**Potential for Immune Reconstitution through G-CSF Treatment of HIV Patients**

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**Abstract.** New treatment strategies for HIV/AIDS are very successful in reducing viral load. However, reconstitution of the immune system takes about one year and may be insufficient or remain incomplete. During this time the patient remains prone to opportunistic infections as a result of the complex immune dysfunction caused by the virus. Recombinant granulocyte colony-stimulating factor (G-CSF) has diverse immunomodulatory properties which may be beneficial in aiding immune reconstitution.

**Key words:** HIV; AIDS; G-CSF; immune reconstitution.

**Introduction**

Acquired immunodeficiency syndrome (AIDS), a disease that was first described in the early 1980’s, is the consequence of an infection with human immunodeficiency virus (HIV). This pandemic is still spreading very fast, especially in Africa and Asia: worldwide, 36.1 million people were living with HIV/AIDS in the year 2000, 5.3 million new infections and 3 million deaths were recorded in that year (http://www.unaids.org/wac/2000/wad00/files/WAD_epidemic_report.htm).

HIV infection results in complex immune dysfunction. Although T helper (Th) cells, which are part of the specific immune system, are the primary targets of HIV infection, cells of the non-specific immune system, such as macrophages and dendritic cells, are also infected. The numbers and functions of neutrophils, monocytes and lymphocytes are pathologically altered in the course of HIV disease. Furthermore, HIV-infected patients develop major defects in the immune signaling system, in particular the cytokine mediator system. Imbalances in the network of cytokines may ultimately trigger the immune system collapse caused by HIV. Abnormal cytokine production may explain not only the immune defects in HIV infection, but also the development of clinical abnormalities, such as cachexia, encephalitis, Kaposi sarcoma and lymphoma.

For a long time, zidovudine (AZT) and other nucleoside analogs were the only type of medication available to slow the replication of HIV. However, they were also associated with severe side effects, including myelosuppression. Recent therapeutic protocols for HIV infection have introduced cocktails of drugs that counteract de novo infection of cells (reverse transcriptase inhibitors) and prevent virus assembly in infected cells (protease inhibitors). This highly active anti-retro-
viral therapy (HAART) not only reduces viral burden drastically, but also affects T cell dynamics\(^5\). The clinical benefits of HAART are demonstrated by the resolving of opportunistic infections and malignancies, as well as declining hospitalization and mortality rates. However, while substantial increases in CD4\(^+\) cell count and functional improvement are observed in patients receiving HAART, normal values are generally still not attained after 2 years of therapy, despite sustained decreases in plasma viremia to below detectable levels\(^3\) (Fig. 1).

**Neutropenia, Neutrophil Dysfunction and Opportunistic Infections**

Neutrophils pose the first line of immune defense against invading pathogens. Studies in cancer patients receiving chemotherapy, which attacks all replicating tissue including leukocytes, have shown that the duration and depth of neutropenia is correlated with the risk of infections. This also holds true for patients with advanced HIV infection\(^6\). Until the advent of HAART, neutropenia was common in the advanced stages of AIDS (75–90%), often caused directly by the retroviral infection, by antiretroviral and other drug therapy, systemic infections, and autoimmune mechanisms\(^19, 25, 68\).

Furthermore, a number of functional defects of neutrophils have been reported for HIV-infected patients, including impaired chemotaxis, phagocytosis, oxidative metabolism, intracellular enzyme activity and killing of bacterial and fungal pathogens and HIV-infected cells, as well as accelerated apoptosis\(^19, 26, 55, 61, 70, 71, 74\).

Apoptosis and functional impairment of neutrophils may further exacerbate the characteristic underlying immune defects and, as with neutropenia, increase the risk of secondary and/or certain opportunistic infections\(^25, 71, 74\). These are, in turn, associated with increased levels of proinflammatory cytokines and concomitant viremia, a vicious cycle of virus replication and cellular activation in response to this replication\(^24, 49, 86\). Infections associated with neutropenia and neutrophil dysfunction in AIDS patients include bacteremia and fungemia, pulmonary aspergillosis, pyomyositis, malignant external otitis, neutropenic enterocolitis and pseudomonas keratitis\(^19, 45, 46, 63, 68\).

**Effects of G-CSF on Neutrophil Counts and Functions and Innate Defense**

The predominant neutrophil-stimulatory activity of G-CSF has been studied most intensively so far. In a number of settings, including various infectious diseases, both the induced neutrophilia and the augmentation of neutrophil function have been found safe and, in some cases, beneficial\(^34\). The role of G-CSF in infection and inflammation and the potential clinical uses of G-CSF compared with those of granulocyte-macrophage colony-stimulating factor (GM-CSF) are discussed in detail in\(^38, 39\).

Data from animal models suggest that G-CSF may also support the immune system in its defense against diseases caused by opportunistic pathogens which are relevant in AIDS patients. Prophylactic administration
of G-CSF before infection with *Streptococcus pneumoniae* significantly increased survival compared with placebo treatment in rats and also in splenectomized mice. This latter study was associated with decreased numbers of viable pneumococci recovered from tracheobronchial lymph nodes from G-CSF-treated mice compared with saline-treated control mice. When G-CSF treatment was initiated after experimental infection with *S. pneumoniae* in rats, survival was significantly improved over control rats. Even immunosuppressed mice which received G-CSF after experimental pulmonary infection with *Pneumocystis carinii* also had increased survival compared with control mice that received a placebo.

Also, the combination of G-CSF with antibiotics or antifungal treatment seems to be more beneficial than either treatment alone. **Lazard** et al. showed that G-CSF in combination with clarithromycin was more effective than clarithromycin alone against *Mycobacterium avium* complex-related lung and spleen infection in mice. In a mouse model of disseminated candidiasis, prophylactic or postinfection treatment with G-CSF and fluconazole resulted in increased survival and reduced kidney tissue counts of *Candida* compared with mice receiving fluconazole or G-CSF alone. The mechanisms underlying the survival benefits seen with G-CSF administration in animal models of infection are most likely multifactorial.

Functional defects of neutrophils from patients with HIV infection have been improved or corrected by *in vitro* or *in vivo* administration of G-CSF. Defective bacteria-killing capacity of neutrophils from patients with AIDS or AIDS-related complex (ARC) was restored *in vitro* as well as *ex vivo*. G-CSF also corrected the impairment of respiratory burst of *P. carinii*-stimulated neutrophils. *Ex vivo*, antifungal activity of neutrophils from HIV-infected patients was significantly enhanced after administration of G-CSF. This correlated with augmented superoxide anion and leukotriene production in response to pathogens. Accelerated spontaneous *ex vivo* apoptosis of neutrophils from AIDS patients was reduced by incubation with G-CSF. Also, cytotoxic function of neutrophils from AIDS patients against HIV-infected cells was markedly augmented by G-CSF *in vitro*.

Filgrastim treatment has been shown to reverse neutropenia in HIV-infected patients and to decrease infection and increase survival without increasing viral load. A retrospective cohort analysis found that G-CSF treatment for neutropenia was associated with prolonged survival in HIV-infected patients with disseminated *M. avium* complex infection. Increased survival was also seen in the group of patients who received clarithromycin and G-CSF versus clarithromycin alone. Furthermore, a recent study showed that regular administration of G-CSF (daily or intermittent) can actually prevent severe neutropenia in patients with HIV infection and that it reduces infection-related morbidity.

### Defects in Monocyte Function in HIV/AIDS

Both HIV-infected bone marrow stroma cells and monocytes have shown a reduced ability to produce G-CSF and other hematopoietic growth factors in response to interleukin 1α (IL-1α) or lipopolysaccharide (LPS). However, secretion of proinflammatory cytokines (IL-1β, IL-6, IL-8 and tumor necrosis factor α–TNF-α) was upregulated in comparison with the response of uninfected control cells. As there was no difference in the frequency or intensity of cell-associated immunocytotoxic cytokine staining between HIV and mock-infected monocyte-derived macrophages, the altered cytokine release seems to result not from dysregulated productive, but rather from defective secretory activity. TNF-α and interferon γ (IFN-γ) have also been shown to suppress proliferation of bone marrow cells and, like IL-6, enhance HIV replication *in vitro*. This shift in the cytokine pattern may contribute to the pathogenesis of HIV-mediated disease, e.g. by exacerbating tissue damage associated with opportunistic infections.

The release of high levels of these proinflammatory cytokines by isolated peripheral blood cells and tissue macrophages from HIV-infected patients cultured *in vitro* was observed in many, though not all, studies, reviewed in. However, *ex vivo* LPS-inducible monokine release in whole blood from HIV-infected patients did not differ from that of normal or high-risk volunteers.

Prolonged periods of afebrile neutropenia in HIV-seropositive individuals did not induce an increase in G-CSF serum levels, which is the normal, adequate response seen in HIV-negative neutropenic patients. However, elevated serum levels of proinflammatory cytokines were detected in HIV-infected patients in some studies, though one study reported normal concentrations of cytokines in serum, discussed in. Chronic production of monokines over the time span of many years may be responsible for clinical manifestations such as cachexia, as demonstrated by the anticausal effect resulting from administration of anti-IL-6 monoclonal antibodies to HIV-infected patients with lymphoma.
Anti-Inflammatory Effects of G-CSF Might Limit HIV Progression

Monocytes express G-CSF receptors and respond to G-CSF in an anti-inflammatory manner. G-CSF administered to healthy volunteers reduced the release of proinflammatory TNF-α, IL-1β and IL-12 per monocyte in response to LPS stimulation of whole blood ex vivo. Furthermore, the release of the anti-inflammatory soluble TNF receptors I and II as well as IL-1 receptor antagonist (IL-1ra) was augmented. The reduction in TNF-α and IL-12 release by monocytes by G-CSF directly results in less activation of lymphocytes to produce the proinflammatory cytokine IFN-γ.

Both HIV itself and opportunistic infections overbalance cytokine regulation towards proinflammatory cytokine production together with diminished growth factor response, as described above. This suggests that supportive therapy with a safe and well-tolerated hematopoietic growth factor such as G-CSF, which has anti-inflammatory properties, might help to rebalance cytokine relations and prevent infections by strengthening the nonspecific defense system, as well as ameliorating morbidity associated with proinflammatory cytokines and breaking the vicious cycle of immune activation and virus replication (Fig. 2). Although the suppressive effect of G-CSF added in vitro on LPS-inducible TNF-α and IFN-γ formation in whole blood of normal volunteers was not found in blood of HIV-infected patients, this may be different when G-CSF is administered in vivo to HIV-infected patients.

Fig. 2. Putative effects of G-CSF on the innate immune system and on HIV replication

Lymphopenia and Lymphocyte Dysfunction in HIV/AIDS

In contrast to CD4+ T lymphocyte depletion, the hallmark of HIV infection, a pronounced expansion of CD8+ T lymphocytes occurs early on during HIV infection. Anti-viral cytotoxic T lymphocytes (CTL) constitute the strongest mechanism by which the immune system can partially control HIV spread during the latency period by two mechanisms: killing of infected cells, e.g. via perforin and granzymes, and releasing soluble mediators which interfere with viral replication, e.g. RANTES, macrophage inflammatory protein 1α and 1β. Paradoxically, however, the CD8+ population becomes unresponsive to T cell signaling in vitro and displays decreased in vitro cloning potential in the course of HIV infection. These findings suggest an increase in anergic or apoptotic CD8+ T cells in HIV-infected persons.

The numbers of both naive and memory CD4+ and CD8+ T cells, including the HIV-specific CTL precursors, decline in the blood during HIV disease progression, reflecting alterations in T cell regeneration capacity and virus-mediated cell death. The loss of memory (specific) CD4+ T cell reactivity against recall antigens, which is an early event in HIV disease progression, results in increased susceptibility to opportunistic infections and tumors. Primary responses of naive CD4+ cells towards new pathogens are suppressed even earlier in the course of HIV disease.

Furthermore, the progression of HIV disease is characterized by a major T cell activation, as assessed by cell surface expression of various T cell activation markers such as CD25 (the α chain of IL-2 receptor), CD38 or HLA-DR, on both CD4+ and CD8+ T cells. Abnormalities in the activation and functional states of B cells, macrophages and dendritic cells have also been observed.

Based on their release of different combinations of lymphokines, the Th cells have been grouped into Th1 and Th2 cells. The Th1 cytokines, IL-2 and IFN-γ, are involved in stimulating the “cell-mediated responses” that help the host eliminate cells infected with pathogens. In contrast, Th2 cytokines, such as IL-4, IL-10 and others, activate antibody production. Attenuated production of IL-2 in advanced HIV infection appears to play a major role in the loss of Th cells. IL-2 is considered to be the primary T cell growth factor. In addition to stimulating the proliferation of CD4+ and CD8+ lymphocytes in response to antigen activation, IL-2 also enhances cytolytic and oncolytic activity of suppressor cells, augments natural killer cell activity, and increases IFN-γ production. Moreover, IL-2 formation by CD4+ T cells appears to be mandatory in maintaining CD8+ T cell control of virus replication. A reduction of Th1 type lymphokine (i.e. primarily IL-2) and concomitant increase of Th2 type lymphokine...
(such as IL-4 and IL-10) production in response to mitogens, such as phytohemagglutinin (PHA), alloantigens, tetanus toxoid, etc., was partially or completely restored certain defective cellular immune responses, including the proliferative response to antigen stimulation, and reduced the spontaneous and activation-induced programmed cell death, but was also associated with increases in HIV replication and spread. The addition of IL-2 in vitro to cultures of peripheral blood mononuclear cells (PBMC) from HIV-infected individuals initiated a polyclonal expansion of CD4+ and CD8+ cells and partially or completely restored certain defective cellular immune responses, including the proliferative response to antigen stimulation, and reduced the spontaneous and activation-induced programmed cell death, but was also associated with increases in HIV replication and spread. Treatment of CD8+ cells from long-term survivors with the Th1 cytokine IL-2 enhanced their anti-HIV activity, whereas exposure of these cells to the Th2 cytokines IL-4 or IL-10 reduced their ability to suppress HIV replication and to produce IL-2.

In a number of clinical trials, the administration of antiretroviral agents plus IL-2 produced either transient (less than 1 year) or sustained (1–3 year) increases in CD4+ T cell numbers in asymptomatic HIV-infected individuals. Among the functional responses reported to be increased during and, in some cases, after cessation of IL-2 treatment were natural killer, delayed type hypersensitivity, major histocompatibility complex (MHC)-restricted HIV-specific cytototoxicity, and in vitro proliferative responses to IL-2 and mitogen. Increases in numbers of eosinophils, CD8+ and CD16+ cells have also been reported. Transient increases in the expression of activation markers (CD38 and HLA-DR) were reported for both CD4+ and CD8+ cells, and an increase of CD25 (α chain of the IL-2 receptor) expression was sustained for months after discontinuation of IL-2 therapy. Sustained increases in viral burden were observed only in HIV-infected individuals with <200 CD4+ T cells/ml, also reviewed in. Although IL-2 treatment seems to hold some benefit for HIV-infected patients, one must consider that IL-2 induces clonal proliferation of lymphocytes in the periphery and not production of new lymphocytes in the bone marrow and thymus. In this sense, it risks inducing the replication of infected cells without promising to patch up the holes in the specific immune system by increasing the diversity of healthy lymphocytes.

**Effects of G-CSF on Lymphocyte Counts and Functions**

We conducted a study where 24 healthy volunteers were randomized to receive G-CSF (filgrastim 75, 150, 300 µg/d) or a placebo for 12 days. Daily blood samples were assayed for IL-2 production and lymphocyte proliferation in response to anti-CD3 antibodies (OKT-3), PHA, or staphylococcal enterotoxin B (SEB). Volunteers receiving filgrastim showed a transient dose-dependent lymphocytosis with a doubling of CD4+ cells by day 8. Twenty four hours after the first injection of filgrastim, ex vivo IL-2 release by OKT-3-stimulated PBMC was increased by 110% in the group receiving 300 µg filgrastim. Lymphocyte proliferation was augmented in response to PHA or OKT-3, though not to SEB, by 50% 24 h after the first injection of filgrastim. At later time points, however, proliferation was suppressed in comparison to placebo values. A suppression of lymphocyte proliferation was also described when serum from healthy volunteers receiving G-CSF was incubated with allogeneic lymphocytes stimulated with PHA and when PBMC from G-CSF-treated breast cancer patients were stimulated with the mitogens PHA, anti-CD3 and concanavalin A. In the latter study, a tendency towards a decreased proliferative response was already noted 24 h after the first injection of G-CSF which was more pronounced (p ≤ 0.05) after 4 days of treatment. No changes in IL-2 production were observed in either of these two studies.

The observation that both the numbers and the proliferative response of lymphocytes were initially augmented but then decreased under prolonged G-CSF therapy suggested that intermittent administration of G-CSF might be more effective in boosting this activity to stable, higher levels. Therefore, we treated 4 groups of 6 healthy volunteers each with different regimens of filgrastim over the time period of 8 weeks. One group received saline only, one group received 300 µg filgrastim once per week, another group was given filgrastim 3 times per week on alternate days, and the last group received filgrastim for 5 consecutive days in week 1 and week 5, otherwise saline. An increase in lymphocyte counts could only be attained by daily injection of filgrastim, not by intermittent treatment. We found that the doubling of lymphocyte counts in the group of healthy volunteers that received filgrastim for 5 consecutive days resulted from proportionate increases in Th, cytotoxic T, natural killer (NK) and B cells, suggesting that G-CSF promotes the production of new cells in the bone marrow, not lymphocyte proliferation in the periphery. The expression of activation or proliferation
markers on T cells was unchanged or slightly decreased and the proliferative response and IL-2 production of isolated mononuclear cells ex vivo were unchanged, thus supporting this hypothesis. Lymphocyte counts had returned to baseline by the next measurement 1 week later. Further studies are required for the adaptation of treatment regimens and doses originally established for neutropenic cancer patients after chemotherapy to the requirements of non-neutropenic HIV patients. Perhaps cyclic treatment schedules, where G-CSF is administered for e.g. 5 consecutive days followed by e.g. 2 days break, or treatment with the new longer-lasting form of G-CSF currently being tested in clinical trials, may offer an effective strategy for maximizing the increase in lymphocyte counts by G-CSF treatment.

In a study conducted by our group, blood samples from 31 HIV-infected patients as well as normal volunteers and 8 subjects at high risk for HIV infection due to intravenous drug use were collected and stimulated with SEB in the presence or absence of G-CSF in vitro. Whole blood from patients with advanced HIV infection showed reduced IL-2 release in the presence of SEB, which was partially restored in the presence of G-CSF.

The phenotype and functional capacity of progenitor cells mobilized by G-CSF were compared with those of unprimed progenitor cells collected from the same patients prior to treatment. Ten HIV patients received 300 µg filgrastim per day for 5 consecutive days. The absolute numbers of immature and T cell progenitors did not increase and the mean number of lymphocytes generated per CD34+ cell in a thymic organ culture decreased. Also, the number of CD4+ cells generated per CD34+ cell was significantly reduced after G-CSF treatment.

Although studies of G-CSF effects in patients with HIV have previously focused on changes in neutrophil levels, an approximate doubling of total lymphocyte count, CD4+ and CD8+ cell numbers, augmented lymphocyte proliferative response, and increased bone marrow cellularity have been reported following the daily use of escalating concentrations of G-CSF alone or in combination with erythropoietin in neutropenic and anemic HIV-infected patients. Although opportunistic infections occurred in 14 of the 22 patients, these were treated successfully with myelosuppressive antimicrobial agents without development of neutropenia. Several other studies have also demonstrated increases in lymphocyte, NK cell, CD4+ and CD8+ counts in neutropenic HIV patients under daily or intermittent (thrice weekly 5 µg/kg subcutaneous) G-CSF therapy. However, in another study with twelve neutropenic patients with AIDS or ARC who received daily subcutaneous injections of G-CSF in a weekly increasing dose (0.4–10 µg/kg) with 4 weeks of subsequent maintenance therapy, neutrophil and monocyte counts increased, but no effects on lymphocyte counts were observed.

In a recent randomized, double blind, placebo-controlled study, filgrastim was given to 30 HIV-infected patients who had been on HAART for at least 6 months but still had a CD4+ lymphocyte count smaller than 350/µl. The patients received either 300 µg filgrastim thrice weekly for 12 weeks or a placebo. A significant increase in absolute numbers of circulating CD34+ cells as well as their colony-forming unit capacity was detected in the treatment group. G-CSF treatment enhanced the total lymphocyte count and increased CD3+, CD4+ and CD8+ T cell counts as well as natural killer cell numbers. The increases in CD4+ and CD8+ cells resulted from increases in CD45RO memory T cells and cells expressing the activation marker CD38. There was no correlation between the numbers of progenitors and the CD4 count. There were also no significant changes in the expression of activation markers CD25 or CD69. Lymphocyte proliferative responses to PHA and Candida antigen decreased, whereas NK cell activity and plasma HIV RNA did not change. Also, there was no change in the mean telomere length in PBMC. All cell counts and immunological parameters had returned to baseline values 12 weeks after cessation of treatment.

In a follow-up study, treatment-naive HIV-infected patients were randomized to receive either a placebo or filgrastim (300 µg, 3 times a week) for 12 weeks and HAART simultaneously. The study was terminated prematurely because one patient developed severe encephalopathy. Until then, 6 patients had been treated with G-CSF and 5 with placebo. CD4+ memory and CD8+ naive and memory T cells increased in response to HAART and there was a trend towards more pronounced increases in the G-CSF group. NK cells increased significantly more in the G-CSF group. However, plasma HIV RNA decrease was less pronounced in the group that received G-CSF and rebounded in 2 patients of the G-CSF group despite compliance with HAART drugs, although it cannot be determined whether this was due to the G-CSF treatment or resistance development. Further investigations of these patients also showed that, although the telomere lengths of PBMC from patients on HAART alone increased, this was not observed in the group that received G-CSF in addition to HAART.

The analysis of the types and capacity of progenitors
recruited by G-CSF and of the telomere lengths in PBMC have indicated that G-CSF may induce production of new lymphocytes, redistribution of lymphocytes from the lymphoreticular system, and proliferation of peripheral lymphocytes. However, the mechanisms by which these diverse actions might be achieved are unknown.

Summary and Outlook

The advent of HAART has revolutionized HIV/AIDS therapy and concomitantly the prospects of HIV-infected patients. Still, our knowledge of the changes in the immune status under HAART is limited. The main factors that are monitored are HIV RNA plasma levels and CD4⁺ cell counts, sometimes also some lymphocyte functions such as the reaction to recall antigens. The innate immune system is the first line of defense against infections. However, we are not aware of studies which additionally monitored the change of status of the innate immune system under HAART, i.e. neutrophil functions and cytokine release. Such data are necessary for a clear definition of the requirements to be fulfilled by adjuvant therapies. Patients who do not respond to HAART or who develop resistance to the drugs should be evaluated separately in their requirements for immune support.

Building on the information available, G-CSF seems a good candidate to aid immune reconstitution. Benefits to patients might include:

- prevention of neutropenia due to HIV infection or myelosuppressive drugs; substitution of impaired G-\textsuperscript{1}CSF production,
- improved defense against opportunistic bacterial and fungal infections by induction of neutrophilia, improvement of neutrophil functions, balancing and strengthening of the non-specific immune system,
- increases in lymphocyte counts, including recovery of CD4⁺ cell counts, and protection against opportunistic diseases by reconstitution of the specific immune system,
- restoration of IL-2 formation and lymphocyte proliferation in response to antigens and mitogens,
- attenuation of virus replication by reduction of pro-inflammatory cytokine release.

To investigate the potential of G-CSF as an adjuvant therapy for immune reconstitution in HIV patients, tailoring of the dosage, application regimen and timing in relation to the onset of HAART drugs is still outstanding. The longer-lasting form of G-CSF may offer new possibilities in this respect. So far, treatment regimens of G-CSF were designed to overcome neutropenia, but despite this focus, promising effects as to a broader immune reconstitution were observed. There findings call for clinical studies exploiting the immunomodulatory properties of G-CSF on various leukocyte subsets. In such trials a comprehensive immune status analysis should be performed to evaluate the effects of G-CSF on both the acquired and the innate immune system, e.g. response of monocytes and neutrophils to bacterial stimuli. Only with all this information can a clear evaluation of G-CSF administration in these settings be made. G-CSF treatment in HIV patients might prove to have impact far beyond fighting neutropenia.

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