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In Vivo Impairment of Neutrophil Recruitment during Lentivirus Infection

Paul Kubes,² Bryan Heit, Guido van Marle, James B. Johnston, Derrice Knight, Adil Khan, and Christopher Power

Evidence indicates that the lentivirus, HIV, infection affects neutrophil response to bacteria and bacterial products in vitro. We used a novel model of rapid onset immunosuppression following infection with a similar lentivirus, feline immunodeficiency virus (FIV), in cats to examine neutrophil function within the microvasculature in vivo and to determine the steps that are impaired in the neutrophil recruitment cascade. In uninfected cats and cats infected neonatally with FIV, the mesentery was exteriorized, but remained autoperfused during intravital microscopy for 4 h. When the tissue was superfused with 10 µg/ml of LPS for 4 h, intravital microscopy displayed a profound increase in neutrophil rolling at both 8 and 12 wk of age in uninfected cats. At 12 wk of age, FIV-infected animals showed a profound decrease in the number of rolling neutrophils. In vitro studies revealed that neutrophils from infected and uninfected animals rolled equally well on surrogate selectin substrata. In addition, in vivo neutrophil adhesion and emigration out of the vasculature were severely reduced, and in vitro neutrophil chemotaxis from FIV-infected animals was significantly impaired in response to fMLP or IL-8. However, FIV infection of neutrophils could not be detected. In summary, in vivo lentivirus infection with immunosuppression leads to a severe impairment in neutrophil rolling, adhesion, and emigration in response to bacterial stimulants potentially involving both endothelial and neutrophil dysfunction. These in vivo studies also indicate that neutrophil dysfunction should be taken into account when treating infections and tissue injury. The Journal of Immunology, 2003, 171: 4801–4808.

Lentiviruses, including HIV, SIV, and feline (FIV) immunodeficiency viruses, are associated with immunological impairment in their respective hosts, and the latter two serve as important models for the study of HIV pathogenesis (1, 2). HIV and FIV share many properties, including genomic organization, replication strategy, cell tropism, and a common mechanism of infection involving the chemokine receptors (2). Both HIV and FIV infections increase the likelihood of secondary bacterial infections, although the mechanism of action remains unclear (1). Although neither HIV nor FIV is thought to infect neutrophils (the primary leukocytes involved in innate immunity), there is a growing body of literature to suggest impairment of neutrophil function from HIV-infected patients in response to bacterial infections despite the preservation of absolute neutrophil counts with advanced immunosuppression. In fact, in the case of HIV, isolated neutrophils have been shown to have decreased oxidant production (3), protease release (3), bacterial killing (4), phagocytosis (5), and chemotaxis (4, 6). Although the increased susceptibility to infections in HIV- and FIV-infected patients may be related to impaired antimicrobial functions related to bacterial killing (oxidant production, protease release, phagocytosis), it might also be associated with impaired neutrophil recruitment (rolling, adhesion, or emigration). To our knowledge, a systematic in vivo assessment of efficacy of recruitment of neutrophils to inflammatory stimuli in lentivirus-infected humans or animals has not been performed.

Neutrophil recruitment to sites of infection is a complex, multistep event that requires the coordinated expression of multiple proteins (7, 8). The first step of the recruitment cascade is the tethering and rolling of neutrophils within postcapillary venules (9). The rapid endothelial expression of presynthesized P-selectin from endothelial stores as well as de novo synthesis of E-selectin allow for leukocyte rolling (10, 11). The rolling event is thought to bring the neutrophils in close proximity to the endothelial surface where integrin-activating molecules, including proinflammatory phospholipids and chemokines, stimulate the rolling neutrophils to firmly adhere (12). This latter event is dependent in part upon β2 integrins, requiring both up-regulation and activation for full adhesive function (13). Once firmly adherent, the neutrophils can emigrate across the endothelium and chemotaxis toward the invading organism. Clearly, lentiviruses could potentially impact at any stage of this multistep cascade of neutrophil recruitment.

Although some aspects of this multistep cascade can be recapitulated in vitro, it is extremely difficult to reconstruct the entire event. Therefore, in this study we used intravital microscopy, a system that allowed us to visualize the postcapillary venules within FIV-infected and uninfected cats, and tested the hypothesis that neutrophil recruitment was impaired by lentivirus infection at one or more steps of the leukocyte recruitment pathway. We tested the ability of FIV-infected and uninfected animals to respond to LPS, the primary cell wall component of Gram-negative bacteria. Our...
results suggest a marked impairment in neutrophil rolling, adhesion, as well as emigration in response to this inflammatory signal.

Materials and Methods

Cell cultures

Feline PBMC used for infection studies and preparation of viral stocks were isolated from blood obtained from specific pathogen-free adult felines by density gradient centrifugation, as described previously (14). DNA was extracted from 10 4.5 50% tissue culture infectious doses/ml, incubated for 2 h (Promega, Madison, WI) and reverse transcribed using Superscript II and RNA. Subsequently, the transcripts were treated with RNase-free DNase I by phenol/chloroform extraction and ethanol precipitation. The amount of RNA was determined by spectrophotometry.

Virus infection

The FIV strain used in this study was the infectious molecular clone, FIV-4. It is derived from the infectious clone p34TF10 by PCR using primers (POL-I, CTC and POL-II, treated with proteinase K (0.2 mg/ml, 0.5% SDS, 50 g/ml of FITC-labeled, isotype-matched murine IgG1 (BD Biosciences) served as controls.

Intravitral microscopic studies

The surgical preparation used in this study is the same as described previously (16, 17). 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, i.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, Oxnard, CA), connected to a catheter in the left carotid artery. A midline abdominal incision was made, and a segment of small intestine was isolated from the ligament of Treitz to the ileocecal valve. The remainder of the small and large intestines was extraperitoneal. Body temperature was maintained at 37°C using an infrared heat lamp. All exposed tissues were moistened with saline-soaked gauze to prevent evaporation. Whole blood was collected in K 3 EDTA tubes and incubated for 5 min at room temperature with anti-CD4 or CD8 mAbs (3 µg/ml, provided by Dr. P. Moore, University of California, Davis, CA). Cells were again washed, incubated for 20 min with FITC-conjugated goat anti-mouse IgG (0.25 µg/ml; BD Biosciences, San Jose, CA), and resuspended in 0.5 ml of 1% formalin in PBS for analysis. Using a FACScan flow cytometer (BD Biosciences), we identified the argon laser excitation set at 488 nm, data were acquired from ~15,000 events for each experimental condition, and results were expressed as a single-parameter log fluorescence histogram. Cells incubated in the absence of Abs or with 1 µg/ml of FITC-labeled, isotype-matched murine IgG1 (BD Biosciences) served as controls.
all neutrophils entering the vessel was measured. A neutrophil was defined as adherent to venular endothelium if it remained stationary for >30 s. Adherent cells were measured at 10-min intervals as described in the experimental protocol and were expressed as the number per 100-μm length of venule. RBC velocity (V_{RBC}) was measured using an optical Doppler velocimeter (Microcirculation Research Institute), and mean V_{RBC} (V_{mean}) was determined as V_{RBC}/D, where D is the venular diameter.

**Experimental protocol: in vivo experiments**

Baseline measurements of blood pressure, SMA blood flow, V_{RBC}, and vessel diameter were obtained, and neutrophil-endothelial cell interactions were determined at 8 and 12 wk of age. Endotoxin (Escherichia coli, 1.0 μg/ml) was 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 indeed endothelial selectin dependent, and the adhesion was dependent upon neutrophil integrins (19, 20).

**Histology**

A section of cat mesentry was removed immediately after intravital microscopy and was placed in 10% formalin. The tissue was embedded in paraffin, sectioned (4 μm), and stained with H&E. Images were captured using an IX70 widefield microscope (Olympus, Melville, NY) and Openlab software (Improvision, Guelph, Canada). Images were taken at ×200 (air) and ×600 (oil immersion). Infiltrating leukocytes were identified via morphology.

**Neutrophil isolation and preparation**

Blood was collected from FIV-infected and uninfected cats. Erythrocytes were removed using dextran sedimentation (6% dextran/0.9% NaCl), followed by two rounds of hypotonic lysis using ddH2O. Neutrophils were isolated from the resulting cell suspension using Ficoll-Histopaque density centrifugation. The entire isolation was performed at 4°C. Purified neutrophils were suspended in HBSS at a concentration of 1.0 × 10^7 cells/ml and were kept on ice until needed. Neutrophils were >95% viable and >97.5% pure, as determined by FACS.

**Under agarose assay procedure**

The under agarose assay was performed as previously described (21–23) with minor modifications. Falcon 35 × 10-mm culture dishes were filled with 3 ml of a 1.2% agarose solution containing 50% H2CO3-buffered HBSS and 50% RPMI 1640 culture medium with 20% heat-inactivated FCS. After the agarose solidified, wells were cut into a straight line in the gel. The gels were allowed to equilibrate for 1 h in a 37°C/5% CO2 incubator. The outer wells were loaded with purified neutrophils, and the central well was loaded with varying concentrations of chemotactic agent. Once loaded, the gels were incubated for 2 h in a 37°C/5% 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 Axiosverter 135 microscope (Zeiss, Thornwood, NY).

**Neutrophil cell counts**

The number of blood neutrophils was determined using a Diff-Quick staining kit (Richard Allen Scientific, Kalamazoo, MI) according to instructions provided. Cells were counted using an Axiosivert microscope (Zeiss) at ×100 magnification. Neutrophils were identified by morphology.

**Statistics**

The data were analyzed using standard statistical analysis, i.e., ANOVA and Student’s t test with Bonferroni’s correction for multiple comparisons when appropriate. Values are the mean ± SE. Statistical significance was set at p < 0.05.

**Results**

**Systemic changes in FIV-infected neonates**

The systemic features of FIV infection were assessed by measuring changes in body weight and peripheral blood cell populations over the 12-wk period. Weekly determinations of body weights indicated that progressive weight gains occurred in both uninfected (control) and FIV-infected animals. However, the mean weight gain of the FIV-infected group was significantly lower than that in control animals (p < 0.05) and accounted for ~10% of the weight difference at 12 wk (data not shown). Analysis by flow cytometry of PBMC isolated at wk 8 PI from FIV-infected and uninfected cats revealed no significant differences between groups in the percentage of CD4+ cells (Fig. 1). In contrast, CD4+ cell levels were significantly lower at wk 12 PI (p < 0.001) in animals infected with FIV-Ch compared with mock-infected cats (Fig. 1A). These differences reflected an increase in the number of CD4+ cells in control animals (80%), while levels in FIV-Ch animals decreased over the same time period (50%). Comparison of CD8+ cells at the same time points indicated that levels were increased significantly in the FIV-infected group compared with controls at wk 8 PI, which declined by 12 wk PI (Fig. 1B). Analysis of CD4+/CD8+ ratios at both time points indicated that for uninfected animals, the ratio was >2.0, while the ratio was <1.0 for the FIV-infected group at each time point. This is similar to lymphocyte ratios among HIV-infected patients with advanced immunosuppression (24). Total leukocyte counts in blood did not show any significant differences among groups at 8 wk of age (Table I). Although a 50% increase in total leukocyte counts was noted at 12 wk in both groups; it did not achieve statistical significance. Neutropenia was not a major feature of FIV infection (Table I), similar to HIV infection.

**Viral quantitation**

To assess viral quantity in blood, a quantitative real-time RT-PCR assay was established (Fig. 2). The plasma viral load of the infected animals was determined from a standard curve, generated using known quantities of RNA from which corresponding cycle thresholds were measured (Fig. 2A). Analysis of plasma viral load revealed detectable viral RNA at both 8 and 12 wk in all FIV-infected animals (Fig. 2B). The viral load ranged from 1.84–4.6 log viral RNA copies/ml of plasma among FIV-infected animals, and no significant difference was observed between 8 and 12 wk groups of FIV-infected animals. In contrast, FIV was not detected in plasma from uninfected controls. Nested PCR, RT-PCR, as well as FACS were also used in an attempt to detect viral load in purified neutrophils. None of these approaches revealed any detectable FIV infection in neutrophils from FIV-infected cats.
Table 1. Total leukocyte counts and neutrophil counts for experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total Leukocyte Count ($\times 10^5$/ml)</th>
<th>Total Neutrophil Count ($\times 10^3$/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected, 8 wk</td>
<td>4</td>
<td>4.72 ± 1.03</td>
<td>2.28 ± 0.67</td>
</tr>
<tr>
<td>FIV infected, 8 wk</td>
<td>4</td>
<td>7.67 ± 2.54</td>
<td>3.65 ± 1.93</td>
</tr>
<tr>
<td>Uninfected, 12 wk</td>
<td>4</td>
<td>7.98 ± 1.26</td>
<td>4.52 ± 1.11</td>
</tr>
<tr>
<td>FIV infected, 12 wk</td>
<td>5</td>
<td>9.38 ± 1.07</td>
<td>3.15 ± 1.00</td>
</tr>
</tbody>
</table>

*a No significant differences were measured between age-matched groups, as determined by a Student’s t test.

Neutrophil recruitment is impaired at 12 wk, but not at 8 wk

Fig. 3 summarizes the number of rolling neutrophils in 25- to 35-μm-long postcapillary venules. At 8 wk of age (Fig. 3A), the number of rolling neutrophils under basal conditions was ~40 cells/min. In uninfected cats the number of rolling leukocytes approximately tripled (120 cells/min) in response to 4 h of LPS treatment. In the FIV-infected group there was no difference from uninfected animals in the number of neutrophils that rolled under basal conditions, suggesting no impairment in neutrophil surveillance of the microvasculature. In FIV-infected animals there was a modest increase in neutrophil rolling, but the response appeared blunted and reached significance only at 3 h. A profound difference between FIV-infected and uninfected groups was seen by 12 wk (Fig. 3B). In the 12-wk-old uninfected cats the number of rolling neutrophils increased >5-fold to 150–175 rolling neutrophils/min. Although similar levels of rolling were observed under basal conditions in 12-wk FIV-infected cats, the ability to increase the number of rolling neutrophils in response to LPS was greatly impaired, such that the number of rolling neutrophils was significantly less in FIV-infected than uninfected cats ($p < 0.05$).

Since much of the neutrophil rolling is dependent on endothelial P-selectin, based on previous experiments (16, 20) we perfused whole blood from FIV-infected and uninfected animals over a set number of platelet-derived P-selectin molecules (using a surrogate platelet monolayer). Fig. 4 demonstrates that the same number of rolling neutrophils from uninfected and FIV-infected cats was present on the inert selectin substratum, suggesting no inherent global impairment in the ability of neutrophils to tether and roll.

Fig. 5 summarizes the number of adherent neutrophils in response to LPS in uninfected and FIV-infected animals. There was a significant increase in the number of adherent neutrophils in both groups of 8-wk-old animals with LPS superfusion (Fig. 5A). By contrast, neutrophil adhesion in 12-wk-old cats was dramatically reduced in FIV-infected animals compared with uninfected animals (Fig. 5B). Over 4 h >30 neutrophils adhered/100-μm-long venule in uninfected animals, whereas fewer than five neutrophils adhered in FIV-infected animals.

Similar results were observed in the number of neutrophils that emigrated out of the vasculature and into the interstitium. In 8-wk-old uninfected animals, 25–30 neutrophils emigrated out of the vasculature per field of view over 4 h in response to LPS (Fig. 6A). Similar numbers of neutrophils emigrated over the same period of time in FIV-infected animals (Fig. 6A). Conversely, very few neutrophils from FIV-infected animals emigrated out of the vasculature in response to LPS in 12-wk-old cats (Fig. 6B). In uninfected 12-wk-old cats the number of neutrophils that emigrated into the surrounding interstitium was >75 cells/field of view (Fig. 6B), which was significantly greater than that in both 8-wk-old and FIV-infected 12-wk-old animals.

Immunohistochemistry was performed to determine the types of cells recruited in response to LPS superfusion. Immediately after the intravital microscopy was completed a section of the mesentery was removed and stained using H&E dyes. Under control conditions few cells are found in the mesentery. Those that are present have a mononuclear morphology (Fig. 7A). After 4 h LPS superfusion the number of emigrated cells increases dramatically. The inflammatory infiltrate consists almost exclusively of polymorphonuclear cells (Fig. 7B).

To further examine whether there is an impairment in neutrophil function per se, we used an under agarose migration assay that allows neutrophils to migrate toward a chemoattractant via an integrin-dependent mechanism (23). Dose responses were conducted to determine the optimal concentrations of chemoattractants (data not shown). The optimal concentrations of chemoattractants were found to be 1.0 μM for IL-8 and 0.1 μM for fMLP. These concentrations are very similar to those published previously using human cells (23). The data clearly demonstrate that in vitro, neutrophils from uninfected cats migrate very effectively toward bacterial products such as fMLP as well as toward endogenous chemokines like IL-8 (Fig. 8). By contrast, neutrophils from FIV-infected animals were not able to respond to either bacterial or endogenous chemoattractants. The cells were found to change shape (data not shown), but simply did not chemotax as effectively, clearly demonstrating an impairment in neutrophil chemotaxis.

Discussion

FIV and SIV viruses have been the two most commonly used in vivo experimental models of HIV infection (1, 2). The similarities...
between HIV and FIV are striking in that CD4+ T cell number and function decline with resulting immunosuppression, viremia, and an increased incidence of acute and chronic opportunistic infections. Based on in vitro experiments, the prevailing view is that neutrophil biology including chemotaxis (4, 6), oxidant production, and bacterial killing (4) are affected in HIV infections, such that the innate immune response dominated by these leukocytes is depressed. Although a key feature of the innate immune response is the rapid recruitment of neutrophils, to date a systematic assessment of the potentially affected mechanisms in this multistep process has not been evaluated in lentiviral infections. In this study we have made use of the FIV model system, which results in rapid immunosuppression with high viral loads, and used intravital microscopy to visualize the behavior of neutrophils in vivo in their normal physiologic milieu (intact blood vessels). These studies demonstrated that each of the steps (rolling, adhesion, and emigration) of the multistep recruitment cascade is impaired in lentivirus-infected animals.

Our data clearly demonstrate that with age and increasing immunosuppression, lentivirus-infected animals developed a significant impairment in the ability of the innate immune system to respond to a bacterial stimulus like LPS. Unexpectedly, the first step of the leukocyte recruitment cascade, i.e., selectin-dependent neutrophil rolling, was reduced in lentivirus-infected cats. The rolling event is regulated by de novo expression of endothelial selectins and constitutively expressed molecules on neutrophils. The impairment of rolling could be a result of either neutrophil or endothelial impairment. A direct in vitro assay revealed that neutrophil tethering/rolling in vivo was not globally impaired as neutrophils from FIV-infected and uninfected animals rolled equally well on equivalent numbers of selectins. Alternatively, impairment in vivo may exist in endothelium rather than the leukocytes.

Presynthesized P-selectin can be rapidly released from prestored pools in endothelial Weibel-Palade bodies. In addition, depending upon the stimulus, P-selectin and E-selectin can also be synthesized to support further neutrophil rolling. Synthesis of E-selectin and P-selectin generally takes 3–4 h. Our present data show that both rapid (first 2 h of LPS) and more delayed (3 and 4 h) neutrophil rolling was reduced in lentivirus-infected animals consistent with FIV affecting endothelial P- and E-selectin production/expression. The virus could affect endothelial adhesion molecule synthesis indirectly, perhaps by reducing proinflammatory cytokine release and/or increasing the release of suppressive molecules from macrophages and CD4+ T cells (or other/infected cells). Although lymphocytes and macrophages are primarily susceptible to infection by HIV, recent literature suggests that endothelial cells per se may also be permissive to HIV infection via CXCR4 and other receptors, independent of CD4 (25).
FIGURE 5. Number of adherent leukocytes in response to LPS in 8-wk-old animals (A) and 12-wk-old animals (B). *, $p < 0.05$ compared with control animals, by single-sample $t$ test. +, $p < 0.05$ compared with LPS control in same time group, by single-sample $t$ test. $n = 4$ for all groups except for 12 wk FIV$^+$, where $n = 5$.

FIGURE 6. Number of emigrating leukocytes in response to LPS treatment in 8-wk-old animals (A) and 12-wk-old animals (B). *, $p < 0.05$ compared with control animals, by single-sample $t$ test. +, $p < 0.05$ compared with LPS control in same time group, by single-sample $t$ test. $n = 4$ for all groups except for 12-wk-old FIV$^+$, where $n = 5$. 
Indeed, FIV infects endothelia in vitro (26), implying a direct mechanism by which the virus can influence endothelial-dependent rolling. Interestingly, the basal neutrophil rolling (pre-LPS administration) appeared to be similar in both groups of animals, suggesting that only the up-regulation of selectins during the inflammatory response was affected by FIV. The basal rolling cells are thought to be surveying the blood vessel environment, and so the surveillance is not impaired in lentivirus-infected animals, but the ability to subsequently respond to an infection is altered.

Although it is well established that a reduction in rolling can affect adhesion and emigration, our data would suggest impaired neutrophil function beyond rolling. For example, there was a clear impairment in neutrophil chemotaxis in vitro (a rolling-independent event), an observation consistent with numerous other in vitro studies using neutrophils from HIV patients (4, 6). In addition, we observed that the basal rolling was not impaired, and previously we observed that basal rolling was sufficient to support subsequent adhesion and emigration in other models of inflammation (16). The fact that neutrophil adhesion and emigration were reduced in the face of sufficient rolling would support the view that events downstream of rolling were impaired in lentivirus-infected animals following challenge with bacterial products such as LPS. In the neutrophil recruitment paradigm, the process of adhesion and emigration requires a series of events, including activation of G protein-coupled chemotactic receptors on neutrophils, that signal to increase both affinity and avidity of the β2 integrin (CD11/CD18), causing firm adhesion of the rolling granulocytes. Our present in vitro data suggest that there was a key impairment in the ability of the neutrophils to respond to chemotactag agents, suggesting that neutrophil function in vivo was impaired. Taken together, our in vivo demonstration of impaired neutrophil recruitment combined with in vitro studies that suggest reduced chemotaxis (4, 6) and impaired fungal and bacterial killing (4, 5) certainly support the view that there is neutrophil impairment in HIV.

Whether the neutrophil dysfunction is related to reduced T cell numbers remain unclear, but it is well appreciated that GM-CSF, a major regulator of neutrophil function, is primarily produced by T cells, and as CD4+ T cells are depleted, levels of GM-CSF decline (27). Whether this impairs neutrophil functions remains uncertain, but addition of GM-CSF to HIV patients, improved clinical outcomes, consistent with the view that decline of the adaptive immune system is associated with dysregulation of the innate immune system (28). Treatment with antiretroviral inhibitors directed against the viral protease improved both CD4+ T cell counts and neutrophil function (29). Although in our study the impaired neutrophil response was associated with increasing immunosuppression, the effect on the innate immune response (90% decrease in adhesion, emigration, and chemotaxis) was disproportionately greater than the impairment of the acquired immune response (50% decrease in the numbers of CD4+ lymphocytes), raising the possibility that other mechanisms may also underline the very severe neutrophil impairment.

The current in vivo data provide credence to the existing in vitro data that there is an impairment in neutrophil biology in lentivirus-infected humans and animals. In fact, our work highlights a significant impairment in the ability of neutrophils to be recruited to...
sites of infection. The impairment includes selectin-dependent rolling and integrin-dependent adhesion and emigration that were consistent with impaired neutrophil migration in an in vitro chemotaxis assay. This study provides further rationale for the use of adjunct immunotherapies in HIV infection that enhance neutrophil function.

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