HIV and Other Lentiviral Infections Cause Defects in Neutrophil Chemotaxis, Recruitment, and Cell Structure: Immunorestorative Effects of Granulocyte-Macrophage Colony-Stimulating Factor

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HIV and Other Lentiviral Infections Cause Defects in Neutrophil Chemotaxis, Recruitment, and Cell Structure: Immunorestorative Effects of Granulocyte-Macrophage Colony-Stimulating Factor

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Patients with HIV infection exhibit deficits in bacterial and fungal clearance, and possibly depressed innate immunity. In this study, we observed that neutrophils from HIV-infected patients have a profound defect in chemotaxis in response to endogenous (IL-8) and bacterial (FMLP) chemoattractants, which was directly correlated with peripheral CD4⁺ lymphocyte levels but not plasma viral load. A similar chemotactic defect was observed in the feline immunodeficiency virus (FIV) model of HIV infection. Intravital microscopy of FIV-infected animals revealed marked impairment in the in vivo recruitment of leukocytes; specifically integrin-dependent neutrophil adhesion and emigration induced by bacterial products. Treatment of FIV-infected animals with GM-CSF re-established both neutrophil recruitment (rolling, adhesion, and emigration) and in vitro chemotaxis to the levels seen in uninfected animals. This restoration of neutrophil responses was not due to GM-CSF-mediated priming. Rather, HIV and FIV infections resulted in defective neutrophil development, with an ensuing reduction in neutrophil granularity and chemotactic receptor expression. GM-CSF therapy restored neutrophil granularity, implying restoration of normal neutrophil development. Together, our findings underscore the fundamental defects in innate immunity caused by lentivirus infections, while also indicating that GM-CSF may be a potential immunorestorative therapy for HIV-infected patients. The Journal of Immunology, 2006, 177: 6405–6414.

Neutropenia and neutrophil defects are common complications of infection by the lentivirus, HIV-1 (1–3). Although HIV does not infect neutrophils (4), HIV-infected patients, particularly those with AIDS, frequently exhibit a variety of neutrophil and monocyte/macrophage defects resulting in impaired bacterial and fungal killing (5–10). These defects are often present without any evidence of neutropenia or other leukopenias. A direct consequence of these defects is increased susceptibility to Gram-negative, Gram-positive, and fungal infections. The underlying mechanism for this increased permissiveness to bacterial and fungal infections is poorly understood, but could be related to some developmental defects in neutrophils and other myeloid cells during hemopoiesis. Potential mechanisms resulting in the formation of defective cells include HIV infection of bone marrow stromal macrophage and resident CD4⁺ cells (11), altered cytokine milieu in the bone marrow (12), direct suppression of hemopoiesis by viral proteins (13, 14), myelosuppressive effects of antiretroviral drugs (15–18), infection of the bone marrow by viruses, bacteria or fungi (19, 20), and possibly even HIV infection of hemopoietic stem cells (21). Some form of general therapy, such as the use of hemopoietic factors that promote the formation of myeloid cells, may be of benefit in restoring normal innate immunity.

CSFs, such as GM-CSF, are cytokine-like molecules that have a diverse range of biological roles. GM-CSF is a supportive factor in the hemopoiesis of neutrophils and macrophages (22). GM-CSF also plays a central role in the inflammatory response, as evidenced by the GM-CSF knockout mice, which have profound defects in their inflammatory responses and are highly susceptible to pulmonary bacterial and fungal infections (23). In addition, GM-CSF has been shown to act as a neutrophil-priming factor, which allows it to amplify inflammatory responses in the periphery (24–26). These diverse biological qualities make GM-CSF an attractive therapeutic option for treatment of HIV/AIDS. Its role as a hemopoietic factor may allow for correction of hemopoietic defects, whereas its effects in the periphery may allow for enhanced function of neutrophils and other phagocytes (5). GM-CSF has been shown to be an effective treatment for HIV-induced neutropenia and HIV-associated infections, but it is unknown whether GM-CSF resolves neutrophil defects in nonneutropenic HIV patients (2, 3, 27–29).

In this study, we used both samples taken from HIV patients, as well as the feline immunodeficiency virus (FIV) model of HIV

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3Abbreviations used in this paper: FIV, feline immunodeficiency virus; SMA, superior mesenteric artery.

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infection (30–32), FIV shares many characteristics with HIV including similar env, gag, and pol genes (33), neurovirulence (34, 35), the use of CXCR4 and CCR5 as coreceptors (36, 37), high viral burden, depletion of circulating CD4+ and CD8+ lymphocytes, and immunosuppression (38, 39). Although FIV is a useful model of HIV infection, and replicates many aspects of HIV infection, there are some notable differences between HIV and FIV; notably, the use of CD134 instead of CD4 as FIV’s primary receptor (40, 41), some differences in regulatory genes (42), and a broader cell tropism (43, 44).

In this study, we investigated the effects of GM-CSF therapy on HIV-mediated neutrophil defects. Using an established lentivirus animal model of HIV/AIDS, FIV (30–32), as well as neutrophils isolated from HIV-infected patients, we identified a consistent and profound defect in the recruitment and chemotaxis of neutrophils to both endogenous chemokines and to bacterial inflammatory stimuli. This defect correlated strongly with the patients’ peripheral CD4+ lymphocyte levels but not plasma viral load, suggesting that this defect is not a direct effect of the virus or viral proteins. GM-CSF treatment of FIV-infected animals, at levels, which had no effect on neutrophils from uninfected animals, was shown to result in a dramatic improvement in neutrophil recruitment and chemotaxis in response to all mediators tested. Interestingly, this improvement appeared to be mediated entirely through corrected neutrophil development, and not through peripheral effects such as priming. Together, our data suggest that GM-CSF therapy may represent a novel supplemental HIV therapy that can be used to enhance antibacterial immunity in HIV/AIDS patients with normal neutrophil counts.

Materials and Methods

Patient blood collection

HIV-1-seropositive patients were recruited from the Southern Alberta Clinic (Calgary, Alberta, Canada), with consent based on approval by the University of Calgary Ethics Committee with no linkage between identity and clinical sample. Age, sex, viral load, CD4 counts, CD8 counts, anti-retroviral regimen, past opportunistic infections, and duration of antiretroviral treatment were not used as selection criteria unless otherwise noted. Neutropenic patients and patients with ongoing opportunistic infections were excluded from the study. A total of 85.2% of patients was on anti-retroviral therapy at the time of the study, with an average treatment duration of 1471 ± 324 days. Note that a viral load of 0 represent either HIV-positive individuals with viral loads below detection, or uninfected controls.

Animals and virus inoculation

Adult specific pathogen-free pregnant cats were obtained from an established breeding colony maintained by the University of Calgary and housed according to University of Calgary Animal Resource Center guidelines. All females were found to be negative for feline retroviruses by PCR analysis according to University of Calgary Animal Resource Committee with no linkage between identity and clinical sample. After birth, all kittens were housed in a temperature-controlled facility (32°C) with a 12-h light/dark cycle. All animals were weaned at 6 wk and were monitored for survival and physical health. The animals were characterized for age, sex, and neutrophil count prior to and immediately following inoculation.

Flow cytometry

Whole blood was collected in K3 EDTA tubes and incubated for 5 min at room temperature with four parts ammonium chloride lysing solution (17 mM NH4Cl, 100 mM KHCO3, and 0.1 mM EDTA) to lyse erythrocytes. Feline PBMCs were pelleted by centrifugation, resuspended in PBS containing 0.1% sodium azide (1 × 106 cells), and incubated for 20 min at room temperature with anti-CD4 or CD8 mAb (3 μg/ml) provided by Dr. P. Moore, University of California, Davis, CA). Cells were then washed, incubated for 20 min with FITC-conjugated goat anti-mouse IgG (0.25 μg/ml; BD Biosciences), and resuspended in 0.5 ml of 1% formalin in PBS for analysis. Human leukocytes were pelleted by centrifugation, resuspended in PBS, and incubated for 20 min at room temperature with primary-labeled anti-CXCR1, anti-CXCR2, anti-CD18, or anti-CD11a (100 μl/106 cells; BD Pharmingen). Using a FACScan flow cytometer (BD Biosciences) with the argon laser excitation set at 488 nm, data were collected from ~15,000 events for each experimental condition, and results were expressed as a single-parameter log fluorescence histogram. Cells incubated with the absence of Ab or with 1 μg/ml FITC-labeled, isotype-matched murine IgG1 (BD Biosciences) served as controls.

Real-time RT-PCR measurement of plasma viral load

A quantitative real-time RT-PCR protocol using primers that detect the FIV pol gene from multiple strains was used to determine the number of copies of viral RNA per milliliter of plasma. Briefly, to obtain a standard curve, in vitro RNA transcripts of the FIV pol region were generated from PBS-FIV pol (32). A standard curve was generated with serial 10-fold dilutions (106 copies/μl to 1 copy/μl) of the in vitro-transcribed RNA. Subsequently, the transcripts were treated with RNAse-free DNaI (Promega) and reverse transcribed using Superscript II and random N6 oligomers to prime cDNA synthesis (Inviron Technologies). Total RNA was extracted by TRIzol (Inviron Life Technologies) from plasma harvested at each time point, and subsequently cDNA synthesis was performed with the cDNA was then amplified with a specific CD134 primer and using an Applied Biosystems 7900HT Fast Real-time PCR system (Bio-Rad). Viral loads in plasma were expressed as copies per milliliter of plasma.

Intravital microscopic studies

The surgical preparation used in this study is the same as described previously (45, 46). Briefly, age-matched uninfected (control) and FIV-infected cats (1.2–2.4 kg) were fasted for 24 h and initially anesthetized with ketamine hydrochloride (75 mg·l, m.). The jugular vein was cannulated, and anesthesia was maintained by the administration of pentobarbital sodium. A tracheotomy was performed to support breathing by artificial ventilation. Systemic arterial pressure was monitored by a pressure transducer (Statham P23A; Gould), connected to a catheter in the left carotid artery. Body temperature was maintained at 37°C using an infrared heat lamp. Heparin sodium (10,000 U; Elkins-Sinn) was administered when an arterial circuit was established between the superior mesenteric artery (SMA) and the left femoral artery. SMA blood flow was continuously monitored using an electromagnetic flow meter (Carolina Medical Electronics). Blood pressures were continuously recorded with a physiological recorder (Grass Instruments).

Animals were placed in a supine position on an adjustable Plexiglas microscope stage, and a segment of midjejunum was exteriorized through the abdominal incision. The mesentery was prepared for in vivo microscopic observation as described previously (45, 46). The mesentery was draped over an optically clear viewing pedestal and transilluminated with white light. The temperature of the pedestal was maintained at 37°C with a constant temperature circulator (model 80; Fisher Scientific). The exposed bowel was draped with saline soaked gauze, while the remainder of the mesentery was covered with Saran Wrap (Dow Corning). The exposed mesentery was suffused with 5% CO2/95% N2, bubbled, warmed bicarbonate-buffered saline (pH 7.4). The mesenteric preparation was observed through an intravital microscope (Optiphot-2; Nikon) with a ×25/0.35NA objective lens (Leitz). The image of the microcirculatory bed (magnification, ×1400) was recorded using a video camera (Digital Sti100, Panasonic) and a video recorder (NYV950, Panasonic).

Single unbranched mesenteric venules (25- to 40-μm diameter, 250-μm length) were selected for each study. Venular diameter was measured using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). The number of rolling and adherent leukocytes was quantified using playback analysis. Rolling leukocytes were defined as white blood cells that moved at a velocity less than that of erythrocytes in a given vessel. The number of rolling leukocytes (flux) was counted using frame-by-frame analysis, with the rolling velocity of all leukocytes being recorded to obtain a complete leukocyte rolling velocity profile. A leukocyte was defined as adherent to venular endothelium if it remained stationary for >30 s. Adherent cells were measured at 10-min intervals and were expressed as the number per 100-μm length of venule. RBC velocity was measured using an optical Doppler velocimeter (Microcirculation Research Institute).

Experimental protocol: in vivo experiments

Animals were infected with FIV for 12 wk before initiation of GM-CSF treatment. Before the first GM-CSF injection, 2.5 ml of blood was drawn.
from all animals to determine baseline hemopoietic parameters, and to perform an in vitro chemotactic assay. A total of 1000 μg/ml GM-CSF was then injected s.c. (day 1), and identical injections were given on days 3 and 5. On day 6, another 2.5 ml of blood sample was taken for measurement of hemopoietic and chemotactic parameters, and then intravital microscopy was performed. Baseline measurements of blood pressure, SMA blood flow, RBC velocity, and vessel diameter were obtained, and neutrophil-endothelial cell interactions were determined. Endotoxin (Escherichia coli, 1.0 μg/ml) was then superfused over the exteriorized mesentery for 4 h. This prevented any systemic, hemodynamic disturbances and permitted the direct assessment of neutrophil-endothelial cell interactions in FIV-infected animals. The microvasculature was videotaped for the last 10 min of every hour. In previous work, we established that the LPS-induced neutrophil rolling was selectin-dependent, and the adhesion was dependent upon neutrophil integrins (47, 48).

**Neutrophil isolation and preparation**

Human and feline neutrophils were isolated using the same technique. Either 2.5 ml of blood (cats) or 10 ml of blood (humans) was collected into heparinized syringes. Erythrocytes were removed using dextran sedimentation (1:1 mixture of blood:6% dextran/PBS), followed by one or two rounds of hypotonic lysis using ddH2O. Neutrophils were isolated from the remaining cell suspension using Ficoll-Histopaque density centrifugation. The entire isolation was performed at 4°C in a 2% CO2 incubator, which allowed sufficient time for neutrophils to migrate the entire distance between the wells. Results were recorded using a video camera attached to an Axiovert 135 microscope and a ×20 objective lens (Zeiss).

To measure the priming effect of GM-CSF treatment on ex vivo neutrophil chemotaxis, neutrophils were isolated from control and FIV-infected animals that had not been given GM-CSF therapy. The neutrophils were then treated with 10 μg/ml GM-CSF for 30 min and then loaded into the under agarose assay. Chemotaxis was then assayed toward 100×, 10×, 1×, 1/10th, and 1/100th optimal concentrations of fMLP and IL-8 (optimal concentration = 0.1 μM and 1.0 μM, respectively).

**Quantification of neutrophil granule content**

To quantify the relative numbers of granules in the control and FIV-infected animals, bone marrow cross-sections were cut from isolated femurs, and pre- and post-GM-CSF blood smears were made. Bone marrow cross-sections were fixed in 10% formalin, decalcified with formic acid, and embedded in paraffin for sectioning. The bone marrow cross-sections and blood smears were stained with Wright-Giemsa stain, and both color and fluorescent images (550 nm excitation/600 nm emission) were taken...
using a filter set on an Olympus IX70 inverted microscope equipped with
digital camera. Images of neutrophils were captured at ×400.30NA mag-
nification, using Openlab software. Neutrophils were identified using the
color images, and then the eosin content was quantified by measuring the
mean fluorescent intensity of the fluorescent image using computer soft-
ware to avoid bias as described below. All neutrophils in each image were
quantified, as well as the eosin staining of a minimum of 10 RBC. For
comparisons between slides, the staining of the neutrophils was expressed
as a ratio compared with the staining of RBC. All analysis was performed
nih.gov/ij/)).

Long-term bone marrow cultures
Bone marrow cultures were made using a protocol modified from Tejero and colleagues (51) and Raza and colleagues (52). Briefly, bone marrow
was flushed from the femurs of uninfected or FIV-infected animals with
ice-cold FBS. Bone marrow was then centrifuged at 1150 rpm, 4°C for 10
min, and then suspended into bone marrow culture medium (RPMI 1640 +
12.5% heat-inactivated FBS, 12.5% heat-inactivated horse serum, 10⁻⁴ M
2-ME, 2 mM L-glutamine, 10⁻⁷ M dexamethasone, and 200 U/ml penicil-
lin/streptomycin). Suspended bone marrow was alloquated into 6-well tis-
sue culture plates, at a density of 1 × 10⁶ cells/well. Cultures were grown
in a 37°C5% CO₂ incubator. After 3 days, the medium and all suspended
cells were swapped for fresh medium. Every 7 days afterward, 50% of the
medium was replaced with fresh medium. When cultures were confluent
(10–14 days) a 50% medium change was made, and the medium was
supplemented with GM-CSF. One week later the suspended cells were
removed, and the adherent cells were trypsinized. Cytospins of the adher-
ent and suspended cells were prepared and stained with Wright-Giemsa
stain. Analysis was performed as described in Quantification of neutrophil
granule content.

Statistics
Statistical analysis was preformed using GraphPad Prism software (Graph-
Pad). ANOVA with a Bonferroni correction for multiple comparisons was
used for analysis of in vivo data. Unless otherwise noted, in vitro data were
analyzed using Student’s t test. Correlations were calculated using a Spear-
man’s correction for nonparametric data. Significance was established as
p < 0.05.

Results
HIV-infection results in defects in neutrophil chemotaxis
Although HIV does not infect neutrophils (4, 53), neutrophils iso-
lated from HIV-infected patients with CD4⁺ counts <400/µL ex-
hibited a severe defect in their ability to chemotax in response to
both endogenous (IL-8) and bacterial (ILP) chemoattractants
(Fig. 1A). We observed an identical chemotactic defect in neutro-
phils isolated from FIV-infected animals (Fig. 1B). Interestingly,
in HIV-infected patients, the severity of this chemotactic defect
was strongly correlated with CD4⁺ lymphocyte levels (Spearman
r > 0.76; p < 0.0001) (Fig. 1C and D), but there was no associ-
ation with plasma viral load (Spearman r < −0.34; p = 0.15)
(Fig. 1, E and F). No correlation was observed between neutrophil
chemotaxis and any single antiretroviral drug, combination of an-
tiretroviral drugs, or duration of antiretroviral therapy (data not
shown).

Previously, we demonstrated that bacterial products can block
neutrophil chemotaxis in vitro (49) and in vivo (54). This inhibi-
tion of chemotaxis was a result of p38 MAPK activation and could be
reversed by inhibiting p38 MAPK. Based on these studies, we
hypothesized that a potential cause of the HIV-induced chemotac-
tic defect was the presence of bacterial or viral products in the
blood of infected patients, resulting in inappropriate p38 MAPK
activation. To determine whether p38 MAPK activation was re-
sponsible for the HIV-induced chemotactic defect, we treated ne-
 utrophils isolated from control and HIV-infected patients with the
p38 MAPK inhibitor SB203580 (Fig. 2A). As we have published
previously (49), p38 MAPK inhibition did not block the chemotax-
sis of neutrophils from uninfected patients to IL-8 (Fig. 2A).
Treatment of neutrophils from uninfected patients with LPS re-
sulted in a defect in neutrophil chemotaxis, which was p38 MAPK-
dependent. In contrast, neutrophils from HIV-infected patients had
severely depressed chemotaxis to IL-8, but p38 MAPK inhibition
had no impact on this chemotactic defect (Fig. 2A). An identical
trend was observed in neutrophils from FIV-infected animals (Fig.
2B). Neutrophils isolated from HIV-infected patients had a sig-
ificant decrease in the surface expression of the IL-8 receptors
(CXCR1 and CXCR2), the β₂ integrin common subunit (CD18),

![FIGURE 2. Effect of p38 MAPK inhibition on LPS-mediated and HIV/
FIV-mediated chemotactic defect of human and feline neutrophils to IL-8.
A. Effect of p38 MAPK inhibition (SB) on the chemotaxis of human neu-
 trophils from uninfected (HIV(−)) and HIV-infected (HIV(+) subjects. B. Effect
of p38 MAPK inhibition on the chemotaxis of feline neutrophils from uninfected
(FIV(−)) and FIV-infected (FIV(+)) animals. There were no significant differences
within the HIV/FIV and HIV/FIV + SB groups. C. Expression of chemotactic receptors
on neutrophils isolated from HIV(+) patients, relative to uninfected controls (dotted
line). Each point represents the mean fluorescence intensity from a single subject’s neutro-
phils, relative to the expression of the same receptor on neutrophils from an
uninfected control. Bars represent the arithmetic mean for each receptor.
Uninhibited (UT) = cells not treated with a p38 MAPK inhibitor, SB =
cells treated with the p38 MAPK inhibitor SB203580. n = 5. †, p < 0.05
compared with cells from uninfected animals (FIV(−)) not treated with the
p38 MAPK inhibitor (UT). ‡, p < 0.05 compared with UT group. Student’s
t test (A and B) or Wilcoxon signed-rank test (C).]
and the β2 integrin LFA-1 (Fig. 2C). Clearly, aberrant p38 MAPK activation is not responsible for the HIV/FIV-mediated defects in neutrophil chemotaxis, and rather the defect appears to be due to decreased expression of receptors required for chemotaxis.

**GM-CSF treatment restores leukocyte recruitment to locally administered LPS**

To determine the effects of GM-CSF on FIV infection-induced defects in recruitment and chemotaxis, FIV-infected and uninfected animals were treated over a 6-day period with 500 µg/m2/day GM-CSF, given as three 1000 µg/m2 injections on days 1, 3, and 5. This concentration of GM-CSF is widely used for treatment of patients with neutropenia (55). FIV-infected animals had a significantly lower percentage of CD4+ counts compared with uninfected animals (Table I), and GM-CSF treatment did not significantly alter CD4+ lymphocyte percentages over the treatment period (Table I). FIV infection resulted in an elevated CD8+ lymphocyte percentage, and GM-CSF therapy did not result in significant changes in CD8+ lymphocyte counts over the treatment period (Table I). Interestingly, GM-CSF therapy suppressed plasma FIV load (Table I); an effect previously observed in several GM-CSF clinical trials among HIV/AIDS patients, possibly due to decreased release of HIV from infected monocytes (56, 57).

We next examined the leukocyte recruitment cascade (58) in vivo in FIV-infected animals. Small amounts of locally administered LPS placed directly on the mesentery caused a significant increase in neutrophil rolling (Fig. 3A), adhesion (Fig. 3B), and emigration (Fig. 3C) over a 4-h period in animals not infected with FIV. This dose of LPS is much lower than the high LPS concentration that blocked chemotaxis in vitro (Fig. 2). We previously reported that the LPS-induced rolling was selectin dependent, whereas adhesion was dependent upon integrins (47, 48). Leukocyte rolling (Fig. 3A), adhesion (Fig. 3B), and emigration (Fig. 3C) in response to 4 h of LPS superfusion was significantly impaired in FIV-infected animals compared with uninfected animals. GM-CSF had no effect on basal levels of rolling, adhesion, or emigration, regardless of whether animals were uninfected or infected with FIV (Fig. 3). GM-CSF completely reversed the FIV-induced defects in rolling, adhesion and emigration (Table I). Interestingly, GM-CSF therapy suppressed plasma FIV load (Table I); an effect previously observed in several GM-CSF clinical trials among HIV/AIDS patients, possibly due to decreased release of HIV from infected monocytes (56, 57).

### Table I. Effect of long-term GM-CSF treatment on percentage of CD4, percentage of CD8, and viral load in FIV-infected animals

<table>
<thead>
<tr>
<th></th>
<th>Uninfected</th>
<th>FIV Infected</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pre-GM</td>
<td>Post-GM</td>
<td>Pre-GM</td>
<td>Post-GM</td>
</tr>
<tr>
<td>Percentage of CD4⁺ cells</td>
<td>27.12 ± 3.28</td>
<td>29.51 ± 6.94</td>
<td>14.31 ± 1.29</td>
<td>9.65 ± 4.06</td>
</tr>
<tr>
<td>Percentage of CD8⁺ cells</td>
<td>11.65 ± 1.29</td>
<td>11.06 ± 2.05</td>
<td>24.63 ± 3.59</td>
<td>27.63 ± 6.26</td>
</tr>
<tr>
<td>Viral load</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2.31 x 10⁴ ± 1.65</td>
<td>1.04 x 10⁴ ± 2.05</td>
</tr>
</tbody>
</table>

*, p < 0.05 compared to pre-GM-CSF; †, p < 0.05 compared to uninfected.
defect in LPS-mediated leukocyte adhesion and emigration (Fig. 3, B and C), but did not have a significant effect on the rolling impairment (Fig. 3A). In uninfected animals, GM-CSF treatment did not enhance rolling, adhesion, or emigration in response to LPS (data not shown).

Next, we assayed the ability of neutrophils from GM-CSF-treated animals to chemotax in the in vitro under agarose chemotaxis assay. FIV infection and GM-CSF therapy had no effect on the basal movement of neutrophils when no chemoattractant was present (HBSS group; Fig. 3D). However, neutrophils isolated from FIV-infected animals had a profound defect in their ability to chemotax to both a bacterial chemoattractant (fMLP) and an endogenous chemoattractant (IL-8). GM-CSF treatment restored the migration of neutrophils from FIV-infected animals to >50% of control levels (Fig. 3D).

Role of GM-CSF priming

GM-CSF is known to act as a neutrophil priming agent, and has been shown to enhance neutrophil responses such that they respond to lower doses of chemoattractants (24–26). Although the GM-CSF concentration used in our in vivo animal studies produced no increase in neutrophil function in uninfected animals, acute administration of GM-CSF primed neutrophils from uninfected animals, resulting in enhanced chemotaxis. Indeed, when neutrophils from uninfected animals were treated acutely ex vivo with GM-CSF, they became responsive to 10-fold lower concentrations of fMLP (Fig. 4A) and IL-8 (Fig. 4B). However, when neutrophils from FIV-infected animals were acutely treated ex vivo with GM-CSF, there was no chemotaxis to any tested concentration of either chemoattractant, demonstrating that priming was not a likely cause of the improvement in neutrophil chemotaxis observed after long-term GM-CSF treatment (Fig. 4, A and B).

We then confirmed these results using neutrophils isolated from HIV patients with CD4$^+$ counts of <400/$\mu l$ (Fig. 4C). As in neutrophils isolated from FIV-infected animals, no improvement in chemotaxis was seen when neutrophils from HIV-infected patients were acutely stimulated ex vivo with GM-CSF, confirming that priming was not responsible for the improvement in recruitment and chemotaxis observed after long-term GM-CSF treatment.

Long-term GM-CSF treatment and hematology

Another possible reason for the observed defect in neutrophil recruitment and chemotaxis during HIV and FIV infections is that lentiviruses may cause aberrant neutrophil development. To test this possibility, blood was collected from all animals immediately before and after GM-CSF treatment, and analysis of the hematocrit, total leukocyte counts, and differential leukocyte counts was performed. Although GM-CSF is a well-described hemopoietic factor, it did not significantly alter the white blood cell count, or hematocrit of uninfected or FIV-infected animals, resulting in enhanced chemotaxis. Indeed, when neutrophils from uninfected animals were treated acutely ex vivo with GM-CSF, they became responsive to 10-fold lower concentrations of fMLP (Fig. 4A) and IL-8 (Fig. 4B). However, when neutrophils from FIV-infected animals were acutely treated ex vivo with GM-CSF, there was no chemotaxis to any tested concentration of either chemoattractant, demonstrating that priming was not a likely cause of the improvement in neutrophil chemotaxis observed after long-term GM-CSF treatment.

Role of altered neutrophil development

There are many conditions in which neutrophil development is impaired without changes in the number of circulating neutrophils
Neutrophils from the blood and bone marrow of FIV-infected animals had significantly decreased granularity compared with neutrophils from uninfected animals. There was no difference between the granularity of blood and bone marrow neutrophils in FIV-infected animals, demonstrating that the decrease in granularity was not due to degranulation in the periphery (Fig. 5A). This is consistent with other studies that demonstrated that HIV infection inhibits, rather than promotes, neutrophil degranulation (60, 61). In vivo GM-CSF treatment had no effect on the granule content of blood neutrophils from uninfected animals (Fig. 5B). However, GM-CSF treatment of FIV-infected animals increased neutrophil granularity by 50% (Fig. 5B), suggesting that GM-CSF may restore granule formation in neutrophils from FIV-infected animals. To further explore this issue, bone marrow from uninfected and FIV-infected animals was cultured in vitro in the presence of varying concentrations of GM-CSF. After 1 wk of in vitro GM-CSF treatment, at all concentrations tested, the granularity of neutrophils from FIV-infected cultures was restored to normal levels, and significantly elevated above untreated FIV-infected cultures (Fig. 5C). This demonstrates that GM-CSF treatment can counter the detrimental effects of FIV on neutrophil development in the bone marrow.

Discussion

Defects in neutrophil function are a well-known complication of HIV infection (2). Indeed, neutrophil dysfunction and neutropenia were identified as clinical signs of HIV/AIDS before the HIV virus had been identified (62). These defects in neutrophil function result in increased susceptibility to bacterial and fungal infections, and are likely a major contributor to the increased morbidity/mortality seen in HIV patients (63–66). Highly active antiretroviral therapy can effectively control HIV infection. However, neutrophil defects and neutropenia are still observed in many HIV/AIDS patients, including those who have responded positively to antiretroviral therapy (2). In this study, we identify a profound defect in the chemotaxis of neutrophils from HIV patients, as well as in the FIV model of HIV infection. Indeed, HIV-infected patients had up to a 90% inhibition in the chemotaxis of their neutrophils to both endogenous chemoattractants (IL-8) and bacteria-derived chemoattractants (MLP; Fig. 1). An identical chemotactic defect is seen in the FIV model (Fig. 3D), and is accompanied by a severe defect in the recruitment of leukocytes to sites of inflammation (Fig. 3). This decrease in recruitment and chemotaxis is accompanied by a decrease in several receptors that are involved in these processes (Fig. 2C and Refs. 49 and 67). Interestingly, mobilization of some adhesion molecules from granules is required for optimal chemotaxis (68), and a decrease in neutrophil granularity was also observed in FIV-infected animals (Fig. 5). These severe defects in recruitment and chemotaxis could be responsible for the increased susceptibility of HIV/AIDS patients to bacterial and fungal infection (63, 66). Our data suggest that supplemental therapies are required to restore the innate immune system of HIV-infected patients.

The mechanism of impaired chemotaxis and the beneficial effects of GM-CSF therapy are not entirely clear. There are clear indications of defective neutrophil development in both HIV(+) patients and in animals with FIV; notably, a decrease in the expression of chemotactic receptors in humans (Fig. 2C) and a decrease in feline neutrophil granularity (Fig. 5). Our data suggest that this defect may be corrected by GM-CSF therapy, and that GM-CSFs’ beneficial effect is at the level of neutrophil development. First, HIV patients have decreased expression of several chemotactic receptors, including the IL-8 receptors and the β2 integrins (Fig. 2C). This is in agreement with other studies that demonstrated decreased expression of other chemotactic and phagocytic receptors on the neutrophils of HIV patients (61, 64). Second, acute application of GM-CSF did not have any beneficial effect (Fig. 4), whereas long-term treatment restored normal neutrophil chemotaxis and recruitment (Fig. 3). Finally, long-term GM-CSF treatment partially restored the granular content of neutrophils from FIV-infected animals, and completely restored the granularity of neutrophils in bone marrow cultures prepared from FIV-infected animals (Fig. 5B and C). Together, these data suggest that the neutrophil defects we have observed are due to a defect in neutrophil development, which results in the production of chemotactically defective neutrophils.

Several mechanisms have been identified by which HIV infection can result in altered neutrophil development, including, HIV infection of bone marrow stem cells, stroma cells, macrophage and resident CD4(+) cells (11, 21), altered cytokine milieu in the bone marrow (12), direct suppression of hemopoiesis by viral proteins (13, 14), myelosuppressive effects of antiretroviral drugs (15–18), and infection of the bone marrow by viruses, bacteria or fungi (19, 20). Inappropriate activation of neutrophils by opportunistic infections is unlikely because none of the patients had an identified opportunistic infection during the study, nor were bacteria or fungi detected in the blood or bone marrow of FIV-infected animals (data not shown). It is unlikely that this defect is solely the result of myelosuppressive antiretroviral drugs, because none of the FIV-infected animals received antiretroviral drugs, and the FIV-infected animals had a chemotactic defect identical with that observed in HIV patients on antiretroviral drugs (Fig. 1, A and B). In addition, no correlation was seen between the chemotactic abilities of neutrophils from HIV patients and any antiretroviral drug, combination of antiretroviral drugs, or duration of antiretroviral therapy (data not shown). Moreover, not all patients were on the same antiretroviral drugs. As such, it is likely that the defect we have observed is directly mediated by lentivirus infection, and not a side effect of antiretroviral therapy. However, even if the antiretroviral drugs contributed to the defect, GM-CSF could still be beneficial to these patients. Taken together, our study strongly suggests that the neutrophil defects we have observed are due to impaired neutrophil development, and, therefore, drugs that impact on the hemopoietic system may represent a viable supportive therapy for patients with HIV/AIDS.
A number of other possible explanations for the positive effects of GM-CSF therapy exist but are less likely. The release of immature neutrophils could potentially explain the low granule content and altered receptor expression seen in neutrophils from FIV-infected animals and HIV-infected patients. However, we did not detect any band cells or other forms of immature neutrophils in FIV-infected animals (Table II). In addition, none of the structural abnormalities observed in neutrophils from HIV-infected patients are consistent with the known structure of immature neutrophils (69–71). A second possibility is that HIV is directly infecting neutrophils. Indeed, Gabrilovich et al. (72) identified HIV genomes in a small percentage of neutrophils from HIV-infected patients. However, using highly purified neutrophils and real-time PCR, we were unable to detect FIV infection of neutrophils (data not shown), and most studies suggest that HIV does not productively infect neutrophils (4, 53, 73, 74). Importantly, the binding of HIV to neutrophils does not appear to result in neutrophil activation, suggesting that inappropriate neutrophil activation by HIV is not responsible for the defects we observed (4). Indeed, we saw no correlation between viral load and the severity of the chemotactic defect (Fig. 1, E and F), suggesting that HIV-mediated neutrophil defects are not a direct result of interactions between neutrophils and virions or viral proteins.

In this study, we report a profound defect in the chemotaxis of neutrophils from HIV-infected patients that was independent of neutropenia and associated with decreased expression of chemotactic receptors. A similar defect was identified in the FIV model of HIV infection, and chronic treatment with GM-CSF dramatically improved neutrophil recruitment and chemotaxis, as well as dramatically reversed the FIV-associated defect in neutrophil granularity. Previous studies have shown that GM-CSF is an excellent therapeutic for the treatment of HIV-associated neutropenia (3, 29, 75), reverses HIV-associated defects in monocyte/macrophage phagocytosis (5), and suppresses HIV replication (56). Considering that HIV patients can have up to a 90% decrease in neutrophil chemotaxis (Fig. 1), as well as defects in monocyte function, our study would advocate the use of GM-CSF regardless of neutrophil counts in HIV/AIDS patients, particularly in the case of advanced disease.

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Disclosures

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References


