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Stimulation of IL-16 Release by Cells Exposed to HIV Virion Proteins

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Abstract

Introduction: The pathogenesis and immune response to chronic viral infection are multifaceted especially for HIV infection. The effect of HIV on the systemic immune environment of the infected individual is profound and triggered by multiple stimuli. While replication-competent viral infection induces a large spectrum of immune responses, such as cytokine release, we focused on what happens when cells are exposed to replication-incompetent virions and their components.

Results: Monitoring the cytokines released from T cell lines exposed to HIV virion components, we found several cytokines were upregulated with IL-16 being the most upregulated. HIV virion components induced Caspase-3 activation which is required for IL-16 release and this IL-16 release is dependent on protein components within the HIV virion.

Conclusion: Overall, this work allows us to see that replication-incompetent HIV virions have an impact on the immune landscape of a person infected by HIV. We see that exposure of T cells to HIV virion proteins leads to Caspase-3 activation and subsequent IL-16 release.

Key words: HIV; AIDS; Innate Immunity; Cytokines, Cytokine; Array; IL-16; Caspase-3.

INTRUDUCTION

As of 2014, it is estimated that 36.9 million people are living with Human Immunodeficiency Virus (HIV) worldwide with about 2 million newly infected per year. HIV is estimated to cause about 1.2 million deaths among adults and children annually. Although there has been much improvement in both prevention and treatment, realization of completely effective therapies, cures or vaccines requires a better understanding of the pathophysiology and immunological consequences of HIV infection.

Like a virus with immune system tropism, HIV induces broad, system-level changes in cytokine levels in an individual infected with HIV [1,2]. While certain major cytokines have been well studied, including those associated with mortality from HIV, few studies have looked at comprehensive cytokine analysis after exposure to HIV [3].

HIV-1 infects CD^{4+} T cells. Prior research on cytokine response to HIV-1 has shown that HIV-1 causes a decrease in Th1 cytokines (IL-2, IFN-gamma) but causes an increase in Th2 and inflammatory cytokines (IL-4/IL-10 and TNF alpha respectively).

Reconstituting Th1 cytokine response has been demonstrated to improve CD8+ T cells and control of the virus [4]. Other cytokines such as IL-16 and IL-2 have an inhibitory effect on HIV-1 and have been suggested as possible therapeutic modalities [5]. During any viral infection, a high level of defective virions is released into the local microenvironment as exemplified in the evolution of Influenza virus [6]. These defective virions are often structurally enough to enter cells.

The goal of this work is to investigate how exposure to HIV virion components modulates normal cytokine release using an unbiased approach. We found that exposure to HIV virion components induces the release

of several cytokines with IL-16 being most induced. We further focused on IL-16 release since levels of IL-16 have been shown to be increased in the initial stages of HIV-1 infection, followed by a decrease as HIV infection progresses [7,8]. This strongly implies that IL-16 is involved in the initial immune response to HIV. Here we also show how HIV exposure induces IL-16 production.

MATERIALS AND METHODS

CEM and Jurkat, both leukemic CD^{4+} T cell lines, were cultured in RPMI + 5% Fetal Bovine Serum (FBS) + 1% Penicillin/Streptomycin (P/S). We use 5% FBS to limit innate immune signaling. Cells were incubated at 37 °C and 5% CO_2 and maintained by diluting to $1x10^5$ cells/mL every 2-3 days to allow growth. Cells were used at a working concentration between $2x10^5$ and $5x10^5$ cells/mL. HEK 293T cells, a transformed human embryonic kidney epithelial cells line that expresses the Large T Antigen from SV40, was grown in DMEM + 5% FBS + 1% Penicillin/Streptomycin and maintained at 20-70% confluence. All cell lines were purchased from ATCC.

Cells were transfected with purified HIV Virion Lysates (ABI) with Lipofectamine 2000 (Invitrogen, Corp) transfection reagent according to a modified manufacturer's protocol as described below. Jurkat and CEM cells were transferred to RPMI + 5% FBS with no P/S at cell concentration of either $2x10^5$ or $5x10^5$ cells/mL in 12-well dishes. To 400 µL of RPMI, 12 µL of Lipofectamine 2000 reagent was added and allowed to incubate for 5 minutes at room temperature. After the incubation, 50 µL of the RPMI/Lipofectamine 2000 reagent mix was added to tubes with 0 µL (vehicle), 1 µL, 3 µL, 6 µL, or 9 µL of HIV lysate and allowed to incubate for 15 minutes at room temperature. After incubation, these mixtures were added to the appropriate cells. Transfected cells were grown for 24-48 hours under the conditions as described above.

Changes in cytokine production were assessed with Human Cytokine Array Panel A (R&D Systems) according to the manufacturer's instructions. In brief, nitrocellulose membranes in a 4-well multi-dish, spotted with antibodies for a panel of cytokines, were blocked to prevent nonspecific binding. Signal produced by the cytokine array was detected and quantitated by a computer-aided gel documentation system.

HIV Virion Lysates were treated with 1µl of RNase A for 20 minutes at 37°C, with 1 unit (0.5 µl) of Ribonuclease H for 20 minutes at 37°C, 10 µl of Proteinase K at 50°C for 30 minutes or mock treated. Enzymes were inactivated at 65°C for 20 minutes. Either 1 ml or 10 ml of treated lysates were transfected into CEM cells for 36 hours and supernatants were analyzed by ELISA for IL-16 production, as described above.

RESULTS

Exposure to HIV Virion Components Induced Modulation of Specific Cytokines

To better understand how CD^{4+} T cells are affected by exposure to HIV virions we utilized a Human Cytokine Array to interrogate the modulation of different cytokines. We used CEM CD^{4+} T cell lines and transfected a lysate from purified HIV virions into the cells. In this manner, we mimicked exposure of the CD^{4+} T cells to defective virions. Supernatants from the transfected CEM cells were analyzed by a Human Cytokine Array to detect levels of cytokine with or without exposure to HIV components (Figure 1).

Cytokine levels were quantitated and displayed in Table 1 along with a list of cytokines that were not detected.

Table 1: Ratio of quantification of cytokine levels in cells exposed to HIV virion lysate or transfection vehicle.

Cytokine	Ratio of HIV Lysate/Vehicle
IL-16	4.47
sICAM-1	2.55
IL-23	1.6
MIF	1.56
CD40L	1.53
IL-32a	1.34
CXCL 12	1.32
MIP-1a	0.72
IP-10	0.67
CCL5	0.37

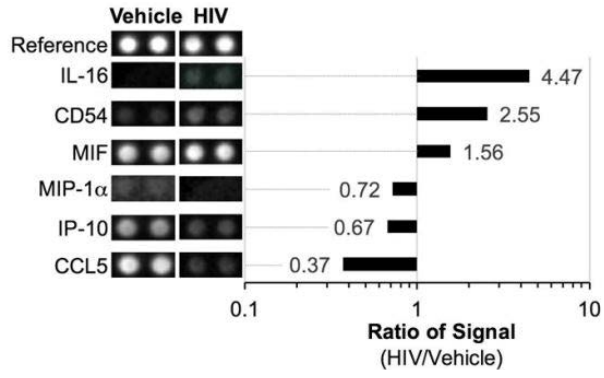

Figure 1.

The cytokine array measures 36 different cytokines and we compared cytokines levels in CEM cells transfected with virion lysates versus CEM cells exposed to vehicle alone. As shown in Figure 1, some cytokines were upregulated while others were downregulated. Each pair of spots corresponding to the different cytokines on the Human Cytokine Array were digitally quantitated and reference spots were used to normalize each of the cytokines. We plotted ratios of signal for each cytokine as iron-exposed cells compared to vehicle alone cells. Several cytokines had increased in expression, most notably IL-16. On the other hand, several cytokines had a decrease in their expression, with CCL5 showing the biggest decrease in expression. Many other cytokines on the panel had no expression by CEM CD^{4+} T cells and all array data is presented in Table 1.

IL-16 is released upon Exposure to HIV Virion Components

IL-16 has been well-known to limit HIV replication, so we were intrigued by the high induction seen when we exposed our samples to HIV virion lysates [9]. To confirm the results from the cytokine array, we exposed CEM and Jurkat CD^{4+} T cell lines to HIV virion lysate and quantitated the levels of IL-16 produced using ELISA. Two separate cell concentrations of CEM CD^{4+} T cells were transfected with different amounts of HIV virion lysate, while Jurkat cells were transfected at one concentration of cells. Supernatants of the cells were assayed to quantitate the level of IL-16 produced.

CEM cells demonstrated increasing release of IL-16 in response to increasing concentrations of HIV Virion Lysate at different cell concentrations (Figure 2a). Jurkat cells demonstrated increasing release of IL-16 in response to increasing concentrations of HIV Virion Lysate at one cell concentrations (Figure 2b). Transfection of HEK 293T cells, which are not CD^{4+} T cells, showed no production of IL-16 (data not shown). In addition, transfection of CEM cells with HIV Virion Lysate showed no production of the cytokine IFN-gamma (as prior studies have found decreased expression), confirming the data from the Human Cytokine Array (Data not shown) [10].

Exposure to HIV induces Caspase Activation

Since IL-16 release is dependent on activation of Caspase 3, we monitored activation of Caspase 3 upon exposure of CEM cells to HIV Lysate [11,12]. Caspase 3 activation can be monitored due to the proteolytic cleavage of Caspase 3

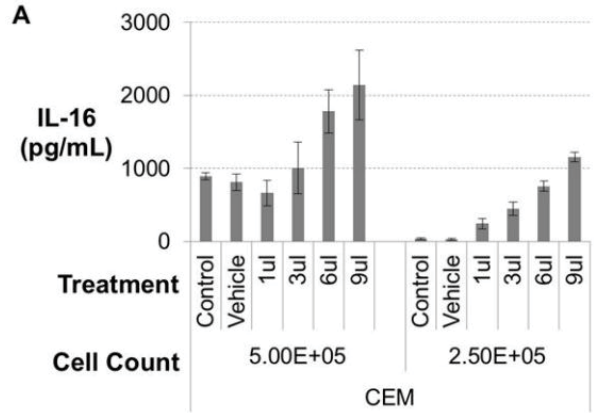

Figure 2a.

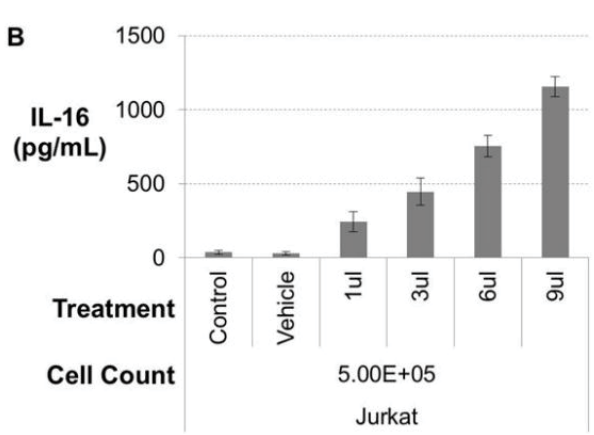

Figure 2b.

into a smaller form. To see this, we used western blotted to produce the smaller form of Caspase 3 during exposure to HIV Lysate. As shown in Figure 3, exposure of CEM cells to HIV Lysate induced a smaller form of Caspase 3 as cells are exposed.

Virion Proteins, but not Virion RNA, induce IL-16

Cytokines are released upon initiation of cellular signaling within cells. To better understand what type of immune signaling is being initiated, we treated virion lysates with enzymes to remove different molecular components. Specifically, we pre-treated virion lysates with RNase to remove RNA, RNase H to remove reverse transcriptase intermediates, or Proteinase K to remove protein components of the virions. The treated virions were then exposed to CEM cells and the levels of IL-16 in the supernatants were assessed by ELISA. The production of IL-16 was below the limit of detection for

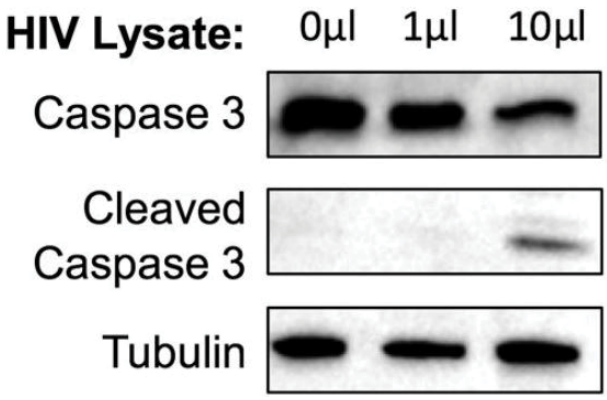

Figure 3.

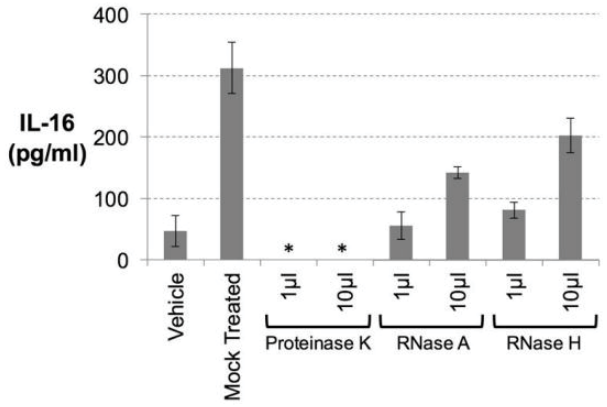

Figure 4.

the supernatant from CD^{4+} T cells transfected with virion lysates treated with Proteinase K. The supernatant from CD^{4+} T cells treated with RNase and RNase H still showed detectable levels of IL-16 production in a dose-dependent manner (Figure 4).

DISCUSSION

HIV has evolved to efficiently persist in a person due to its ability to intricately manipulate the host immune response as well as its viral proteins [13,14]. HIV appears to manipulate the delicate balance of cytokines essential in normal immune function towards its goal of immune evasion and a heightened ability to spread [14]. To model this process, we transfected CD^{4+} T cells with lysates from purified HIV virions. We used Human cytokines Arrays to interrogate a set of cytokines and how they are affected by exposure to HIV virions. The HIV Virion Lysate transfected CEM cells yield both induced and blocked expression of cytokines.

Induced Cytokines

We saw that levels of IL-16, a chemoattractant for immune cells, were greatly increased after CD^{4+} T cells were exposed to HIV Virion Lysate [15]. Previous studies have found that IL-16 can induce human T-cells to be resistant to HIV infection [16,17]. As the HIV Virion Lysate represents the components of HIV virions, and not functionally-replicating virus, an actively-replicating virus may not induce IL-16 or may induce it at lower levels, allowing for replication. There may be a mechanism, possibly involved with blocking the cleavage of the pro-IL-16 form, to suppress the normal release of IL-16 in patients living with HIV infection and should be further studied. However, this may also indicate an initial induction of IL-16, as has been demonstrated in early infection of a cohort of patients living with HIV infection, with blockage of IL-16 occurring at later stages of the infection [18]. These temporal aspects of IL-16 release may be a fitting target for future therapies.

We also observed that sICAM-1 (a soluble form of an intracellular adhesion marker), which plays a role in immune system activation, was increased. This trend has also been demonstrated in patients living with HIV infection [19]. Furthermore, we saw an increase in MIF (macrophage migration inhibitory factor) when exposed to HIV virion components. This is observed in patients with HIV, where MIF activates signaling pathways that aid in HIV-1 replication [20].

Down-regulated Cytokines

Cytokines such as CCL5 (RANTES), which inhibits HIV-1 by binding and blocking the CCR5 HIV-1 co-receptor, were decreased in the HIV Virion Lysate transfected samples [21]. Additionally, IP-10 (CXCL-10), which is secreted in response to IFN-gamma and aids in the recruitment of cytotoxic T-cells also decreased. This could help limit the cytotoxic T-cell recruitment and killing of HIV-infected cells.

Mechanism of IL-16 Release

Our experiments show a positive correlation between increasing HIV lysate concentrations and IL-16 production levels. Without any HIV lysate, IL-16 productions levels were minimal-to-undetectable in our control cells. Therefore, we can conclude that when CD^{4+} T cells are transfected with HIV Virion Lysate, they increase IL-16 production.

When we enzymatically removed the protein components of HIV Virion Lysate, IL-16 production levels (of the CD4+ T cell supernatant) fell below the detectable range. Eliminating any other part of the HIV Virion Lysate did not significantly decrease the level of induced IL-16. This may indicate that CD4+ T cells recognize a protein segment of HIV-1 and we conclude that HIV proteins stimulate CD^{4+} T cells to produce IL-16. IL-16 is expressed in high abundance in CD4+ and CD^{8+} T cells, as well as in a larger precursor protein in some neuronal cells. In CD^{4+} T cells, antigen or mitogen has been shown to stimulate the translation of the pro-IL-16 protein, which is then cleaved by active Caspase 3 to the active IL-16 form. The pro-IL-16 molecule is translated and positioned in the perinuclear area of the cytoplasm in lymphocytes. In response to stimulus, Caspase 3 cleaves this molecule releasing the C-terminal portion (including a PDZ domain) as active IL-16. The N-terminal portion has a nuclear translocation signal which directs it into the nucleus where it subsequently induces arrest of the cell cycle in G0/G1. There appears to exist some unknown mechanism to prevent localization of the pro-IL-16 molecule to the nucleus prior to cleavage thus preventing premature cell cycle arrest. This perhaps is related to the PDZ domain in the C-terminal portion or possibly related to the size of the pro-molecule preventing localization to the nucleus. Induction of IL-16 changes signaling within the cell and appears to have antiviral mechanisms that are both intra- and intercellular.

Located in both the cytoplasm and nucleus of T cells as a pro-molecule, IL-16 has many functions. In the cytoplasm, Pro-IL-16 is cleaved by Caspase-3 and the activated IL-16 has a chemoattractant property for predominantly Th1 T cells (having binding properties to CD4 receptors and triggering the secretion of IL-2). In addition, IL-16 has been found to protect CD^{4+} T cells from activation-induced cell death. IL-16 has multiple regulatory properties on mast cells and basophils including making them less susceptible to M/R5-tropic strains of HIV-1. The cytokine also has inhibitory properties of Th2 T cells, direct inhibitory properties to HIV, and the nuclear cleaved products of pro-IL-16 induce cell cycle arrest, as mentioned above [9]. These activities may be the reason why IL-16 levels have been shown to be decreased in progression to AIDS as the viral levels increase however- further studies have demonstrated elevation of IL-16 levels in correlation with anti-protease activity (protease inhibitor therapy) and some HIV-1 long-term nonprogressor patients. Unlike protease inhibitor therapy, IL-16 levels have been observed to remain grossly unchanged after structured interruption of HAART therapy. It is possible that HIV must inhibit IL-16 to propagate, to ultimately lead to AIDS.

IL-16 direct inhibition of HIV-1 appears to have multiple possible mechanisms. The first mechanism involves IL-16 interaction with CD4 receptors. IL-16 binding to CD4 receptors was mapped to the D4 immunoglobin-like loop of the receptor-however this region is not involved in binding MHC II molecules or gp120 proteins of HIV-1 [5]. Instead, IL-16 has been found to form homodimers initially, then use disulfide bonds in conjugation to CD4 dimers to form a tetramer complex. This complex has anti-HIV activity. The tetramer may lower the level of CD4 available to interact with the co-receptor CCR5, ultimately lowering the affinity of both receptors to gp120, resulting in less viral activity. The second mechanism does not involve blocking viral entry (mediated via gp120 binding sites) but instead appears to down-regulate the long-term repeat promoter activity of HIV-1.

IL-16 has been shown to increase in the initial stages of HIV-1 infection and may represent an adequate immune response to inhibit the virus. At later stages, as the viral load increases, IL-16 levels sharply decline. However, some long-term non-progressors (without symptoms or detectable viremia) fail to show an increase in IL-16 levels, as they may lack the initial stimulus for IL-16 levels to increase, possibly viral load [18].

IL-16, as previously discussed, has properties of a CD4 ligand, inhibiting HIV-1 replication and entry into CD^{4+} T cells, monocyte-derived macrophages, and dendritic cells. IL-16 is also a chemoattractant for T cells, monocytes, and eosinophils. In parallel to its effect on IL-16, HIV-1 infection causes a decrease in the secretion of Th1 cytokines such as IL-2, IFN-gamma, but causes an increase in the secretion of Th2 cytokines such as IL-4, IL-10, and inflammatory cytokines such as TNF-alpha, IL-1, IL-6, IL-8. As a result, cytotoxic T cells do not get activated, as would normally be seen in a Th1-mediated response to an intracellular virus. HIV-1 causes this paradoxical viral response cytokines while limiting the effectiveness of cell-mediated immunity.

Of further interest is the ability of IL-16 to enhance the IL-2 receptor alpha and HLA-DR expression, as well as suppress HIV-1 promoter activity in CD^{4+} T cells. Serum levels of IL-16 initially remain normal or slightly

increased in asymptomatic patients living with HIV, but serum levels of IL-16 then undergo significant depletion as HIV infection progresses. IL-2 treatment for two weeks has been shown to dramatically increase the level of IL-16 in patients living with HIV infection [21]. IL-16 stimulates CD^{4+} T cells respond to IL-2 by up-regulating the expression of the IL-2 receptor, CD25. Co-treatment of PBMCs with IL-16 and IL-2 expands the CD^{4+} T cell population [5].

CONCLUSION

Exposure of CD^{4+} T cells to HIV Virion Lysate was found to induce IL-16 release - moreover, a protein component of the lysate was found to be the active component that induced this cytokine release. Although IL-16 levels have been found to be elevated in the early stages of HIV-1 in vivo infection, IL-16 levels decrease as viral load increases. Induction of IL-16, a known

HIV-1 inhibitor appears to run contrary to sustained HIV-1 infection. This may indicate that HIV-1 has a mechanism of blocking sustained IL-16 release to avoid the inhibitory effect of higher levels of the cytokine. Identifying this unknown protein as well as discovering the mechanism by which HIV-1 evades the IL-16 response is crucial. Further studies looking for this mechanism are paramount, as well as exploration of administration of IL-16 as a therapeutic approach to inhibition of HIV-1 in patients.

Additionally, understanding the mechanisms and patterns of cytokine dysregulation may lead to targeted therapies that include the administration or inhibition of certain cytokines to maximize the normal immune response to HIV-1. Also, prior research has shown progressive destruction of CD^{4+} T cells during HIV infection, causing loss of associated cytokines, but the extent of overall cytokine dysregulation is unclear and warrants further investigation. As further research is also looking into using immunotherapy and therapeutic vaccines to combat HIV, a combination approach with targeted cytokine modification could be beneficial as a multi-prong treatment. Comparing HIV-caused cytokine dysregulation to the response in other viral infections, such as dysregulation by West Nile Virus, may hold a clue to augmenting cell-mediated immunity to HIV-1.

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