. Consequently, to evaluate the significance of CCR5 blockade in the CAR functioning, we used the therapeutic drug maraviroc. As mentioned, maraviroc prevented the α7-nAChR upregulation (Fig. 2B). Here, we tested whether blockade of CCR5 with maraviroc would help restore the CAR measuring cytokines produced by MDMs. Results showed that maraviroc did not reduce any of the cytokines measured (Fig. S4).

gp120JRFL-mediated upregulation of α7-nAChR does not trigger apoptosis in MDMs

Several studies have proved that calcium overload through α7-nAChR (19, 22) and its uncontrolled influx induced by HIV-1 soluble proteins (23) can promote apoptosis in neurons (19, 22, 23). Considering that gp120JRFL can trigger

Figure 1. HIV-1 R5 gp120JRFL upregulates the α7-nAChR in MDMs. MDMs from uninfected subjects were used to evaluate α7-nAChR’s expression. A, MDMs were treated with 0.15, 15, and 150 nM of gp120JRFL followed by α-BuTX addition. Scale bar: 20 μm. The total number of MDMs analyzed is in parenthesis at the top of each column. ****p ≤ 0.0001, ***p = 0.0002 (n = 5 subjects). B, fourteen donors were evaluated for α7-nAChR levels after gp120JRFL (0.15 nM) exposure. Mean fluorescence intensity measurements show a significant increase in α7-nAChR expression (*p= 0.0034). C, comparison of gp120JRFL (p = 0.0210) and gp120ADA. (**p = 0.0034) effects on MDMs showed that α7-nAChR levels are higher in those incubated with gp120JRFL (0.15 nM). Untreated counterparts were normalized to 1, (n = 3 subjects). D, nine donors were evaluated for α7-nAChR levels after gp120ADA (0.15 nM) addition. Confocal imaging showed a significant increase in α7-nAChR levels expression (*p = 0.0273). E, nicotine outcompetes α-BuTX binding in MDMs demonstrating α-BuTX selectivity for α7-nAChRs, dots in graph represent snapshots, (n = 4 subjects; control n = 132 and nicotine-treated = 134 cells, *p = 0.0274). Each subject was identified using distinctive dots. F, immunoblot assays revealed significant increase in α7-nAChR protein levels relative to their untreated counterparts normalized to 1 (n = 3 subjects). Incubation time for gp120JRFL was 72 h. Total protein amount was normalized to GAPDH signal. Lane 1: molecular weight markers, lane 2: untreated (ctrl) sample, and lane 3: gp120JRFL-treated samples. Statistical analysis for panel A consists of a paired Student’s t-test, and for panels B, C, and D analysis consists of a Wilcoxon’s signed-rank test. Error bars represent mean with 95% CI for panels A, C, F, and E. Raw values were employed for panels A, B, D, and E while normalized values were used in panels C and F.
intracellular calcium signaling in macrophages through CCR5 stimulation and that $\alpha_7$-nAChRs are highly permeable to calcium, we studied possible changes in calcium after gp120JRFL exposure (24, 25) (Fig. S1D). As shown in Figure 4, A and B, gp120JRFL induced a significant increase in calcium basal levels in MDMs; however, the addition of the chelating agent, EGTA, or adding $\alpha$-BuTX (an $\alpha_7$-nAChR blocker) did not result in a significant difference compared with responses to gp120JRFL alone (Fig. 4B). Furthermore, acetylcholine (ACh)-induced calcium mobilization after gp120JRFL addition, in MDMs upregulated for $\alpha_7$-nAChR, was not observed (data not shown). These findings suggest that $\alpha_7$-nAChR upregulation did not trigger apoptosis of macrophages, which is consistent with the antiapoptotic signature observed in HIV-infected monocytes (26, 27) and macrophages (28). Therefore, MDMs upregulated for the $\alpha_7$-nAChR did not undergo apoptosis upon ACh stimulation.

**gp120JRFL disrupts the cholinergic anti-inflammatory response (CAR) in MDMs**

Acute HIV-1 infection is associated with the induction of cytokine cascades, including interferon-α (IFN-α), interferon-γ (IFN-γ), inducible protein 10 (IP-10), TNF-α, IL-6, IL-10, and IL-15 (29). As a consequence, HIV-infected patients experience sustained and chronic inflammation that affects their immune status (30). Given the vital role of $\alpha_7$-nAChR in regulating innate immune responses through the CAR activation (31), we evaluated the cytokine profile of MDMs upregulated for $\alpha_7$-nAChR. Secretion of cytokines (interleukins and chemokines) was measured in MDMs challenged with LPS and exposed to gp120JRFL (Fig. S1E). As expected, ACh reduced the release of proinflammatory cytokines in LPS-treated MDMs (Fig. 5, A and B; green bars) not exposed to gp120JRFL, indicating that the CAR is functional. Of
note, ACh was able to reduce TNF-α production in LPS-treated MDMs upregulated for α7-nAChR (Fig. 5A) but not for IL-6. Also, as expected, CAR activation did not alter IL-10 levels (7). In terms of chemokines, ACh did not reduce the production of either GRO-α or I-309 in LPS-treated MDMs exposed to gp120JRFL (Fig. 2B). Therefore, in the presence of gp120JRFL (α7-nAChR upregulation), a CAR disruption was observed for the majority of cytokines tested. Overall, these results suggest that gp120JRFL negatively affected the CAR functioning in MDMs, thereby contributing to the inflammatory state present in HIV-infected subjects.

The α7-nAChR partial antagonist, bupropion limitedly restores the CAR

Considering that the α7-nAChR function is critical for proper CAR functioning (5), we next sought to determine whether it is possible to rescue the disrupted CAR pharmacologically. To this end, we pretreated MDMs with the α7-nAChR partial antagonist bupropion (32, 33) (Fig. S1F). We have previously shown that bupropion attenuates the production of some chemokines (MCP-1, RANTES, and GRO-α) in MDMs upregulated for α7-nAChR upon gp120IIIB addition followed by LPS challenging (8). As shown in Figure 6, bupropion significantly attenuated IL-6 levels while no reduction was observed in the other cytokines.

**gp120JRFL-induced α7-nAChR upregulation does not involve cross talk between the CCR5/CXCR4 coreceptors signaling**

Macrophages can be infected by M-tropic strains that use only the CCR5 receptor or by dual-tropic (X4/R5) strains that use both receptors. Even though analysis of newly infected individuals has shown that HIV-1 R5 virus strains are predominant at the early stages of infection in 80% of patients, X4 strains appear later during the development of the disease in 40–50% of patients (34, 35). Studies conducted in CD4/CXCR4-expressing cells transfected with CCR5 have demonstrated that both receptors heterodimerize and associate with CD4, suggesting that this interaction may modulate HIV-1 coreceptor function. To evaluate possible signaling cross talk between both coreceptors, MDMs were exposed to gp120IIIB and gp120JRFL in the presence of their correspondent antagonists AMD3100 (CXCR4) and maraviroc (CCR5). MDMs treated with both gp120s showed an increase in α7-nAChR’s expression similar to the upregulation induced by gp120IIIB or gp120JRFL alone (Fig. S4, A and B). However, antagonizing the CCR5 with AMD3100 or the CXCR4 receptor with maraviroc did not result in a reduction of α7-nAChR upregulation (Fig. S4B). These results suggest that gp120s effects on MDMs were not mediated via cross talk between CCR5 and CXCR4 coreceptors. However, we cannot rule out the possibility that there might some kind of cross talk via their respective signaling cascades.

**Discussion**

The most successful available pharmacological strategy to fight HIV infection is antiretroviral therapy (ART). Although ART has been beneficial in reducing the risk of developing AIDS, it is not effective at reducing the continuous inflammatory immune response mounted against soluble viral proteins (e.g., gp120) and other HIV constituents. Moreover, even though certain combinations of ART have shown certain effectiveness in reducing inflammation, it is not sufficient to reinstate basal levels to those displayed by HIV-uninfected people (36) or to eradicate residual inflammation in this...
population (37). Recent results have shown that soluble gp120IIIB from an X4-tropic HIV-1 could disrupt an innate immune response (8, 9) tailored to decrease inflammation, the CAR, which operates through the α7-nAChR expressed by macrophages (5).

HIV soluble viral proteins such as gp120 circulate in the body of infected people and influence its physiology by interacting with receptors of immune cells, ultimately supporting and increasing the inflammatory process. In this study, the addition of gp120JRFL in concentrations similar to those present in HIV-infected patients (0.002–2.5 nM) (10–13) caused the upregulation of α7-nAChR in uninfected MDMs that depends on cells’ basal expression levels. This upregulation was observed using pathophysiological (0.15 nM) and supraphysiological (15 and 150 nM) concentrations of gp120JRFL (Fig. 1A). Nevertheless, the extent of the α7-nAChR upregulation for the three concentrations tested proved to be similar among them (Fig. 1A), suggesting that upregulation reached a plateau. Interestingly, we did not observe statistically significant differences on α7-nAChR after applying an intermediate concentration (15 nM) of gp120JRFL. This type of response is very similar to that observed before for gp120IIIB (8); however, this result does not influence the interpretation since both 15 and 150 nM are supraphysiological concentrations. On the other hand, the upregulation of α7-nAChR in MDMs using gp120s with different tropisms (X4: gp120IIIB and R5: gp120ADA and gp120JRFL) was similar among them (Fig. 1, C and D). Interestingly, our Z-stack imaging studies and nicotine competitive binding assays confirm an observation previously made by other groups. We observed that the application of nicotine did not eliminate the fluorescent α-BuTX signal completely. This has been observed previously as α7-nAChR tends to accumulate intracellularly close to the perinuclear area in macrophages (5) and other cells expressing α7-nAChR (38–40). Overexpression of this high-calcium-permeable channel produced an increase in intracellular calcium levels but did not cause apoptosis, a finding similar to what has been observed for gp120IIIB (8) but opposite to what has been shown in neuronal cells (22). Thus, this may imply that neuronal cells are more susceptible to changes in calcium levels, suggesting...
that the upregulation of α7-nAChR in the central nervous system has more serious consequences than it does in blood-derived macrophages. Our results suggest that the upregulation of α7-nAChR coincides with a greater calcium accumulation that does not come from extracellular sources, since chelation with EGTA did not decrease calcium entry. Similarly, the observed increase of MDM’s intracellular calcium does not occur through α7-nAChR because blocking with α-BuTX did not prevent it.

Because extracellular calcium entry into MDMs does not occur via α7-nAChR, it is possible to occur through other mechanisms. Along these lines, recent studies demonstrated that α7-nAChR could interact with G-protein-coupled receptors once stimulated with acetylcholine (41–43). For instance, in T cells, mobilization of calcium through the α7-nAChR is not necessarily required for the nicotine-induced release of calcium from the internal stores (44). Moreover, channel-independent signal transduction has been related to the role of α7-nAChR in inflammation (45, 46). Another possibility is that the activation of CCR5 by gp120RFL also allows calcium to accumulate in MDMs from intracellular stores, the latter has been demonstrated previously (25, 46). The fact that this increase in calcium was observed after adding gp120RFL suggests that this glycoprotein may be activating CCR5, which is known to increase intracellular calcium levels (24). Nevertheless, this resistance to apoptosis is consistent with the antiapoptotic signature observed in MDMs exposed to gp120HIB (8, 28), HIV-infected macrophages (47), macrophages/microglia serving as HIV reservoirs (48), and monocytes recovered from HIV-1-infected patients (27).

Together, these results imply that regardless of whether gp120 originates from X4 or R5 strains, both are capable of inducing the overexpression of α7-nAChR in human macrophages that seem to be a distinctive element of HIV infection (8), chronic inflammation (49–52), and bacterial infection (53). Finally, the upregulation of α7-nAChR was not accompanied by any significant increase in Egr1 levels during the first 24 h of incubation; therefore, there is still the possibility that it will experience changes later.

We focused our study on measuring α7-nAChR levels because this receptor is the cornerstone of CAR functioning (5). In our studies we employed a specific antagonist (α-BuTX) of α7-nAChR, to measure its surface expression. Several nAChRs have been detected in murine macrophages, including α9-nAChR, which also recognizes α-BuTX; however, this receptor has not yet been identified in human macrophages (5, 54, 55). Conversely, α7-nAChR has been overwhelmingly identified in human macrophages from cell lines and primary cultures. Nonetheless, α9-nAChR must be studied in the future to rule out any participation in the gp120-induced upregulation of α7-nAChR in macrophages or its expression itself. In summary, these results confirm previous observations regarding the α7-nAChR upregulation and substantially expand it through the evaluation of additional gp120s (gp120RFL and gp120ADA) from R5 HIV strains that use the CCR5 coreceptor to infect CD4-expressing cells.

The gp120RFL used in this study has R5 tropism and binds to the CD4 receptor and CCR5 coreceptor (1) present in MDMs. To determine whether the α7-nAChR upregulation occurs via these receptors, CD4 and CCR5 were blocked with DIDS and maraviroc, respectively. Results showed that antagonizing CD4 and CCR5 significantly blocked the upregulation of α7-nAChR in MDMs. The reduction in α7-nAChR levels after blocking CD4 and CCR5 demonstrates that both compounds were able to avoid the interaction between gp120RFL and these receptors, thus abrogating their respective activations and preventing α7-nAChR upregulation. Moreover, the fact that both compounds were able to significantly inhibit the upregulation of α7-nAChR suggests that both CD4 and CCR5 may be involved in the intracellular signaling that leads to the upregulation of α7-nAChR in human macrophages. This hypothesis is supported by studies demonstrating that RANTES enhancement of HIV replication in T-cells induces colocalization of CD4 and CXCR4 on immune cells, an effect that is also known to be triggered by gp120 (56–58) in CCR5-expressing cells (59). On the other hand, interestingly, the addition of two endogenous agonists produced by macrophages (60), RANTES and MIP-1β, just before addition of gp120RFL, substantially prevented the upregulation of α7-nAChR in MDMs to the levels found in the control cells (Fig. 2B). RANTES and MIP-1β are chemoattractant chemokines that play important roles in chronic and acute inflammation in uninfected subjects (61, 62), and inflammation is particularly elevated in HIV-infected subjects (63), mainly in the serum, lymph nodes, and cerebrospinal fluid (64–66). Results obtained in the current study are contrary to what has been observed for SDF-1α, a natural ligand of CXCR4, which
Figure 7. Proposed mechanism for gp120-induced upregulation of the α7-nAChR in MDMs. A, activation of CCR5 or CD4 by gp120JRFL causes activation of the Ras-Raf-MEK pathway that leads to modest activation of Egr1, a known transcription factor for the α7-nAChR gene (CHRNA7). B, the viral glycoprotein gp120JRFL induces the upregulation of α7-nAChRs. C, activation of the cholinergic anti-inflammatory response in macrophages demonstrates that gp120JRFL alters the macrophage phenotype, imparting a proinflammatory character because the activation of CAR is unable to reduce inflammatory cytokine levels.
has been reported to induce α7-nAChR upregulation in MDMs in vitro (8). Taken together, these results suggest that even though both gp120s promote similar upregulation of α7-nAChR, the stimulation of the CXCR4 and CCR5 coreceptors works differently as far as α7-nAChR upregulation in MDMs is concerned.

To dissect and better understand the mechanisms behind the upregulation of α7-nAChR induced by gp120 JRFL, MDMs were treated with PD98059 to determine if MAPK signaling was involved in transmitting the gp120 JRFL signaling, which primarily leads to α7-nAChR upregulation. According to our results, the inhibition of MEK1 attenuated the upregulation of α7-nAChR induced by gp120 JRFL, but did not restore it to levels comparable to the control (Fig. 3A), suggesting that MEK1 may participate in the upregulation process but not all responses rely on this signaling molecule. Since PD98059 alone is capable of increasing α7-nAChR levels (Fig. 3B), it is possible that the expected inhibitory effect was masked by the stimulating action of PD98059 on α7-nAChR. Therefore, other signaling molecules could participate in this process (67). Overall, it cannot be ruled out that other proteins may contribute to the signaling that leads to α7-nAChR upregulation in MDMs, but MAPK signaling seems to be involved.

To further investigate the mechanisms that govern the upregulation of α7-nAChR in MDMs, we decided to evaluate whether there is any cross talk between CXCR4 and CCR5, the coreceptors used by gp120 IIIB and gp120 JRFL/gp120 ADA, respectively, to upregulate α7-nAChR in MDMs. The CXCR4 coreceptor has previously been shown to participate in α7-nAChR upregulation once stimulated by gp120 IIIB (8), but in the current study, we found that CCR5 stimulation is capable of inducing α7-nAChR upregulation once stimulated by gp120 JRFL or gp120 ADA. Current results show that blocking of either CXCR4 or CCR5 does not influence the signaling of the other, resulting in upregulation of α7-nAChR (Fig. S5). That is, for example, even when CXCR4 is blocked, it does not prevent gp120 JRFL from stimulating α7-nAChR upregulation through CCR5 activation. Consistent with this idea, the upregulation of α7-nAChR is not potentiated by the simultaneous addition of gp120 IIIB and gp120 JRFL (Fig. S5). In summary, the upregulation of α7-nAChR induced by gp120 JRFL does not involve cross talk between CXCR4 and CCR5. Also, these results suggest that upregulation mediated by gp120 JRFL and gp120 ADA recapitulates the cellular events that occur during the early stages of HIV infection with R5 tropic strains. On the other hand, the α7-nAChR upregulation induced by gp120 IIIB can be indicative of the events observed in the chronic phase of the infection in which R5X4 and X4 strains are predominant (68).

HIV infection is characterized by the appearance of inflammatory processes that last throughout the patient’s life. In the present work, similar to what was previously observed for gp120 IIIB (8), gp120 JRFL was able to induce the upregulation of α7-nAChR; however, it produced a different inflammation profile. Here, we show that gp120 JRFL caused a CAR disruption for IL-6, GRO-α, and I-309, which is consistent with what was previously observed for gp120 IIIB (8). However, here we found that CAR activation could reduce TNF-α levels successfully, something not observed in experiments performed with gp120 IIIB. This suggests that, although gp120 JRFL produces a CAR disruption for some cytokines, activation of the CAR may still successfully reduce the levels of TNF-α in our experimental setting. Something similar was observed previously, but for MCP-1, a chemokine (8). Finally, gp120 JRFL maintained levels of IL-10, an anti-inflammatory interleukin, similar to those produced by LPS alone, a finding contrary to what was observed in the case of gp120 IIIB, which stimulated a significant release of this anti-inflammatory molecule (8). This observation may imply that gp120 JRFL may be more proinflammatory than is gp120 IIIB because it does not allow a substantial release of IL-10 to counteract inflammation, although this does not seem always to be the case, at least for the chemokines (69). Our results show that the net phenotype of macrophages exposed to gp120 JRFL is proinflammatory. In summary, gp120 JRFL produces a CAR disruption for IL-6 and for the two chemokines evaluated (GRO-α and I-309). The CAR disruption detected here has serious implications in HIV-infected patients because it sheds light on cellular processes occurring in them that contribute to the chronic inflammation they experience. Another aspect that should not be ignored is the possibility that other immune cells expressing α7-nAChR play some role in the inflammatory alterations described above.

To counteract the proinflammatory effects caused by gp120 JRFL, MDMs were treated with bupropion, a partial α7-nAChR antagonist that (1) has proved to be safe for HIV-infected patients (70, 71), (2) has been used in HIV-infected patients (70, 72, 73), (3) does not interfere with antiretroviral therapy used by HIV-infected patients (71, 74), (4) exhibits anti-inflammatory properties (75–81), and (5) is FDA-approved (82). Those cytokines that did not respond to CAR activation were evaluated. Our results showed that bupropion effectively reduced the levels of IL-6, but not those of GRO-α and I-309. In summary, the application of bupropion proved to have limited anti-inflammatory action, thus underscoring the need for better medications focused on α7-nAChR to counteract HIV-induced inflammation.

Altogether, our findings suggest an important link between CCR5 and CD4 activation and the surface expression of the α7-nAChR in human macrophages. According to our results, these alterations cause CAR activity to be limited, which ends up contributing to the inflammation experienced by HIV-infected subjects. Undoubtedly, more research with a pharmacological approach is needed to rescue the activity of CAR under inflammation scenarios such as HIV infection. Moreover, this work demonstrates the complexity behind the upregulation of α7-nAChR induced by gp120 JRFL and gp120 IIIB, which provides more avenues for research. The precise molecular mechanisms behind the upregulation of α7-nAChR in the presence of gp120 remain elusive. However, our results together with those of other groups suggest that chemokines (RANTES and MIP-1β) and gp120 may initiate both common and unique, ligand-specific signaling pathways. Indeed, signals induced by chemokine’s binding to
correspondent receptors do not always mimic the interaction of the HIV-1 envelope with the chemokine receptors (83). Importantly, the relevance of restoring the CAR is not limited to the field of HIV but has important consequences in the recent pandemic caused by SARS CoV-2, the etiological agent causing COVID-19 in humans. Recent studies show that macrophages play a critical role in the appearance of the cytokine storm (84) and that α7-nAChR may be involved in this process (85). Furthermore, the strongest evidence of the role that α7-nAChR can have in this process is the amount of literature that has emerged showing that patients who self-administer nicotine (smokers) are less likely to have COVID-19, which has led the scientific community to consider therapeutic options including nicotine (86, 87), an agonist of α7-nAChRs. Certainly, the α7-nAChR expressed in human macrophages is emerging as an attractive pharmacological target to alleviate the extensive inflammation experienced by patients suffering from COVID-19.

Experimental procedures

Reagents and antibodies

Nicotine, RANTES, MIP-1β, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), LPS, acetylcholine, bupropion, and maraviroc, were acquired from Sigma-Aldrich. Alexa-488 α-bungarotoxin was purchased from Invitrogen. gp120_{IRFL} and gp120_{ADA} were purchased from MyBioSource. gp120_{mib} was from Fitzgerald Industries International. Monoclonal α7-nAChR antibody (Cat. # sc-5544, Santa Cruz Biotechnology), monoclonal GAPDH antibody (Cat. # ab8245, Abcam), and goat anti-mouse IgG HRP (Cat. # sc-2005, Santa Cruz Biotechnology) were obtained from Santa Cruz Biotechnology. An anti-goat secondary antibody-labeled HRP-conjugated were obtained from Sigma Aldrich (Cat. # AP307P, EMD Millipore).

Study subjects

All donors enrolled in this study signed the informed consent approved by the Institutional Committee for the Protection of Human Participants in Research (IRB number: 0910-033). All experiments were performed per University of Puerto Rico guidelines and regulations. Phlebotomy to obtain peripheral blood mononuclear cells was performed on healthy uninfected volunteer donors bled at the University of Puerto Rico, Río Piedras Campus for the studies depicted in all figures. The human subject studies abide by the declaration of Helsinki Principles.

Cell culture of human monocyte-derived macrophages (MDMs)

Whole blood from all subjects was processed as described elsewhere (88) (Fig. S1A). Peripheral blood mononuclear cells were counted by a countless automated cell counter (Invitrogen) adjusted to 1–2 × 10^6 cells/ml and cultured into four-well Lab-Tek II Chambered Coverglass (Nalgene) for confocal microscopy. For western blots, 1.0 × 10^6–1.0 × 10^8 cells/ml were cultured in cell culture Petri dishes (Fisher Scientific) as previously described (88). For qRT-PCR, 1 × 10^7 cells per ml were cultured in six-well cell culture plates, and for ELISA 400 μl of the supernatant was obtained from 1 × 10^7 cells per ml seeded in 24-well cell culture plates were assayed (Corning CoStar). Monocytes were separated from lymphocytes by adherence. After separation, cells were differentiated for 7–8 days in RPMI-1640 supplemented with 20% inactivated fetal bovine serum, 10% inactivated human serum, and 1% PenStrep. All cultures were maintained at 37 °C with 5% CO_2.

All experiments were performed with cells cultured from a single donor; blood or cells from different donors were not mixed. Cultures, buffers, and reagents were endotoxin-free. The HIV-1 glycoprotein gp120_{mib} manufacturer certified that endotoxin levels were ≤100 EU/mg. The manufacturer of gp120_{IRFL} and gp120_{ADA} certified that endotoxin levels were <0.01 EU/μg as determined by LAL test. Also, for all of them, ≥95% purity was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Immunoblot

After treatment, MDMs were lysed using lysis buffer (mercaptoethanol diluted in phosphate-buffered saline [PBS 1×] to a final concentration of 2.5% and supplemented with a protease inhibitor cocktail [Thermo Scientific; pH 7.4]). Protein sample quantification was performed using an iMark Microplate Reader (BioRad). Total homogenate samples, 50 μg, were loaded onto a 10% polyacrylamide gel and run for 1 h at 30 V and then at 90 V until completion. After electrophoresis, gels were transferred to a PVDF membrane (Bio-Rad) using a wet system (Bio-Rad) for 2 h at 100 V. After this, membranes were incubated in a blocking solution (5% non-fat dry milk, Tris-buffered saline [TBS, 1×] for 1 h at room temperature). Subsequently, primary antibody incubation for α7-nAChR, diluted 1:200 (cat. No.: H-302; Santa Cruz Biotechnology), was performed overnight at 4 °C. After three to six consecutive washes (5 min each) with TBS-T 1× and a final wash with TBS (1×), an anti-goat secondary antibody labeled with horse-peroxidase-conjugated and diluted 1:2000 (cat. No.: AP307P; Millipore) was added and incubated for 1 h at room temperature. As a loading control, a mouse monoclonal [6C5] antibody for GAPDH diluted to 1:500 (cat. No.: ab8245; Abcam) was used. Membranes were processed using a chemiluminescence assay (Super Signal West Dura Extended Duration Substrate; Thermo Scientific) following the manufacturer’s instructions. Molecular weight markers employed did not contain horseradish peroxidase; therefore, the image corresponding to the molecular weight markers lane was taken separately and eventually aligned with their respective immunoblots. Relative intensities of the blots were evaluated using Image Lab Image Capture and Analysis Software (Bio-Rad). The band quantifications are presented as the relative quantity of protein (experimental condition/untreated ratio).

Quantitative RT-PCR

Real-time PCR was performed following a previously described method with minor modifications (19). Total RNA samples were isolated from MDMs using the TRIzol Reagent
Cholinergic anti-inflammatory response altered by gp120JRFL

Possible genomic contamination from extracted RNA was treated with DNase using Ambion’s DNA-free kit (Ambion). The RNA integrity was assayed in 1% electrophoresis agarose gel. Quantification of total RNA was performed using a NanoDrop system (Thermo). The cDNA synthesis was carried out using 2 μg of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc) following the manufacturer’s instructions. Real-time PCR experiments were done using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc) using a Mastercycler Ep realplex Thermal Cycler (Eppendorf, Hauppauge, NY), 10-μM concentration of forward and reverse primers. The following primer pairs were used: fwd=5’-GCTCTCTGTC TCCTGTTTC-3’ and rev=5’-GACTCCGACCTTCACCTCC-3’ for GAPDH, fwd=5’-AGCACCTTTCAACCCCTCA-3’ and rev= 5’-AGTCGAGTGGTTGCGT-3’ for Egr1. Real-time qPCR was performed in a 25-μl reaction containing 12.5 μl SYBR Green PCR Master Mix (Bio-Rad), 1 μl of each primer (10 μM), 2 μl cDNA, and 8.5 μl water. PCR cycling conditions were 95 °C for 10 min followed by 45 cycles of 94 °C for 25 s, 60 °C for 25 s, and 72 °C for 40 s. Expression of Egr1 was normalized to that of GAPDH using the ddCt method.

Confocal imaging

After differentiation, MDMs were incubated and maintained in media supplemented with either gp120JRFL or gp120ADA (both glycoproteins expressed in 293 cells), or full-length monomeric glycosylated gp120IIIb expressed in baculovirus, for 72 h. After incubation, the media was removed and MDMs were washed with PBS 1× (pH 7.4), followed by fixation with 4% formaldehyde for 15 min at room temperature, washed twice with PBS 1×, and labeled with Alexa-488-α-BuTX (Invitrogen) for 1 h at 70 μg/ml final concentration in buffer (NaCl, 120 mM; KCL, 4 mM; KH2PO4, 1.2 mM; MgSO4, 1 mM; HEPES, 15 mM (pH 7.4); CaCl2, 1 mM; bovine serum albumin, 2%; and glucose, 1%). After α-BuTX labeling, MDMs were washed with PBS 1× to remove unbound α-BuTX, followed by the addition of Mounting Media (H-1000, from Vector Labs Biotechnology Company) to be finally studied under confocal microscopes (Zeiss LSM Meta 510 and AR1 Nikon) at the Neuroimaging and Electrophysiology Facility (NIEF), Molecular Sciences Research Center (http://nief-upr.com). The remaining bound α-BuTX was excited at a wavelength of 488 nm (0.2%), and an Argon laser, 2 μm, was used. Emission was acquired at 520 nm using a BP 505–550 filter, 64-μm pinhole using a Plan-Apochromat × 40/0.8 M27 objective for experiments performed on Zeiss LSM Meta 510. For the experiments performed on AR1 Nikon, the excitation wavelength was 487.5 nm using an LU-N3 laser, and emission was acquired at 525 nm using 450/50-550/50-595-50 filter, 24.27-μm pinhole using S Fluor 40× Oil DIC H N2 objective. Images were acquired by random snapshots at 2048 × 2048 dpi on Zeiss LSM 510 and at 1024 × 1024 dpi on AR1 Nikon followed by background subtractions. Relative fluorescence intensity analyses of each MDM were performed using the LSM 510 program or Nikon AR Nis Elements software. Each slide was analyzed generating five Z-stacks of confocal microscopy images (snapshots). Then, intensities were averaged for each cell and each snapshot. Relative intensities were averaged and plotted as a raw value unless otherwise specified in figure legends.

Nicotine competitive binding assay

The competitive binding assay was performed by adding nicotine to a final concentration of 500 μM before addition of Alexa-488-α-BuTX (2 μg/ml). MDMs were incubated for 15 min at 4 °C in the dark and washed with RPMI-1640 non-supplemented base. Cells were then fixed with 4% formaldehyde–PBS solution (pH 7.2) for 15 min at room temperature. After fixation, MDMs were washed three times with PBS 1× (pH 7.2). Finally, VectaShield (Vector Labs) was added for visualization and examination by confocal microscopy. Images were collected in Z-stacks at a magnification of 40× and analyzed. Three random snapshots were performed, and individual MDMs were analyzed for mean intensity and averaged.

Multiplex ELISA assay

Peripheral blood mononuclear cells from donors were cultured (7–8 days) in 24-well plates, differentiated into MDMs (Fig. S1A), and assayed for interleukins and chemokines production (IL-6, IL-10, TNF-α, GRO-α, and I-309) by Quansys Biosciences. After differentiation, media was changed for fresh media, and gp120JRFL was added for 72 h (to induce α7-nAChR upregulation), followed by three consecutive fresh media washes to remove gp120JRFL. To study the cholinergic anti-inflammatory response, LPS was added as an inflammation inducer, according to the experimental condition tested. The cholinergic anti-inflammatory response experimental treatments consisted of LPS (100 ng/ml) challenges using Escherichia coli O111:B4 (Sigma), followed by the addition of ACh (30 μM). The acetylcholinesterase inhibitor pyridostigmine (1 mM) was added 10 min before the ACh application to avoid ACh hydrolysis. In the case of bupropion (70 ng/ml) containing assays, to partially antagonize α7-nAChR, bupropion was added 10 min before LPS or ACh application. For maraviroc (100 nM) containing assays, it was added as a cotreatment with LPS and/or ACh. Supernatants were collected 4-h posttreatment and stored at −80 °C for further analysis. All supernatants were sent to a contract laboratory (Quansys Biosciences, Logan, UT, USA) for cytokines quantification using the multiplex ELISA technology. Samples were analyzed in triplicate. Donors considered for each cytokine were independently evaluated, and those who did not exhibit CAR activity were not considered for analysis. A gp120JRFL control was included on each donor evaluated, and because cytokine induction was minimal, they were not plotted.

Basal calcium levels determination

After differentiation, MDMs were incubated and maintained in media supplemented with gp120JRFL for 72 h. After incubation with gp120, the media was removed and MDMs were washed with RPMI-1640 (fresh complete media), followed by
incubation with α-BuTX (100 nM) for 45 min to 1 h. After α-
BuTX incubation, cells were washed twice with RPMI-1640
(no supplemented media/without fetal bovine serum (FBS)
or human serum). Then RPMI-1640 was removed and cells
were incubated with Fluo-4 AM in RPMI-1640 base, final
concentration of 10 µM for 1 h in dark. After that, cells were
washed twice with RPMI-1640 and kept in this solution to be
finally studied under confocal microscopes (Zeiss LSM Meta
510) at the Neuroimaging and Electrophysiology Facility
(NIEF), Molecular Sciences Research Center (http://nief-upr.com). Cells were excited at a wavelength of 488 nm (0.2%)
using an Argon/2 laser, and its emission was acquired at
520 nm using a BP 505–550 filter, 64-µm pinhole using a
Plan-Apochromat × 40/0.8M27 objective. Images were ac-
quired by random snapshots at 2048 × 2048 dpi on Zeiss LSM
510 at 20× magnification followed by background
subtractions.

Statistical analysis
All statistical analyses were performed using GraphPad
(Graph Pad). For small sample sizes, nonparametric statistics
were used. Wilcoxon signed-rank test was performed for
paired analysis, differences between paired groups were
made by using the Wilcoxon matched-pairs signed-rank test,
and the one-sample t-test was used to compare a group
mean to a hypothetical value=1. Quantitative variables are
presented as a standard error of the mean (SEM) ± SD; a p-
value < 0.05 was considered significant. Grubbs’ test
(alpha = 0.05) from GraphPad QuickCalcs, also called the
ESD method (extreme studentized deviate), was used to
determine outliers.

Data availability
All the data described is contained in the article and in the
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Supporting information—This article contains supporting
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Abbreviations—The abbreviations used are: ACh, acetylcholine; α-
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