Int J Clin Exp Pathol (2008) 1, 260-275
www.ijcep.com/IJCEP708004

Original Article

HIV-1 TAT Inhibits Microglial Phagocytosis of Aβ Peptide

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Received 2 Aug 2007; accepted with revision 10 Sept 2007; available online 20 Sept 2007

Abstract: Human immunodeficiency virus (HIV)-associated dementia (HAD) is a subcortical neuropsychiatric syndrome that has increased in prevalence in the era of highly active antiretroviral therapy (HAART). Several studies demonstrated increased amyloidosis in brains of HIV patients and suggested that there may be a significant number of long-term HIV survivors with co-morbid Alzheimer's disease (AD) in the future. We show HIV-1 Tat protein inhibits microglial uptake of Aβ1-42 peptide, a process that is enhanced by interferon-gamma (IFN-γ) and rescued by the STAT1 inhibitor (-)-epigallocatechin-3-gallate (EGCG). It is hypothesized that reduced Aβ uptake occurs through IFN-γ mediated STAT1 activation. This process promotes a switch from a phagocytic to an antigen presenting phenotype in microglia through activation of class II transactivator (CIITA). Additionally, we show that HIV-1 Tat significantly disrupts apolipoprotein-3 (Apo-E3) promoted microglial Aβ uptake. As Tat has been shown to directly interact with the low density lipoprotein (LRP) receptor and thus inhibit the uptake of its ligands including apolipoprotein E4 (Apo-E4) and Aβ peptide in neurons, we further hypothesize that a similar inhibition of LRP may occur in microglia. Future studies will be required to fully characterize the mechanisms underlying IFN-γ enhancement of HIV-1 Tats disruption of microglial phagocytosis of Aβ and Apo-E3.
Key Words: HIV-associated dementia (HAD), inflammation, AIDS dementia complex (ADC), Interferon-gamma (IFN-γ), green tea, (−)-epigallocatechin-3-gallate (EGCG)

Introduction

Marked by impairment in cognition, affect, emotion, and motor skills, HIV-associated dementia (HAD) represents the most severe form of HIV related neuropsychiatric impairment [1]. It is a relatively common sequela of advanced HIV disease, occurring in some 20% of patients in the era preceding highly active antiretroviral therapy (HAART) [2]. Although the incidence in the HAART era has halved relative to pre-HAART, the prevalence has roughly doubled, due to the life-extending effect of HAART [3, 4]. Moreover, HAD is commonly characterized by amyloid-beta (Aβ) accumulation and other associated Alzheimer's disease (AD)-like neuropathology [5, 6]. This will likely complicate management of HIV by requiring greater provisions for long-term care of HIV-infected patients with dementia [7].

Amyloid deposition in the brain occurs with aging and is an important pathological finding in AD and HAD. Aβ peptide is neurotoxic and its accumulation in brain has been implicated in the associated neurodegeneration [8]. The first study to identify AD-like changes in HIV patients was reported by Esiri and colleagues who compared prevalence of argyrophilic amyloid plaques in 97 AIDS cases dying at ages 30-69 years with that in 125 age matched, non-HIV infected controls. They found that Aβ plaques formed at an earlier age and in greater amounts in the AIDS group. Moreover, there was a significantly greater prevalence of plaques in the AIDS group as a whole (29%) and in those in the fourth decade (18%) than in control subjects (13% and 0% respectively) [9]. In a recent study, 4G8 and 6E10 antibody staining demonstrated that significant deposition of amyloid occurred in the frontal cortex of almost half of 162 autopsied AIDS brains studied [6]. Similar but less abundant deposition was detected in the hippocampus and basal ganglia [6]. In accord, another study of postmortem human brain sections from patients with HIV-1 infection (n = 14; 31-58 years old) demonstrated a significant increase in Aβ, compared to controls (n = 5; 30-52 years old) [10].

Several factors could produce this increasing prevalence of AD pathology in the HIV population. Because of HAART, more HIV patients will live to an age where AD commonly presents [11-13]. Additionally, a recently characterized phenomenon in patients receiving HAART, known as immune reconstitution syndrome (IRS), may increase the incidence of AD in long-term HIV survivors [7]. IRS is an autoimmune condition occurring when reconstituted T cell populations attack opportunistic pathogens which proliferated during T cell suppression by HIV. It is characterized by connective tissue disease symptoms and vasculitis [14-16]. As inflammation has been linked to AD [17-19] patients with IRS would seem to be at an elevated risk of developing AD pathology [7]. The same would apply to patients who demonstrate lipodystrophic and metabolic effects of HAART, which cause hyperlipidemia, alterations in distribution of body fat to metabolically inactive regions, insulin resistance and coronary artery disease; all known AD risk factors [11, 20-22]. Furthermore, HIV itself produces neurotoxicity from chemokines, cytokines such as interferon-gamma (IFN-γ) [23], and from excitoxic effects of the secreted proteins including HIV-1 transactivator of transcription (Tat) protein [1, 24-28]. Here we examined the effects of two these factors, specifically IFN-γ inflammatory signaling and HIV-1 Tat protein.

HIV-infected cells secrete Tat protein which is taken up by adjacent uninfected cells [29-32]. It is uncertain, however, whether Tat is present within CNS of HIV-infected patients at sufficient concentrations to directly produce acute neurotoxicity [33]. It has been shown that nanomolar concentrations of Tat protein induce gene expression, cell proliferation, differentiation, adhesion and morphological changes without detectable cytotoxic effects [32, 34-36]. Importantly, increasing evidence suggests that HIV-1 Tat protein may directly lead to increased Aβ deposition in the HIV infected brain. Pulliam and colleagues demonstrated neprilysin interacts with the cysteine-rich domain of Tat. Neprilysin functions as a type II plasma membrane zinc metallopeptidase; representing one mechanism for its clearance [10, 37]. It has also been demonstrated that binding of HIV-1 Tat protein to low-density lipoprotein receptor-related protein (LRP) resulted in substantial inhibition of neuronal binding, uptake and degradation of physiological ligands for LRP, including apolipoprotein E4 (Apo-E4), and Aβ [33]. In the following experiments we tested the hypothesis that Tat inhibition of Aβ uptake occurred in microglia, a principle mechanism for the removal of Aβ from the brain parenchyma [38].

Previous investigations have demonstrated a critical role of microglia in Aβ plaque clearance [39, 40]. Microglia, functioning as resident brain macrophages, represent the initial defense against invading pathogens and are a critical link between the central nervous system (CNS) and the immune system [17]. In normal adult brain, microglia are quiescent, but in reaction to CNS injury they actively phagocytose cellular debris and apoptotic cells [41]. This has the effect of reducing pro-inflammatory cytokine production and minimizes injury in the inflamed brain [42].

In AD clinical cases as well as animal models, increased activation and recruitment of microglia to areas of cerebral amyloidosis are observed. However, these activated microglia are unable to clear Aβ deposits in AD mice [43]. Strategies which augment microglial phagocytic activity are able to ameliorate cerebral Aβ load in AD mouse models as evidenced by a report demonstrating that transforming growth factor-β over-expression promotes phagocytic clearance of cerebral Aβ [44]. Thus augmentation of this capacity for microglia to clear amyloid plaques, by opposing the effects of HIV-1 Tat protein, may prove therapeutic.

Materials and Methods

Reagents

Aβ 1-42 and FITC-conjugated Aβ1-42 were obtained from Biosource International (Camarillo, CA). Recombinant HIV-1 Tat protein was obtained from the NIH AIDS Research and Reference Reagent program. Recombinant IFN-γ was purchased from R&D Systems. Purified rat anti-mouse major histo-compatibility (MHC) class II antibody was obtained from PharMingen (San Diego, CA). (-)-Epigallocatechin-3-gallate (EGCG) was purchased from Sigma-Aldrich.

Mouse Primary Cell Culture

Breeding pairs of C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in the animal facility at the University of South Florida Health Science Center. Mouse primary cultured microglia were isolated from mouse cerebral cortices and grown in complete Roswell Park Memorial Institute (RPMI) 1640 medium according to previously described methods [45]. Briefly, cerebral cortices from newborn mice (1-2 days old) were isolated under sterile conditions and were kept at 4ºC prior to mechanical dissociation. Cells were plated in 75-cm^2 flasks, and complete medium was added. Primary cultures were kept for 14 days so that only glial cells remained, and microglial cells were isolated by shaking flasks at 200 rpm in a Lab-Line™ Incubator-Shaker. More than 98% of these glial cells stained positive for MAC-1 (Boehringer Mannheim, Indianapolis, IN). Additionally, between 85% and 95% of microglia stained positive for CD45 by fluorescence-activated cell sorter (FACS) analysis as described previously [45].

Microglial Phagocytosis Assays

Fluorimetric analysis: Primary mouse microglia were seeded at 1×10^5 cells/well (n=6 for each condition) in 24-well tissue culture plates containing 0.5 mL of complete RPMI-1640 medium. These cells were treated for 60 minutes with "aged" Aβ1-42 conjugated with fluorescein Isothiocyanate (FITC; Biosource International) [46]. In the presence of FITC- Aβ 1-42, microglia were then co-treated with HIV-1 Tat or control (heat-inactivated Tat protein) in the presence or absence of IFN-γ and/or EGCG. Microglia were then rinsed three times in Aβ-free complete medium, and the media were exchanged with fresh Aβ-free complete medium for 10 minutes both to allow for removal of non-incorporated Aβ and to promote concentration of the Aβ into phagosomes.

Extracellular and cell-associated FITC-Aβ was quantified using a SpectraMax multi-detection reader (Molecular Devices) with an emission wavelength of 538 nm and an excitation wavelength of 485 nm. A standard curve from 0 to 500 nM of FITC-Aβ was run for each plate. Total cellular proteins were quantified using the Bio-Rad protein assay. The mean fluorescence values for each sample at 37ºC and 4ºC at the indicated time points were determined by fluorimetric analysis. Relative fold change values were calculated as: mean fluorescence value for each sample at 37ºC / mean fluorescence value for each sample at 4ºC. In this manner, both extracellular and cell-associated FITC-Aβ was quantified. Considering nonspecific adherence of Aβ to plastic surface of cultured plates, an additional control without cells was carried out through all of experiments above. An incubation time of less than 4 hours did not change the amount of Aβ peptide detected in the supernatant, which is consistent with a previous report [47]. In order to determine the extent to which cell death might have influenced the phagocytic activity in the various treatment groups, we performed the LDH assay on the relevant supernatant. Data showed that there was no significant cell death occurring over the 3-h time frame in any of the treatment groups (data not shown, $p>0.05$).

Fluorescence microscope examination: "Aged" FITC- Aβ 1-42 was prepared according to methods described above. In the presence of FITC-Aβ1-42, microglia were then co-treated with HIV-1 Tat or control (heat-inactivated Tat protein) in the presence or absence of IFN-γ, Apo-E3, and/or EGCG at 37ºC for 60 min. In addition, in parallel 24-well tissue culture plates, microglia were incubated at 4ºC with FITC-conjugated Aβ (50 nM) in the presence or absence of IFN-γ, Apo-E3, and/or EGCG for 60 min. Following treatment, these cells were washed 5 times with ice-cold phosphate buffered saline (PBS) to remove extracellular Aβ and then stained with 4', 6-Diamidino-2-phenylindole (DAPI) at 4ºC for 15 min. After washing three times with ice-cold PBS, these cells were fixed in 4% paraformaldehyde, mounted, and then viewed under an Olympus IX71/IX51 microscope equipped with a digital camera system.

Results

HIV-1 Tat Inhibits Microglial Phagocytosis of Aβ1-42 Peptide

HIV-1 Tat protein has been positively associated [10, 33, 37] with increased brain deposition of Aβ/β-amyloid; a common pathologic feature in HIV-positive patients [6, 8, 9, 11, 48-50]. Since microglial phagocytosis of Aβ has been considered a principle mechanism for the removal of Aβ from the

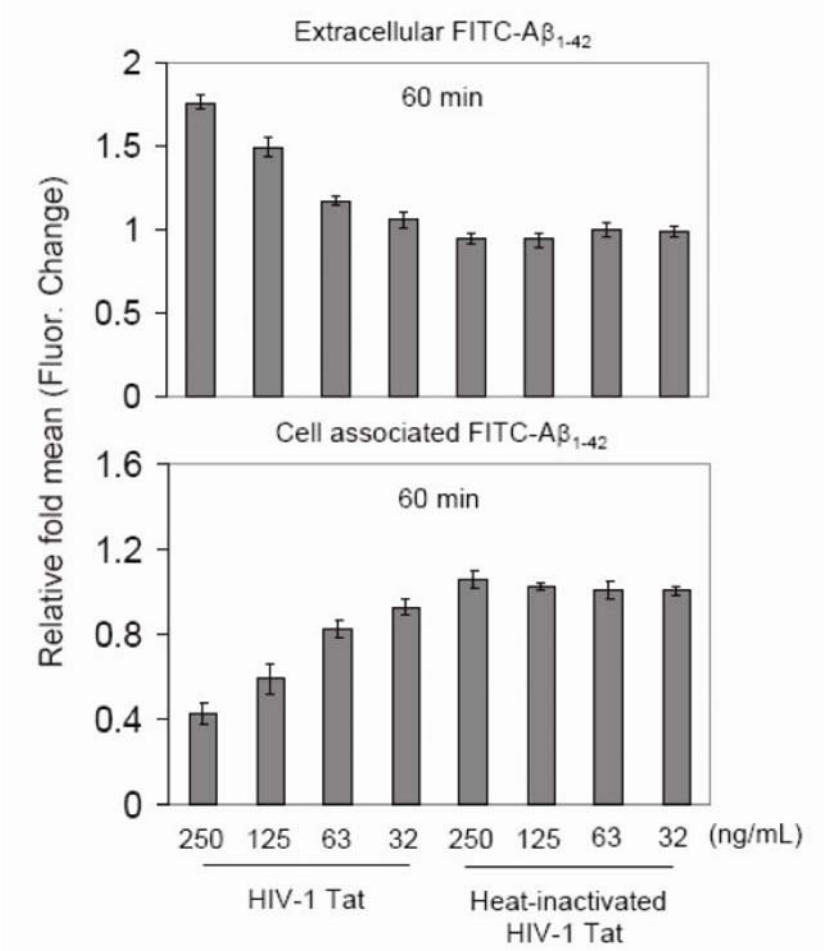

Figure 1 HIV-1 Tat protein inhibits microglial phagocytosis of Aβ 1-42 peptide. Primary cultured microglia (1×10^5 cells/well in 24-well tissue culture plates) were treated with "aged" FITC-tagged Aβ1-42 (300 nM) in complete medium for 60 min in the presence of either recombinant HIV-1 Tat protein (NIH AIDS Research and Reference Reagent Program; 32, 63, 125, 250 ng/mL), or heat inactivated recombinant Tat protein (32, 63, 125, 250 ng/mL). As a control for nonspecifically incorporated Aβ, microglia were incubated at 4ºC with the same treatment as above in parallel 24-well tissue culture plates. Cell supernatants and lysates were analyzed for extracellular (top) and cell-associated (bottom) FITC- Aβ using a fluorimeter. Data are represented as the relative fold of mean fluorescence change (mean ± SD), calculated as the mean fluorescence for each sample at 37ºC divided by mean fluorescence at 4ºC (n=6 for each condition presented). When measuring FITC-tagged Aβ 1-42 in cell supernatants or lysates, one-way ANOVA followed by post-hoc comparison showed a significant difference between HIV-Tat compared to heat inactivated HIV-1 Tat (control) dose dependently from 63-250 ng/mL ($p<0.001$), but no significant difference between HIV-Tat compared to heat inactivated HIV-1 Tat (control) at the 32 ng/mL concentrations ($p>0.05$).

brain parenchyma [38], we evaluated whether Tat could inhibit microglial uptake of Aβ. "Aged", FITC-tagged Aβ1-42 was added to primary cultured microglia for 60 min in the presence of varying concentrations of recombinant HIV-1 Tat at varying concentrations or heat inactivated HIV-Tat (negative control). As shown in **Figures 1** and **2**, addition of HIV-1 Tat protein significantly and dose-dependently decreased microglial uptake of Aβ1-42 compared to heat inactivated Tat (negative control; $p<0.05$) for each of the doses of Tat tested.

IFN-γ Enhances HIV-1 Tat-mediated Reduction in Microglial Phagocytosis of Aβ1-42 Peptide

A pro-inflammatory environment characterizes the HIV-infected and AD brain, respectively [5, 7, 51-61]. Importantly, there is evidence of microglial cell loss of functional capacity to phagocytose Aβ associated with inflammation

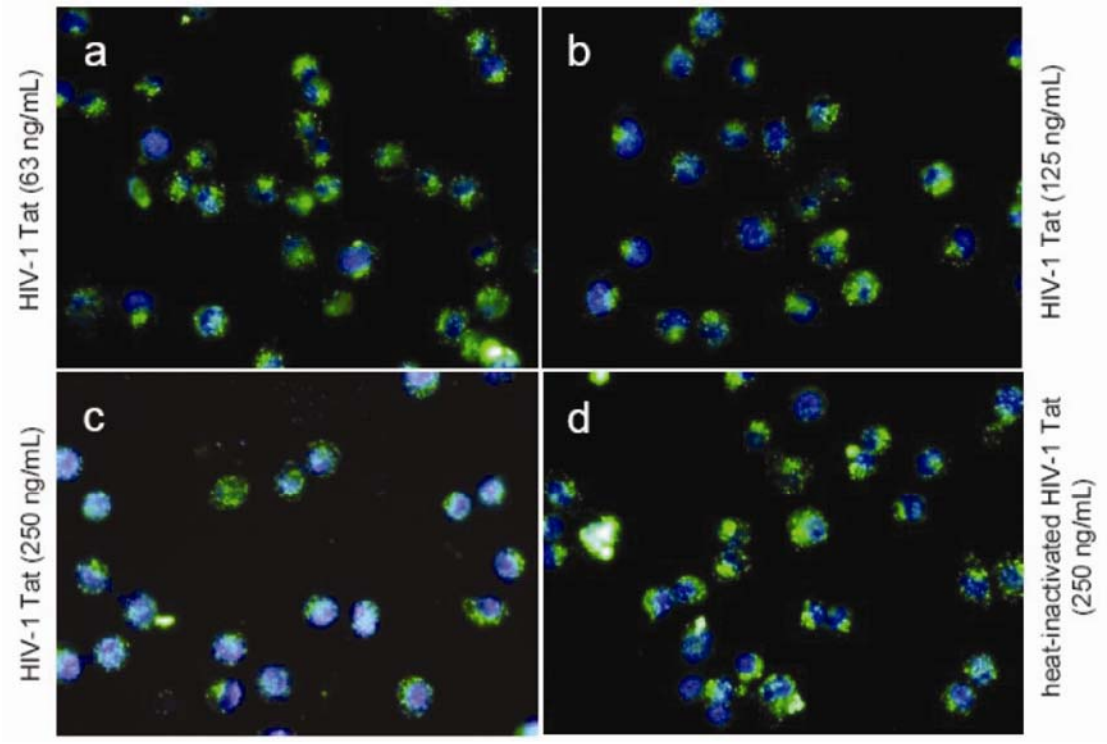

Figure 2 HIV-1 Tat protein inhibits microglial phagocytosis of Aβ 1-42 peptide. In order to examine microglia Aβ co-localization on the microglia surface, primary cultured microglia were treated with "aged" FITC- Aβ 1-42 peptide (50 nM) in the presence of either recombinant HIV-1 Tat protein (NIH AIDS Research and Reference Reagent Program; 63, 125, 250 ng/mL), or heat inactivated recombinant Tat protein (250 ng/mL) for 60 min and then stained these cells with DAPI. HIV-1 Tat dose-dependently inhibited microglia - Aβ co-localization as examined by fluorescence microscopy (**a**, **b**, **c**). As noted, green indicates Aβ1-42-positive; blue indicates microglial nuclei. Addition of HIV-1 Tat protein at 32 ng/mL yielded similar results as heat inactivated Tat (data not shown; **d**). Original magnification = 20×.

[17, 62]. Considered a pro-inflammatory cytokine, IFN-γ is elevated in the brains of HIV-infected patients as well as those patients who go one to develop HAD [1]. This holds clinical relevance as positive associations between severities of cognitive dysfunction in HAD with expression of inflammatory cytokines have been demonstrated [26]. This microglial loss of function has been associated with co-stimulatory cytokine signaling which shifts the functional state of microglia from being primarily phagocytes to antigen presenting cells (APC) [17]. In the phagocytic phenotype, microglia engulf and degrade Aβ peptide, preventing accumulation of Aβ peptide to neurotoxic levels in the brain. Conversely, switching to the APC phenotype inhibits this phagocytic ability and occurs in the presence of pro-inflammatory cytokines, leading to chronic microgliosis [17, 63-65]. This may act to exacerbate both AD and HAD neuropathology [5, 26, 66-68].

To investigate whether IFN-γ augments the inhibited phagocytic ability of microglia in the presence of FITC-Aβ1—42, and HIV-1 Tat, IFN-γ (50U/mL) or its denatured form (negative control) were added in the presence or absence of Tat (63 ng/mL [minimal dose which significantly inhibited Aβ uptake, **Figure 3**]) for 60 min at 37°C. Denatured Tat was not used in this paradigm as the denatured form was shown to be inactive in preventing microglial Aβ uptake (**Figure 1**). Data shows IFN-γ significantly augmented HIV-1 Tat-mediated decreased microglial phagocytosis of Aβ 1-42 (**Figure 3**, left panel) compared to control.

EGCG Inhibits IFN-γ Enhancement of Tat-mediated Reduction in Microglial Phagocytosis of Aβ1-42 Peptide

We have previously shown IFN-γ synergistically enhances neuronal injury induced by Tat in

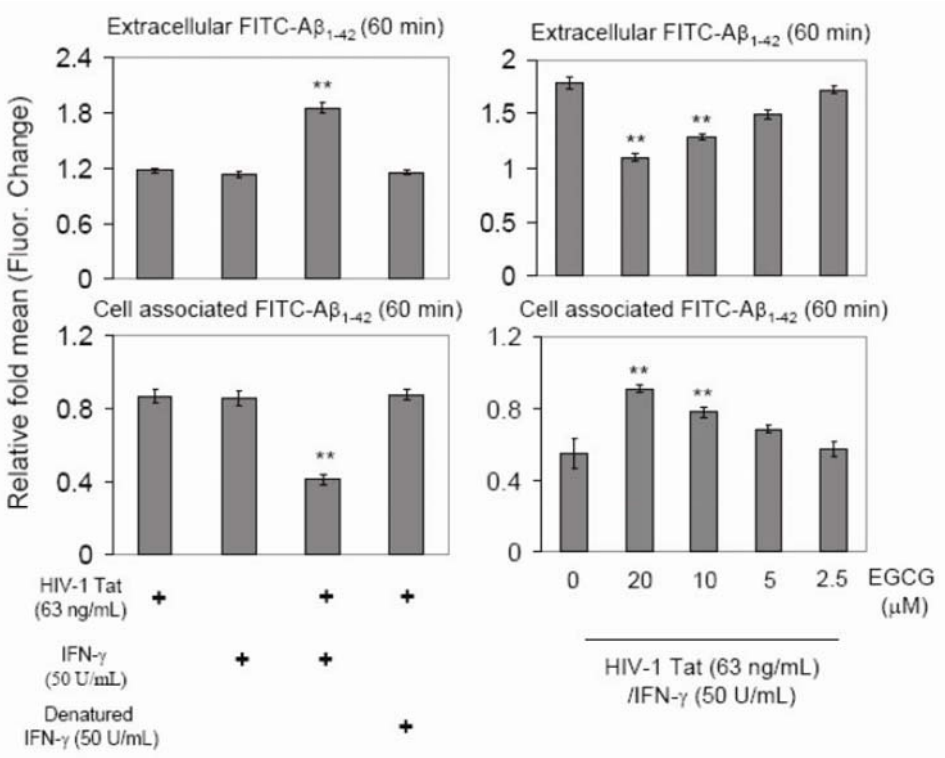

Figure 3 HIV-1 Tat-mediated reduction in microglial phagocytosis of Aβ1-42 peptide is enhanced by IFN-γ and opposed by EGCG. **Left panel**: IFN-γ significantly enhances HIV-1-Tat mediated reduction in microglial phagocytosis of Aβ1-42 peptide (left panel). Primary cultured microglia (1×10^5 cells/well in 24-well tissue culture plates) were treated with "aged" FITC-tagged Aβ 1-42 (300 nM) in complete medium for 60 min in the absence (control) or presence of either HIV-1 Tat (63 ng/mL, minimal effective concentration), active or denatured IFN-γ (50U/mL). As a control for nonspecifically incorporated Aβ, microglia were incubated at 4ºC with the same treatment as above in parallel 24-well tissue culture plates. Cell supernatants and lysates were analyzed for extracellular and cell-associated FITC- Aβ using a fluorimeter. Data are represented as the relative fold of mean fluorescence change (mean ± SD), calculated as the mean fluorescence for each sample at 37ºC divided by mean fluorescence at 4ºC (n=6 for each condition presented). When measuring FITC-tagged Aβ 1-42 in cell supernatants or lysates, one-way ANOVA followed by post-hoc comparison showed a significant difference between Tat alone, IFN-γ alone, or denatured IFN-γ + Tat compared to the IFN-γ + Tat control ($p<0.001$). **Right panel**: EGCG significantly inhibits IFN-γ enhancement of HIV-1Tat-mediated reduction in microglial phagocytosis of Aβ 1-42 peptide. Primary cultured microglia (1×10^5 cells/well in 24-well tissue culture plates) were treated with "aged" FITC-tagged Aβ 1-42 (300 nM) in complete medium for 60 min with addition of HIV-1 Tat (63 ng/mL) and IFN-γ (50 U/mL) in the presence of EGCG (0 [control], 2.5, 5, 10, or 20 μM). As a control for nonspecifically incorporated Aβ, microglia were incubated at 4ºC with the same treatment as above in parallel 24-well tissue culture plates. Cell supernatants and lysates were analyzed for extracellular and cell-associated FITC- Aβ using a fluorimeter. Data are represented as the relative fold of mean fluorescence change (mean ± SD), calculated as the mean fluorescence for each sample at 37ºC divided by mean fluorescence at 4ºC (n=6 for each condition presented). When measuring FITC-tagged Aβ 1-42 in cell supernatants or lysates, one-way ANOVA followed by post-hoc comparison showed a significant difference between EGCG (10 or 20 μM) compared to control ($p<0.001$), but no significant difference between EGCG at the 2.5 and 5.0 μM dose compared to control ($p>0.05$).

vitro and in vivo via activation of the JAK/STAT1 signaling pathway which is specifically attenuated by EGCG inhibition of STAT1 activation [24]. Other investigations have also shown that EGCG inhibits IFN-γ mediated STAT1 signaling as well [69-71]. Thus we evaluated whether EGCG could oppose the IFN-γ mediated enhancement of Tat down-regulation of microglial uptake of Aβ. Again, "aged" FITC-tagged Aβ 1-42 was added to primary cultured microglia with IFN-γ (50 U/mL) and Tat (63 ng/mL) for 60 min at 37ºC in the presence of varying concentrations of EGCG (0, 2.5, 5, 10, and 20μM). As shown in **Figure 3** (right panel), data indicated EGCG dose-dependently increased microglial cell

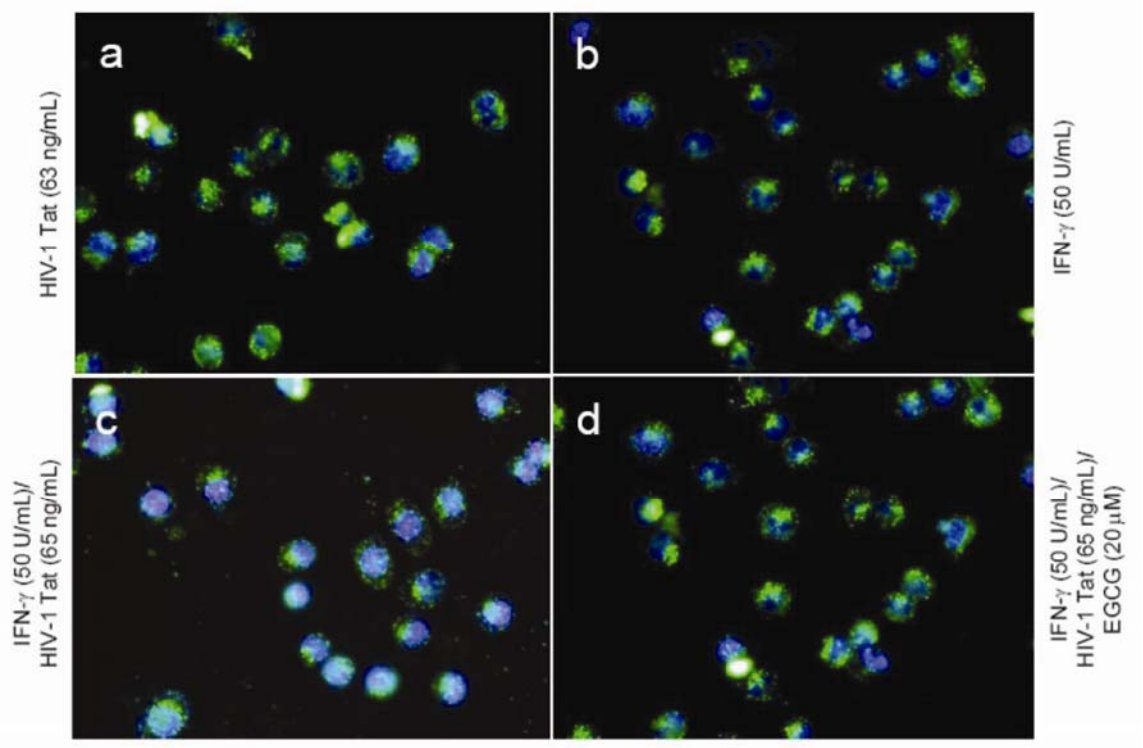

Figure 4 HIV-1 Tat-mediated reduction in microglial phagocytosis of Aβ1-42 peptide is enhanced by IFN-γ and opposed by EGCG. EGCG significantly inhibits IFN-γ enhancement of HIV-1 Tat-mediated reduction in microglial phagocytosis of Aβ 1-42 peptide. In order to examine microglia Aβ co-localization on the microglia surface, primary cultured microglia were treated with "aged" FITC-tagged Aβ 1-42 (50 nM) in complete medium for 60 min with addition of HIV-1 Tat (63 ng/mL), IFN-γ (50 U/mL), or HIV-1 Tat (63 ng/mL) + IFN-γ (50 U/mL) in the presence or absence of EGCG (20 μM) and then stained these cells with DAPI. HIV-1 Tat + IFN-γ (**c**) significantly inhibited microglia - Aβ co-localization as compared to HIV-1 Tat (**a**) or IFN-γ alone (**b**) as examined by fluorescence microscopy. As noted, green indicates Aβ1-42-positive; blue indicates microglial nuclei. Addition of EGCG alone yielded similar results as addition of PBS alone (data not shown). Original magnification = 20×.

associated Aβ 1-42 in the presence of IFN-γ and HIV-1 Tat. The minimal effective concentration was 10μM. As shown in **Figure 4**, HIV-1 Tat + IFN-γ (**c**) significantly inhibited microglial phagocytosis of Aβ peptide as compared to HIV-1 Tat (**a**) or IFN-γ alone (**b**) as examined by fluorescence microscopy.

HIV-1 Tat Opposes Apo-E3-promoted Aβ1-42 Microglial Phagocytosis

Apolipoprotein E (Apo-E) is a constituent of amyloid plaques in the brains of AD patients and is genetically polymorphic. Three frequent alleles exist at the Apo-E gene locus: E2, E3, and E4 [72]. The E4 polymorphism strongly affects the risk of developing both HAD and AD [57]. Several hypotheses have been put forward to account for the association. When Aβ and Apo-E are co-incubated, E4 forms denser monofibrillar structures than E3 [73]. Further, Apo-E4 is more effective than E3 in enhancing the rate and amount at which fibrils are generated from soluble amyloid in vitro [74]. In addition, Apo-E4, but not E3, is neurotoxic; reducing neurite sprouting [75-77] and enhancing depolymerization of microtubule [78, 79]. Conversely, under normal conditions, Apo-E3 promotes microglial clearance of Aβ [80, 81]. In support, cell associated Aβ is approximately half that observed in the presence of heat-inactivated Tat (control; **Figure 1**) compared to Apo-E3 conditions where cell associated Aβ is nearly doubled (**Figure 5**). Moreover, other groups have shown native E3 has been shown to form an stable complex with Aβ that is more abundant than the Apo-E4:Aβ complex [80, 81]. Additionally, exogenous Apo-E3 but not E4 prevents Aβ-induced neurotoxicity by a process that requires Apo-E receptors [80].

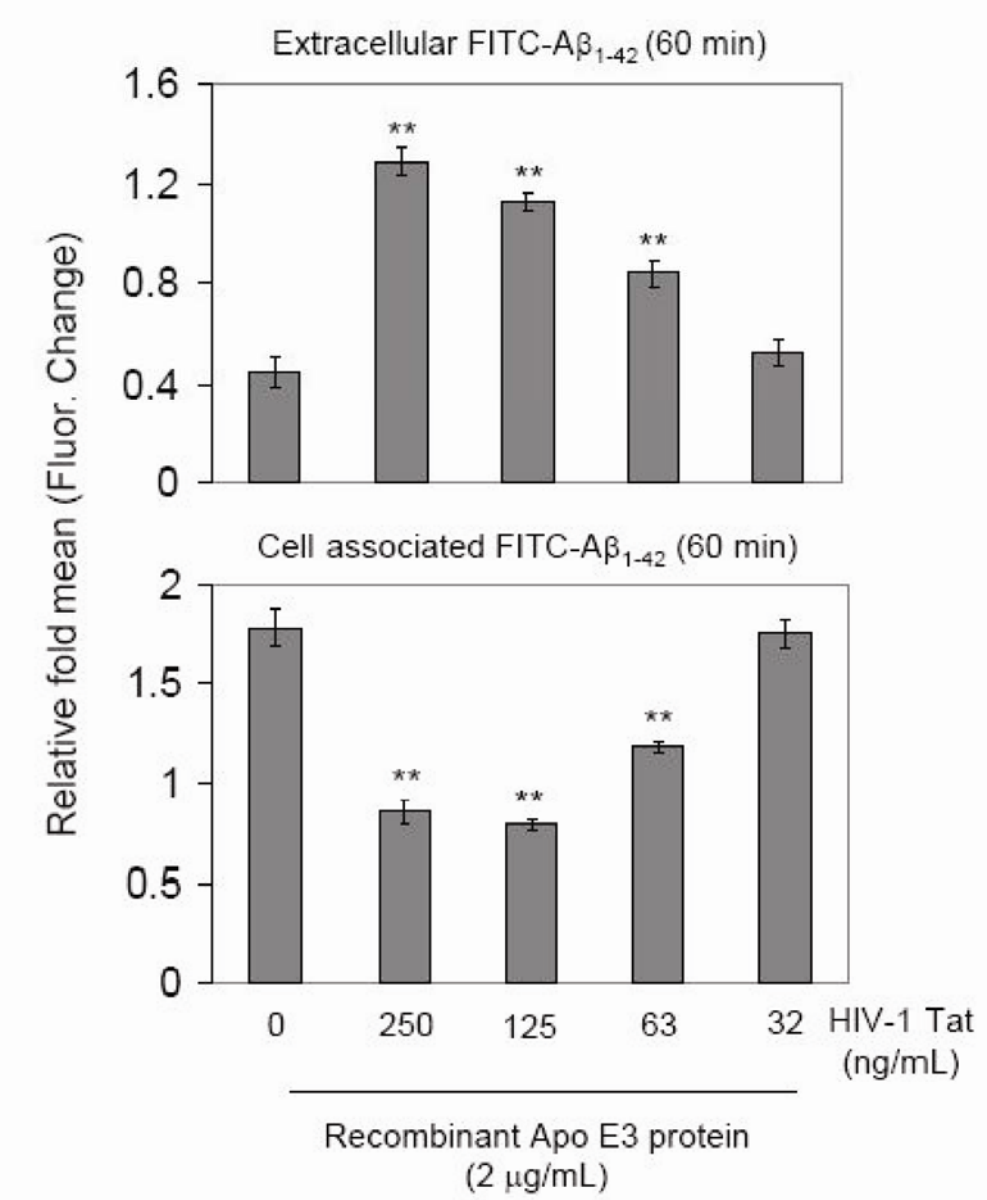

Figure 5 HIV-1 Tat protein opposes Apo-E3 promoted microglial phagocytosis of Aβ1-42 peptide. Primary cultured microglia (1×10^5 cells/well in 24-well tissue culture plates) were treated with "aged" FITC-tagged Aβ 1-42 (300 nM) in complete medium for 60 min in the presence of recombinant HIV-1 Tat protein (NIH AIDS Research and Reference Reagent Program; 0, 32, 63, 125, 250 ng/mL) as well as Apo-E3 (2μg/mL). As a control for nonspecifically incorporated Aβ, microglia were incubated at 4°C with the same treatment as above in parallel 24-well tissue culture plates. Cell supernatants and lysates were analyzed for extracellular and cell-associated FITC-Aβ using a fluorimeter. Data are represented as the relative fold of mean fluorescence change (mean ± SD), calculated as the mean fluorescence for each sample at 37°C divided by mean fluorescence at 4°C (n=6 for each condition presented). When measuring FITC-tagged Aβ 1-42 in cell supernatants or lysates, one-way ANOVA followed by post-hoc comparison showed HIV-1 Tat dose-dependently inhibited Apo-E3 enhanced microglial phagocytosis of Aβ peptide ($p<0.05$).

Thus to investigate whether Tat could inhibit this Apo-E3 promoted microglial uptake of Aβ, recombinant Apo-E3 protein (Sigma; 2μg/mL) was added to primary cultured microglia for 60 min in the presence of varying concentrations of recombinant Tat protein (0, 32, 63, 125, and 250 μg/mL) and "aged" FITC-tagged Aβ1-42. As shown in **Figure 5**, the Tat protein

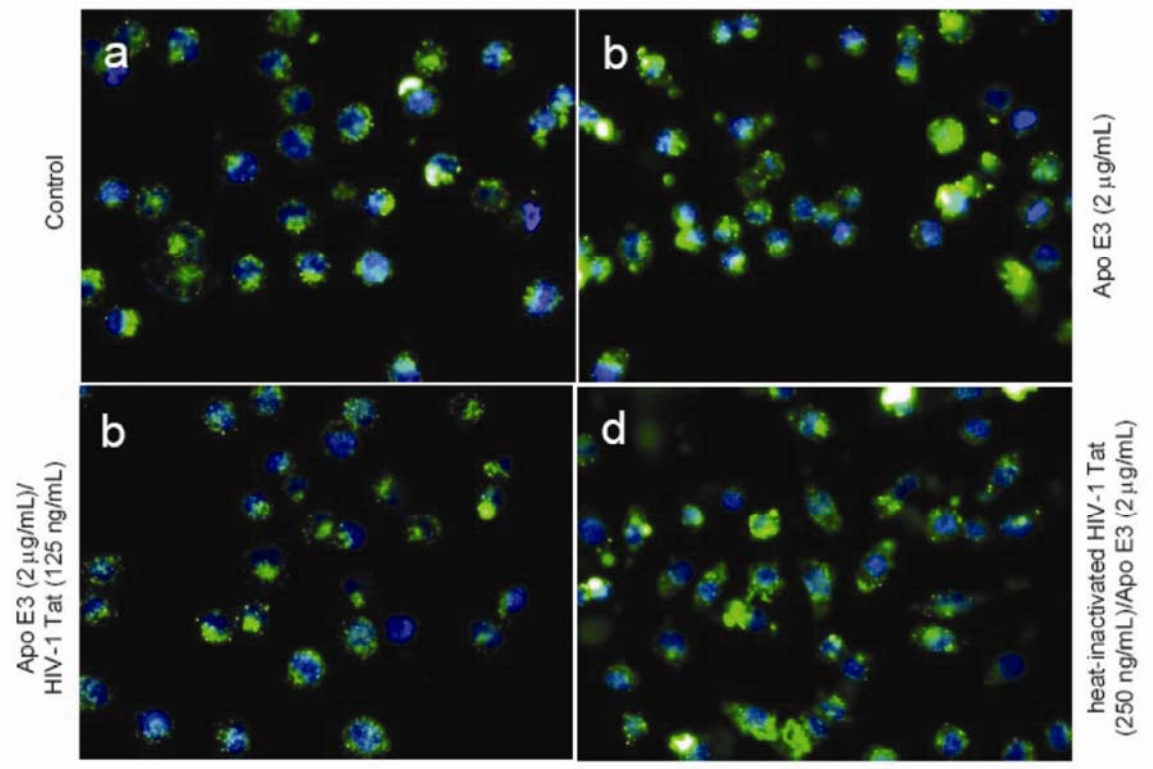

Figure 6 HIV-1 Tat protein opposes Apo-E3 promoted microglial phagocytosis of Aβ1-42 peptide. In order to examine microglia Aβ co-localization on the microglia surface, primary cultured microglia were treated with "aged" FITC- Aβ 1-42 peptide (50 nM) in the presence PBS (**a**; control), Apo-E3 (**b**; 2 µg/mL), Apo-E3 (2 µg/mL) + HIV-1 Tat (125 ng/ml; **c**) or heat inactivated HIV-1 Tat (250 ng/mL) + Apo-E3 (2 µg/mL; **d**) and then stained with DAPI. HIV-1 Tat significantly inhibited Apo-E3 enhanced microglial phagocytosis of Aβ (**c**) as compared to control (**a**), Apo-E3 alone (**b**), or heat inactivated HIV-1 Tat + Apo-E3 (**d**) conditions as examined by fluorescence microscopy. As noted, green indicates Aβ1-42-positive; blue indicates microglial nuclei. Addition of HIV-1 Tat protein at 32 ng/mL yielded similar results as heat inactivated Tat (data not shown). Original magnification = 20×.

significantly and dose-dependently decreased Apo-E3 promoted microglial uptake of FITC-tagged Aβ 1-42. As shown in **Figure 6**, HIV-1 Tat significantly inhibited Apo-E3 enhanced microglial phagocytosis of Aβ as compared to control (**a**), Apo-E3 alone (**b**), or heat inactivated HIV-1 Tat + Apo-E3 (**d**) conditions as examined by fluorescence microscopy.

Discussion

Interactions between HIV and mechanisms underlying AD may cause accelerated and severe dementias [8]. Such interactions may occur at several levels. First, it has been most recently demonstrated that the cysteine-rich domain of Tat interacts with neprilysin, a type II plasma membrane zinc metallopeptidase which cleaves the toxic secreted Aβ peptide [10, 37, 82, 83]. Second, Tat can directly inhibit uptake of Aβ and Apo-E. In the case of neurons, this occurs through inhibition of the LRP receptor [33]. As shown in **Figures 1** and **2**, Tat protein dose-dependently decreased uptake of Aβ1-42 in microglia as well. We suggest extracellular enzymatic degradation of the peptide by neprilysin does not account for this increased clearance of Aβ as endopeptidase activity is generally non-existent or minimal at 4ºC [84]. Indeed microglia incubated at 4ºC in parallel cell culture plates under the same treatment conditions described above displayed similar results to those incubated at 37ºC. In further support, our system involved whole recombinant Tat protein whereas Daily and colleagues in 2006 reported that peptides derived from the Tat protein, but not Tat protein itself, inhibit neprilysin [37]. Additionally, we suggest cell death due to the neurotoxic properties of Tat [24, 85-87] did not significantly influence the phagocytic

activity in the various treatment groups as determined by LDH assay on the relevant supernatants. Indeed results showed that there was no significant cell death occurring over the 3-h time frame in any of the treatment groups (data not shown, $p>0.05$). Therefore our data point to a possible role for a receptor-mediated inhibition of Aβ uptake by Tat. As will be discussed below, these phenomena may be occurring through inhibition of microglial LRP by HIV-1 Tat, as has been previously demonstrated with neuronal LRP [33]. Further studies using the specific antagonist of LRP, receptor associated protein (RAP), are required to test this hypothesis.

IFN-γ may enhance inhibitory effect of HIV Tat through a microglial switch to the non-phagocytic APC phenotype. This remains to be validated in future works focusing on microglial APC markers. However, several past reports strongly indicate that IFN-γ promotes the APC phenotype in a variety of immune cells including microglia and astrocytes through activation of class II transactivator (CIITA). Indeed both constitutive and inducible MHC class II gene expression requires CIITA [88-91]; a scaffolding protein which interacts with itself and other transcription factors to activate the MHC class II promoter [92, 93]. CIITA transcription is modulated by at least three promoters: pI, pIII and pIV each of which generates unique CIITA transcripts [90, 91, 94]. All three promoters can be induced in macrophages by IFN-γ although the major form is transcribed from pIV [90, 91, 94]. In turn, pIV is regulated by three major cis-acting elements: an IFN-γ activation sequence (GAS), an E box, and an interferon regulatory factor (IRF) element, which bind the transcription factors, among them are signal transducer and activator of transcription (STAT1) and interferon regulatory factor-1 (IRF-1) [94]. IFN-γ is known to activate tyrosine Janus-associated kinase (JAK)1 and JAK2, yielding phosphorylated STAT1 in many cells including microglia [95, 96]. Phosphorylated STAT1 then dimerizes and migrates to the nucleus, where it binds to the GAS element in pIV [97-100]. STAT1 also regulates IRF-1 expression, which in turn activates the CIITA promoter [101, 102]. In further support, Aloisi and colleagues demonstrated IFN-γ induces functional expression of MHC class II I-A and I-E molecules [103]. Additionally astrocytes display IFN-γ-induced MHC class II expression upon IFN-β stimulation [104, 105]. Moreover, IFN-γ mediates induction of MHC class II molecules in cultured rat astrocytes [106]. Therefore it is our hypothesis that IFN-γ mediated STAT1 activation leads to pIV activation in turn causing induction of CIITA and the APC phenotype. This yields a minimal number of cells acting as functional phagocytes. To further explore STAT1 activation in these observed results, we next examined the effect of a specific STAT1 inhibitor, the Green tea-derived flavonoid, EGCG.

IFN-γ exerts effects in many cells including microglia [107, 108] via phosphorylation of JAKs which then go on to activate STAT proteins which then migrate to the nucleus where they can activate CIITA. Although STATs are not exclusively activated by JAKs, one of the best studied pathways for STAT activation is through the JAK; a system termed as the JAK/STAT pathway. Importantly, chronic HIV-1 infection among individuals with progressive disease has been correlated with a 6 to 10 fold increase in levels of activated STATs in peripheral blood mononuclear cells after exposure to virions [109]. Our results suggest EGCG, a specific STAT1 inhibitor, attenuates the IFN-γ mediated augmentation of the anti-phagocytic properties of HIV-1 Tat in microglia. We therefore hypothesize that STAT1 mediated activation of CIITA and thus the APC phenotype, is attenuated. Importantly, EGCG has been suggested to be safe since administration of EGCG to rats or dogs for 13 weeks was not toxic at doses up to 500 mg/kg/day [110]. Additionally the compound does not confer genotoxicity in rats [111]. Furthermore, EGCG has been suggested safe in humans in doses up to 800 mg daily [112].

Uptake of Aβ by microglia is mediated by various receptors, including class A scavenger receptors (SR-A) [113], Fc receptors [38], and LRP, provided that Aβ is complexed to α2-macroglobulin (α2M) [114], lactoferrin [114], or Apo-E [115], which are ligands of LRP. Of these ligands, the epsilon-4 allele of Apo-E is the primary risk factor for AD pathology [116, 117]. We hypothesize that LRP is inhibited by HIV-1 Tat, resulting in elevated Aβ/β-amyloid deposition. This is supported by several lines of evidence. First, it has previously been shown that HIV-1 Tat inhibits binding and uptake of Aβ and Apo-E at the LRP receptor in neurons [33]. Second, Aβ binds to heparan sulfate proteoglycans (HSPG) on the cell

surface from where they are subsequently transferred to LRP for cellular uptake [118-126]. Third, genetic and biochemical investigations demonstrated that cell membrane HSPG are the receptors for extracellular Tat internalization as well. Indeed Tyagi and colleagues in 2000 demonstrated Tat uptake is inhibited by heparin and that cell treatment with lyases specific for HSPG, but not for chondroitin sulfates, blocks Tat internalization, and that cell lines genetically deficient in the cellular pathway involved in the production of sulfated HSPG fail to internalize Tat [127]. It has been suggested this phenomenon underlies the ability of HIV-1 Tat to enter into a wide array of human, rodent, and simian cell lines and that extracellular Tat enters most of the exposed cells [127]. Finally, among the above named receptors for uptake of Aβ by microglia, it seems that LRP is more important than SR-A or Fc receptors. In studies of microglia using competitive ligands of SR-A, it has been shown that SR-A was not involved in the recognition of amyloid peptide deposits, whereas LRP specifically recognized deposits of Aβ 1–42 and mediated their uptake [128].

Taken together, these experiments suggest HIV-1 Tat inhibits microglial uptake of Aβ peptide, a phenomenon which is exacerbated by IFN-γ and attenuated by green tea derived EGCG. Our data may partially explain a past report indicating HIV-infected subjects with the E4 allele for Apo-E have excess dementia [57]. Specifically, it has been shown that twice as many E4(+) subjects were demented (30% compared with 15%; $P < 0.0001$) compared to E3(+) subjects by Corder and colleagues (1998). Given that the other Apo-E isoforms are more efficient at promoting microglial uptake of Aβ via LRP, and that the promotion conferred by E3 is dampened in vitro by HIV-1 Tat (**Figures 5** and **6**), it is plausible that having the double risk of both HIV infection and the E4 allele leads to a very severe deficiency in the ability of microglia to uptake and degrade Aβ, which may in turn result in a more severe dementia. In conclusion, we have shown that HIV-1 Tat inhibits microglial phagocytosis of Aβ peptide in vitro, a process which is enhanced by IFN-γ, and opposed by the STAT1 inhibitor EGCG. Secondarily these data point to the microglial LRP and HSPG as sites of HIV-1 Tat inhibition of microglial Aβ phagocytosis. Future investigations are required to fully characterize the receptor(s) and full intracellular signaling mechanisms responsible for these observed phenomena.

Acknowledgments

This work is supported by grants from the NIH/NINDS (JT, BG) and NIH/NIGMS (PS).

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