

# Endogenous Inhibitors of HIV: Potent Anti-HIV Activity of Leukemia Inhibitory Factor

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**Abstract:** The correlates of protective immunity in HIV-1 infection include the endogenous production of compounds with anti-HIV-1 activity. These compounds can be produced independently of specific humoral or cellular immune responses. A model of compartmental inhibition of HIV-1 infection is the placenta, an organ that prevents transmission of HIV-1 to the fetus in the majority of HIV-1 pregnancies. Studies of this organ elucidated new compounds and mechanisms for prevention and treatment of HIV including the potent inhibitor of HIV-1, leukemia inhibitory factor (LIF).

Besides coordinating the humoral and cellular immune responses, cytokines such as IFN- $\gamma$  exhibit intrinsic antiviral activity that represents the first line of defense against pathogens prior to the development of a specific immune response. The study of antiviral factors is particularly important in HIV/AIDS because of the direct destruction of the immune system by HIV-1. In this report, we focus on the identification and mechanism of endogenously produced anti-HIV factors and the overall function of these factors in the prevention and treatment of HIV/AIDS.

**Key words:** HIV, LIF, cytokines, Jak, Stat, reverse transcription, transcription, placenta.

## ENDOGENOUS INHIBITORS OF HIV

Since the first description of a CD8 derived anti-HIV-1 factor [1], numerous studies have identified a role for endogenous factors in controlling HIV-1 disease. Since many of these factors remain unidentified, our laboratory has used molecular techniques to at least define the site of action for several of these factors including the recently described leukemia inhibitory factor [2-6]. Current therapies targeting the HIV-1 lifecycle inhibit viral entry, pre-reverse transcription, reverse transcription, integration, protease function, and viral budding [7-15]. Using blocking ligands or antibodies to map HIV-1 entry [4], quantitative real-time DNA PCR to detect early (LTR U5-R) and late (LTR U3-gag) reverse transcripts [2-4,6], alu-PCR to determine proviral integration [16], and quantitative real-time RTPCR for unspliced (gag-pol) and multiply spliced (tat) HIV-1 transcripts [17], we are able to determine the general site of inhibitory activity and group factors into categories based on this information (Table 1, Fig. 1). These data will be useful when these factors become part of therapeutic strategies

aimed at inhibiting multiple sites of the HIV-1 lifecycle.

## $\beta$ -Chemokines

Lymphocyte activation and chemotaxis during immune responses involve chemokines and cytokines [18], which are known to alter expression of chemokine receptors that, in general, function with CD4 as HIV-1 receptors [19-27]. Chemokines are chemoattractants for a variety of leukocytes and achieve their function through interactions with receptors that can be specific, shared, promiscuous, or viral [18]. Chemokine ligands bind to receptors in the context of a presentation molecule and migration is attained through signaling via a seven transmembrane G-coupled complex [18]. When the C-C chemokines RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  were found to inhibit entry of macrophage tropic (R5) but not T-cell tropic (X4) HIV infection of PM1 cells in vitro [28], the indelible relationship of HIV-1 infection to inflammation and immune activation was established. Stromal-derived factor (SDF-1) was identified as a ligand for LESTR/fusin [29]. SDF-1 blocks entry of HIV-1 isolates that use CXCR4 (X4), the other major HIV-1 coreceptor [19, 30, 31]. Later, RANTES and MCP-3 were found to inhibit T-tropic (X4) isolates of HIV-1 following binding of gp120 to CD4 [32]. Similarly, macrophage chemotactic protein (MDC) was also demonstrated to inhibit HIV-1 entry [33].

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Table 1. Scheme to determine the sites of anti-HIV-1 activity.

Inhibitor	Viral Entry	Reverse Transcription		Integration	Transcription		Viral Release
		Early LTR R-U5	Late-LTR U3-gag	alu-PCR	Unspliced	Multiply spliced	p24/RT
Chemokines	-	-	-	-	-	-	-
LIF	+	-	-	-	-	-	-
Influenza	+	-	-	-	-	-	-
Factor							
Allo-induced	+	-	-	-	-	-	-
Factor							
CAF	+	+	+	+	-	-	-
IL-16	+	+	+	+	-	-	-

A positive sign (+) indicates the presence of that step in the viral life-cycle and a negative sign (-) indicates an impairment of that step.

$\beta$ -chemokines are specific inhibitors of HIV-1 infection both in lymphoid tissue as well as *in vitro* cultured cells from HIV-1-infected individuals [34, 35]. The inhibitory concentrations were determined to be approximately 100 ug/mL which have been disputed to be of physiologic significance. More recently, RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  levels in blood were

not associated with either HIV clinical status or plasma viral load [36, 37]. Other studies found that  $\beta$ -chemokines affect non syncy?? induction (NSI) HIV-1 replication and influence HIV infection and pathogenesis [38]. Although the role of chemokines in inhibiting HIV-1-infection in blood and lymph nodes is well described, our preliminary data indicate

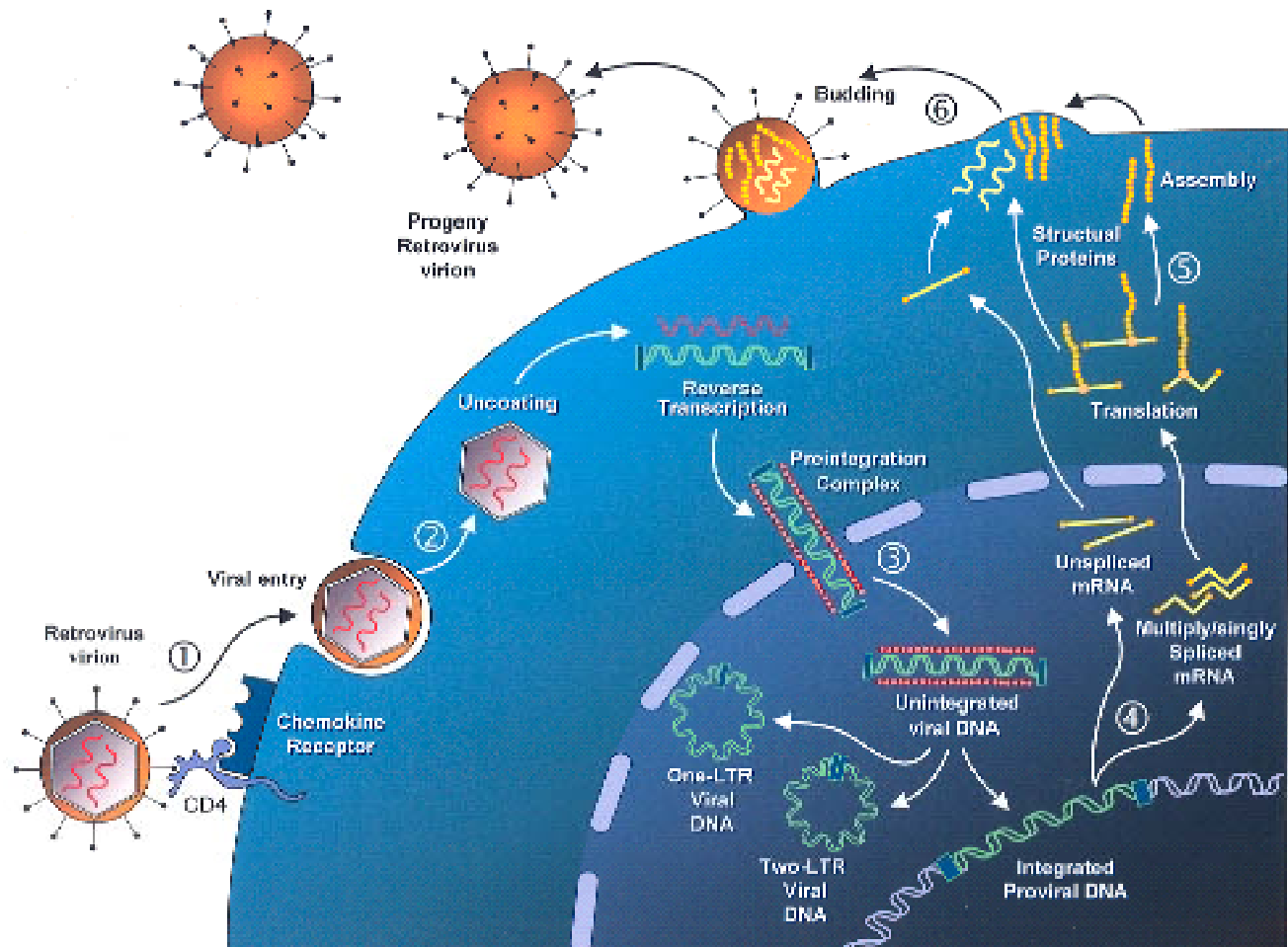


Figure 1. Schematic of the HIV-1 lifecycle with known sites of inhibition including viral entry (1), pre-reverse transcription (2), reverse transcription.integration (3), transcription (4), protein processing (5), and viral budding (6).

that RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  are weakly expressed in some tissues such as the female genital tract (data not shown) in HIV-1 seropositive women and healthy HIV-1 seronegative controls. These data suggest possible sites of deficiency in endogenous HIV inhibitory activity attributed to  $\beta$ -chemokines.

### CD8 Antiviral Factor

Shortly after the discovery that HIV-1 was the causative agent in AIDS, Walker and colleagues demonstrated that a soluble factor in CD8 T-cells suppressed viral replication [1] and was associated with long-term non-progression, favorable clinical status and increased CD4 counts [39-41]. CD8 antiviral factor (CAF) controls viral replication in a MHC class 1-unrestricted manner which is optimal when the CD8 effector cells and CD4 target cells are syngeneic [39]. CAF is active in inhibiting HIV-1 replication in primary CD4+ T-lymphocytes and CD4+ T-cells lines [42, 43]. Studies by Copeland et al suggest that CAF may have the opposite effect on HIV-1 replication in monocytes [44]. As has been demonstrated in collaboration with our laboratory, CAF inhibits HIV-1 replication at the level of transcription inhibiting the synthesis of both unspliced gag-pol transcripts and multiply spliced tat transcripts [6]. Viral entry, reverse transcription (both early and late), and proviral integration are unaffected by CAF. Preliminary studies indicate that CAF reduces the mRNA  $t_{1/2}$  for both gag and tat transcripts (B.K. Patterson, C. Mackewicz, J.A. Levy, unpublished data). This post-HIV integration site of activity makes CAF distinct from  $\beta$ -chemokines, alloantigen- and influenza-induced factors, and leukemia inhibitory factor but similar to IL-16 [13, 45]. CAF activity has been shown to be distinct from IL-16 since levels of IL-16 in CD8 T lymphocyte

supernatants does not correlate with CAF activity nor do IL-16 neutralizing antibodies abolish CAF activity [13, 45].

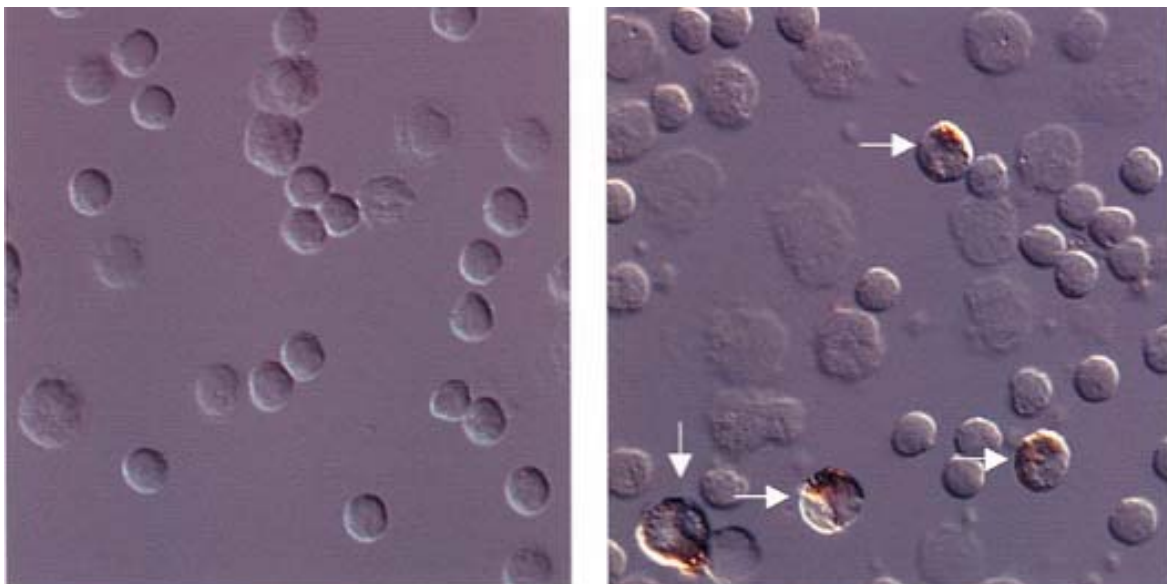
### Alloantigen/Influenza Induced Anti-HIV Factor

Collaborative studies from our laboratory have also demonstrated that alloantigen stimulation and influenza stimulation of T cells produces a factor or factors that inhibit(s) HIV-1 replication [2, 3]. Studies similar to those performed with CAF and LIF demonstrate that this allo/flu anti-HIV-1 activity can be localized to a site prior to early reverse transcription [2, 3]. This site of action is similar to IFN- $\alpha$  and IFN- $\gamma$  [46, 47], however, neutralizing antibodies to these interferons did not abrogate the anti-HIV activity of allo- or flu-induced supernatants. In subsequent experiments, supernatants from influenza-stimulated T-cells contained a 4-fold increase of LIF compared to untreated controls while supernatants from allo-stimulated T-cells demonstrated a 10-fold increase in LIF compared to controls (G. Shearer, B.K. Patterson, unpublished data). In other studies from our laboratory, allo-stimulated T-cells demonstrated a characteristic pattern of LIF expression when visualized microscopically following immunohistochemical staining for LIF (Fig. 2). Whether LIF alone accounts for the activity of allo- and flu- stimulated factors remains to be completely elucidated.

### ANTI-HIV ACTIVITY OF THE PLACENTAS A MODEL OF ENDOGENOUS RETROVIRAL INHIBITION

#### Placenta Histology Relevant to HIV-1 Infection

Many cell types are found in the placenta including fetal capillaries, placental macrophages



**Figure 2.** Peripheral blood mononuclear cells unstimulated (left) or stimulated with alloantigen (right). Alloantigen stimulation results in upregulation of LIF protein (arrows).

(Hofbauer cells) and cytotrophoblasts surrounded by syncytiotrophoblasts to form a functional subunit, the placenta villous. Maternal and fetal circulation is separated by only a few cell layers yet greatly influences HIV-1 transmission. These layers predominately consist of syncytiotrophoblasts, Hofbauer cells and loose connective tissue stroma. It remains controversial whether syncytiotrophoblasts are susceptible to HIV-1 infection although syncytiotrophoblasts are infected *in vitro* by co-culture with virus-infected maternal lymphocytes; a process enhanced by antibody [48-51]. Trophoblasts grown in primary culture were, however, not infectable by free virus but supported replication of virus introduced by transfection [52]. Since syncytiotrophoblasts seem not to be infectable by free virus, other mechanisms such as structural disruptions in the syncytiotrophoblasts, endocytosis of viral particles, or active transport of HIV-1 immune complexes via Fc or complement receptors have been suggested as mechanisms for transplacental passage of HIV-1 [53-55]. Interestingly, functional gene transfer of HIV-1 DNA by uptake of apoptotic bodies, independently of free virus and HIV-specific receptors, was demonstrated by our group [56]. This mechanism might explain transplacental passage/infection of syncytiotrophoblasts by HIV-1. Following penetrating of the trophoblast layer, HIV-1 encounters cells susceptible to HIV-1 such as lymphocytes, dendritic cells, and macrophages. Placental Hofbauer cells support HIV-1 replication of both CCR5-using (R5) and CXCR4-using (X4) isolates as well as primary isolates [57, 58].

### Potential Mechanisms of HIV-1 Inhibition in the Placenta

Many mechanisms that inhibit vertical transmission have been proposed. These mechanisms include maternal HIV-1 infected cells being destroyed by MHC-incompatible cells in the placenta [59] or by interactions with TNF-related apoptosis-inducing ligand/Apo-2L (TRAIL) [60]. Similarly, Fas ligand (FasL) expression on mouse trophoblasts has been shown to inhibit the trafficking of activated lymphocytes into the placenta [61]; an activity that could potentially protect against HIV-1-infected cells migrating into the placenta in humans. Second, selection for non-transmitted HIV-1 variants may occur in placentae from non-transmitting women [62]. Third, maternal production of HIV-1-specific neutralizing antibody or CTL may inhibit HIV-1 replication during pregnancy [63, 64]. Last, expression of soluble factors that inhibit HIV-1 replication has been previously described [65].

### Endogenous Inhibitors of HIV-1 in the Placenta

Studies in HIV-1 transgenic mice have demonstrated that the pregnancy-related hormone human chorionic gonadotropin ( $\beta$ -hCG) exerts an

HIV-1 inhibitory effect [66]. Subsequent work proposed that an unidentified factor associated with  $\beta$ -hCG inhibits HIV-1 replication whereas highly purified preparations of  $\beta$ -hCG demonstrated no HIV-1 inhibitory effects [67].

## IN VIVO ACTIVITIES OF LEUKEMIA INHIBITORY FACTOR

### Inhibition of HIV-1 by LIF and the Association between Decreased LIF Expression and Vertical Transmission

Successful pregnancy is thought to be determined at least in part by the balance of type 1 (Th1) and type 2 (Th2) cytokine responses (Fig 3) [68]. Strong Th2 responses inhibit fetal allograft rejection whereas Th1 responses are associated with recurrent abortions [69]. Progesterone, one of the hormones essential for pregnancy, promotes the development of T-helper cells that produce Th2-type cytokines such as IL-4 and IL-10 [70, 71]. IL-4, in turn, upregulates the production of LIF by T-cells. Defective production of LIF by decidual T-cells has also been linked to unexplained recurrent abortion [70, 71]. The high incidence of recurrent abortion in HIV-infected mothers [72] is consistent with a defect in IL-4, IL-10 and LIF production. We hypothesize that the immunologic milieu of the placenta is a strong determinant of maternal-fetal transmission (Fig. 3). Studies from our laboratory revealed that placentae from non-transmitting women maintained a strong Th2 cytokine milieu whereas placentae from transmitting women showed a Th1 predominance [73]. Consistent with previous reports and our hypothesis, we demonstrated a linkage between Th2 cytokine (IL-4) expression and LIF production [4]. LIF produces a potent, HIV-1-coreceptor-independent inhibition of HIV-1 replication. Further, the  $IC_{50}$  of LIF in PBMCs and in tissue explant cultures is well below the level of LIF produced by  $CD4^+$  T-cell clones derived from deciduae of normal women and 1000-fold lower than the inhibitory concentrations of the  $\beta$ -chemokines [28]. This discovery is consistent with reports that HLA discordance [59] protects against HIV-1 transmission to the fetus and our recent data demonstrating that alloantigen stimulation upregulates LIF expression. LIF has also been demonstrated to activate HIV-1 replication in chronically infected monocytic cell lines [74]. This would be an added benefit of therapies using LIF as LIF might purge HIV from latent reservoirs while inhibiting infection of new cells.

### The Jak-Stat Signal Transduction Pathway

Leukemia inhibitory factor is a ubiquitous cytokine consisting of 202 amino acids [75]. LIF is produced in bone marrow stromal cells [76], CD4 and CD8 T-cells [77] and monocytes/macrophages [78]. LIF was first described for the ability to differentiate acute

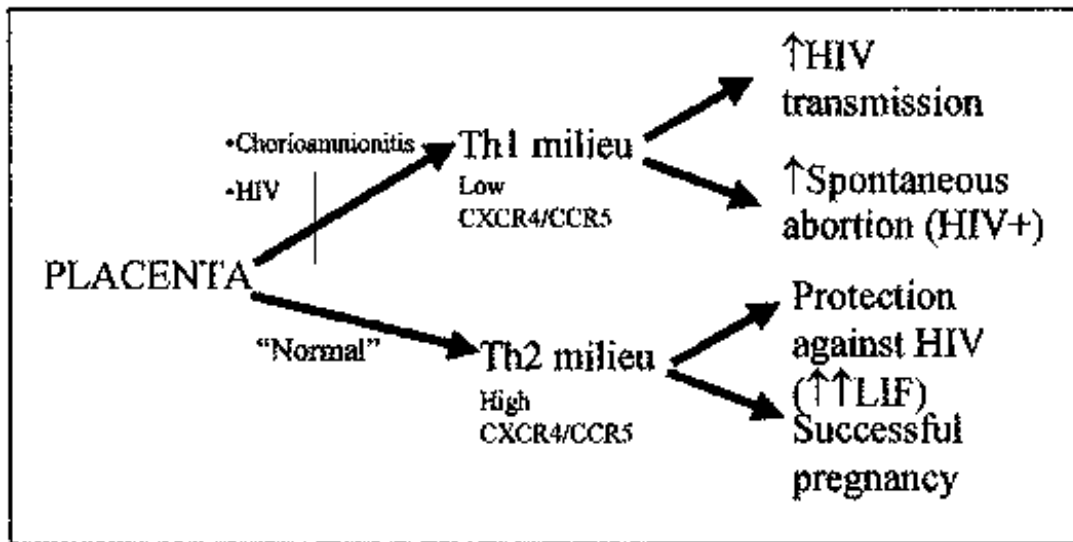


Figure 3. Anti-HIV hypothesis in the placenta as it relates to reproductive outcome.

myelogenous leukemia (AML) M1 cells [79] and was classified as a member of a cytokine family that includes ciliary neurotropic factor (CNTF), IL-6, IL-11, and oncostatin M [79, 80]. These cytokines bind to common cell surface receptor forming homo- and heterodimers that activate the Janus kinase-signal transducer and activator of transcription (Jak-Stat) signal transduction pathway (Fig. 4) [81, reviewed in 82, 83]. This pathway is common to members of the cytokine receptor superfamily including interleukins,

interferons, and other growth factors such as G-CSF and GM-CSF involved in hematopoiesis [83].

Specificity for Jak-Stat responses is extremely important as many different cytokines activate this pathway [84]. One explanation is that specificity is conferred through the recruitment and activation of different Stats through specific Src homology 2 (SH2) domains (Fig 4). Studies in knockout mice have demonstrated tremendous diversity of activities by

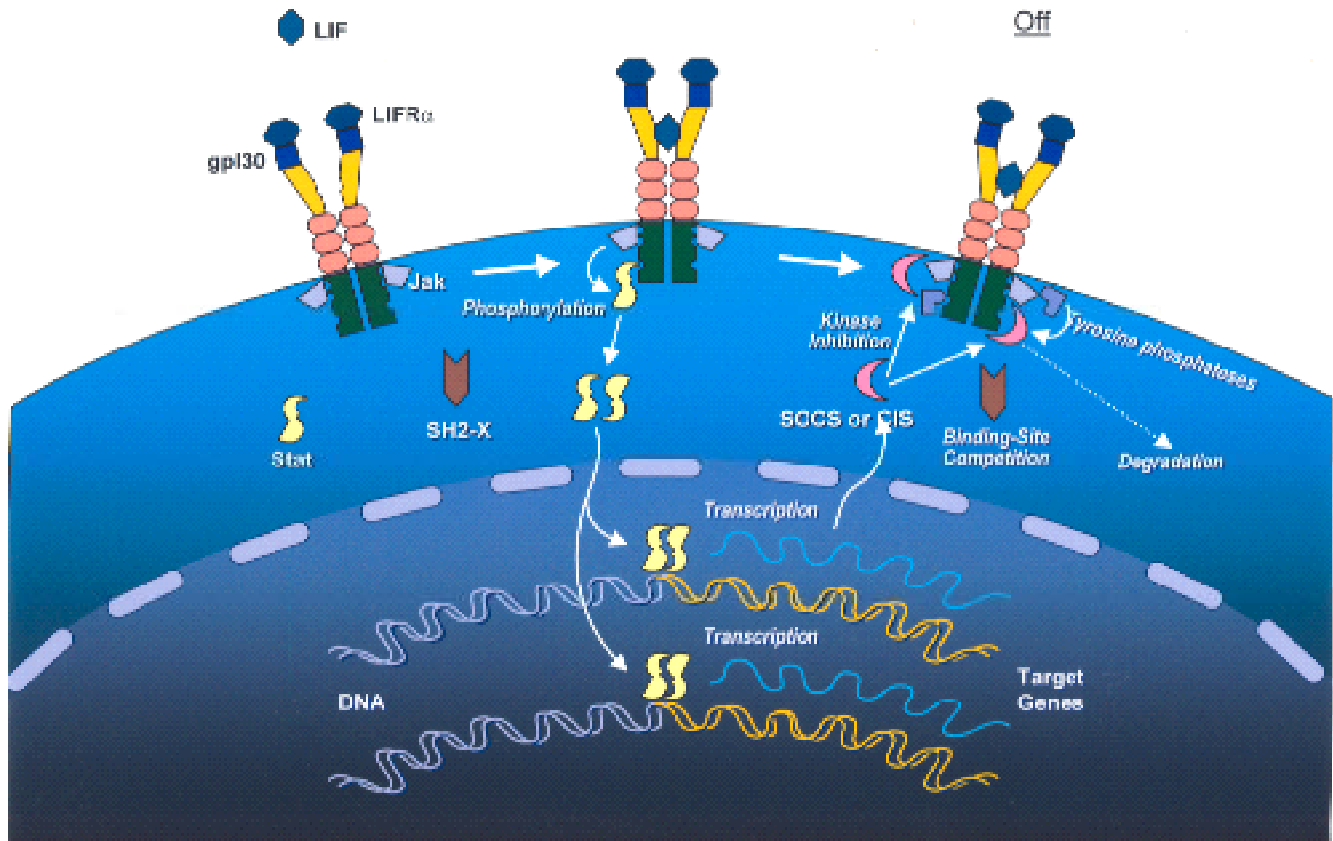


Figure 4. Schematic of cytokine (LIF)-induced Jak-Stat activation and deactivation by SOCS, CIS, and tyrosine phosphatases. Figure adapted in part from ref. 82.

the different Stats [85-91]. Stat 1 has been demonstrated to mediate antiviral and anti-bacterial immune responses generally associated with the interferon response genes while Stat 2 similarly has been shown to mediate responses to IFN- $\alpha/\beta$ . Stat 3 knockout mice demonstrated an early embryonic lethal phenotype. Stat 4, which is activated by IL-12, was shown to be required for the production of Th1 T-cells while Stat 6 was shown to be necessary for production of Th2 T-cells. Stat 5 has a role in cell proliferation and lactation.

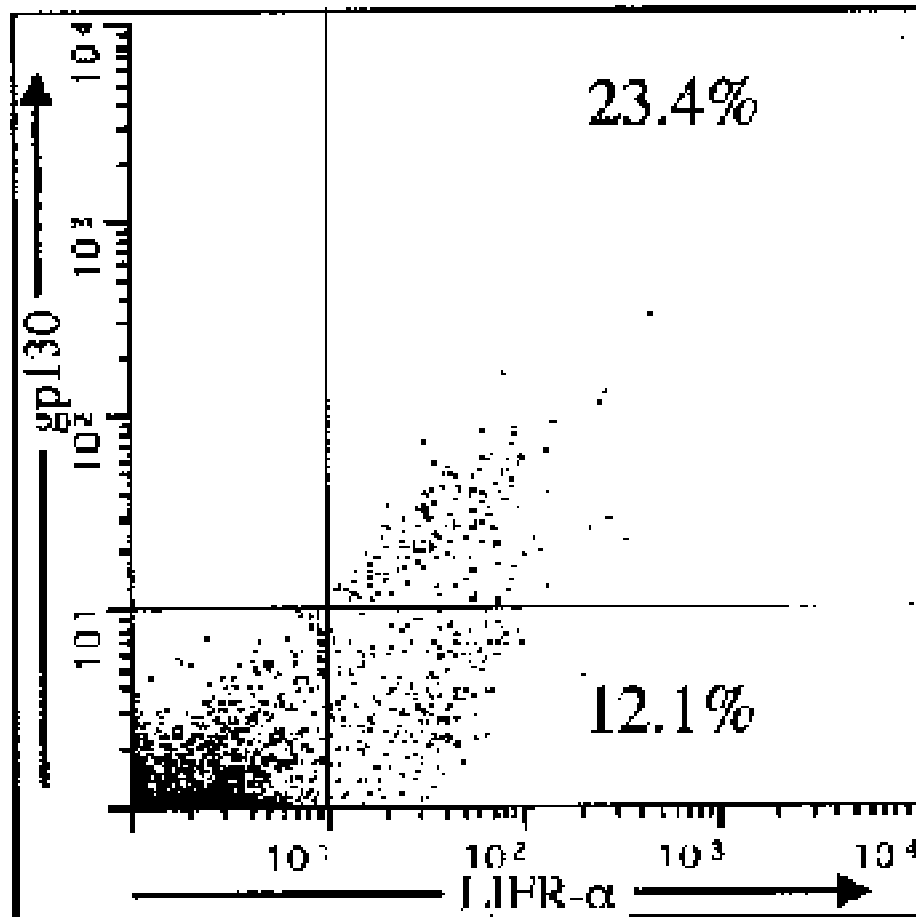
Another mechanism of Jak/Stat specificity is the binding of Stats to specific DNA response elements [92-94] (Fig 4). The formation of Stat homo- and heterodimers as well as the kinetics of Stat activation all influence the pattern of transcriptional response [84]. Further, the recognition sites for the Stats are not identical so a variety of genes are induced by different Stats and transcriptional repression can occur at specific promoters [95, 96].

Negative regulation of the Jak/Stat pathway also influences the overall response of its activation by cytokines. Suppressors of cytokine signaling (SOCS), cytokine-inducible SH2-containing protein (CIS) and

tyrosine phosphatases inhibit the tyrosine phosphorylation of tyrosine kinases including the Jak family members [97-100]. For example, Stat 3 phosphorylation and differentiation of AML M1 cells by LIF has been shown to be inhibited by SOCS-1 and SOCS-3 [100].

Last, the expression of appropriate ligand receptors on specific cell types regulates the global response of Jak/Stat activation. LIF binds to gp130 and LIFR- $\alpha$  to form a heterodimer that engages Jak 1 [101]. The LIF receptors gp130 and LIFR- $\alpha$  are found on a variety of cell types including CD4<sup>+</sup>, CD45RO<sup>+</sup> T-cells (Fig. 5) a cell type demonstrated by our laboratory to be the major productively infected cell type in vivo [102] and in thymocytes (data not shown), a critical cell type involved in immune reconstitution in HIV-1 infected individuals.

The specificity of the Jak-Stat pathway in the context of inhibiting HIV-1 replication is an important issue as other members of the LIF cytokine family such as oncostatin M, IL-11, and CNTF lack anti-HIV-1 activity while still other members of this cytokine family, namely IL-6, stimulate HIV-1 production [103].



**Figure 5.** Flow cytometry dot-plot of T-cells stained with 2-color antibodies against the LIF receptor heterodimer gp130 and LIFR- $\alpha$ . Cells expressing both gp130 and LIFR- $\alpha$  and cells expressing only LIFR- $\alpha$  were identified. Backgating revealed that expression of these cell surface receptors correlated with the activation state of the cells based on the expression of HLA-DR in separate 3- and 4-color experiments.

## MECHANISM OF LIF ANTI-HIV ACTIVITY

Interferon- $\alpha$  (IFN- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) were the first cytokines demonstrated to activate the Jak-Stat signal transduction pathway [104]. Subsequent studies showed that these interferons were potent regulators of Stat 1, a Stat now recognized to have broad antiviral activity [105]. Studies have shown that phosphorylated Stat 1 induces the transcription factor IRF-1 that has been shown to be critical for resistance from and/or inhibition to vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMCV), and Newcastle virus (NDV) [106, 107]. Recent studies have also implicated the Stat 1-IRF-1 pathway in the inhibitory effects of IFN- $\gamma$  on HIV-1 replication [105]. Another possible mechanism for inhibition of HIV-1 by IFN- $\gamma$  is the Stat 1-dependent or Stat 1-independent induction of the transcription factor ISGF3 which induces the broad anti-viral activity of 2'-5' oligoadenylate synthetase [105].

Like the interferons, LIF activates the Jak-Stat pathway. LIF, however, induces the phosphorylation of Stat 3 and to a much more limited extent Stat 1. LIF induces Stat 3 phosphorylation in HIV-1 target cells such as peripheral blood mononuclear cells (PBMCs) and in HeLa-CD4-CCR5 cells (Fig. 6). Studies are currently underway to determine the possible role of Stat 3 phosphorylation in the pre-reverse transcription site of LIF anti-HIV-1 activity. The possibility exists that the small increase in phosphorylated Stat 1 may inhibit HIV-1 in much the same way as suggested by Bovelento and colleagues [105]. A more plausible explanation would be that the phosphorylation of Stat 3 inhibits phosphorylation of the matrix protein of HIV-1, a protein that requires phosphorylation to promote translocation of the reverse transcriptase complex from the membrane to the cytoplasm and subsequently to the nucleus [108]. Another possible explanation is that phosphorylated Stat 1 or Stat 3 compete for factors or repress the promoters required for the transcription of host or viral genes involved in the migration of the HIV particle into the cell or involved in the initiation of reverse transcription such as the cytoskeleton [109]. A similar mechanism has been demonstrated in the

adenovirus system. Stat 1 binds to a domain of CREB binding protein (CBP) and p300 protein that also bind the E1A protein of adenovirus [110]. Stat 1 competition with E1A for factors such as CBP or p300 required for transcription initiation has been proposed to account for the anti-adenoviral effects of Stat 1 [82].

## CONCLUSIONS

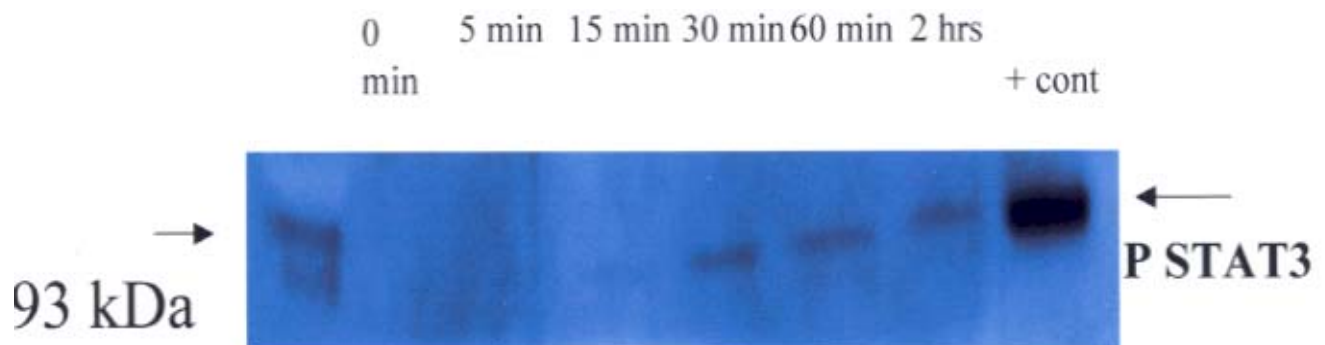
The study of endogenous anti-HIV factors such as LIF is extremely important as we attempt to expand our armamentarium of therapeutics combating the HIV/AIDS epidemic. LIF, in particular, has potent anti-HIV activity with low inhibitory concentrations in experimental models (tissue explant culture) that closely mimic human in vivo conditions. LIF (Enfilermin) has progressed through phase I/II clinical trials for post-chemotherapy neuropathy and is safe in humans at dosages that far exceed the concentrations needed for HIV-1 inhibition [4]. Similarly, strategies that naturally upregulate LIF such as treatment with influenza antigens or progesterone should be investigated as cost effective means to control HIV/AIDS. Last, study is also necessary to elucidate the exact anti-HIV mechanism in the LIF pathway to discover other potential sites for therapeutics.

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**Figure 6.** Whole cell extracts from HeLa-CD4-CCR5 cells were run on a SDS-PAGE gel and a Western blot analysis was performed using antibodies specific for phosphorylated Stat 3. Cells were stimulated with HIV-1 inhibitory concentrations of LIF (100 pg/mL) for the time periods shown.

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