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HIV-1-infected peripheral blood mononuclear cells enhance neutrophil survival and HLA-DR expression via increased production of GM-CSF. Implications for HIV-1 infection

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Abstract

Objective—HIV-1 bound to intact neutrophils efficiently infects activated peripheral blood mononuclear cells (PBMC). Here, we evaluated the effect of the local milieu created by activated PBMC before and after HIV-1 infection on neutrophil survival and HLA-DR expression, with emphasis placed on a role for GM-CSF.

Methods—PBMC of healthy adult individuals were activated by phytohemagglutinin (PHA) or anti-CD3/anti-CD28 and were subsequently cultured without (HIV-1[−]) or with HIV-1 (HIV-1⁺). The effects of the culture supernatants or recombinant GM-CSF on survival and HLA-DR expression by neutrophils of healthy adult individuals and of HIV-1-infected individuals were evaluated using flow cytometry.

Results—Conditioned medium from PHA-activated PBMC (HIV-1[−] and HIV-1⁺) increased neutrophil survival and induced HLA-DR expression by neutrophils of healthy individuals in a GM-CSF dependent fashion. HIV-1 infection variably, but consistently, increased GM-CSF production by PHA-activated PBMC but not GM-CSF production by anti-CD3/anti-CD28-activated PBMC. The latter was correlated with a loss of CD3⁺GM-CSF⁺ cells after infection. Neutrophils of elite controllers exhibited a diminished HLA-DR response to GM-CSF in culture, whereas neutrophils of HIV-1⁺ subjects having a low viral load on anti-retroviral therapy or subjects with a high viral load exhibited a range of HLA-DR responses.

Conclusions—GM-CSF production within the mucosa or draining lymph nodes may promote HIV-1 infection by facilitating sustained contact between viable neutrophils with bound HIV-1 and CD4 lymphocytes. The minimal effect of GM-CSF on HLA-DR expression by neutrophils of elite controllers provides indirect support for this conclusion.

Keywords

HIV-1 infection; neutrophils; viability; HLA-DR; GM-CSF; elite controllers

INTRODUCTION

A role for neutrophils in HIV-1 disease has been considered most often in the context of the increased susceptibility of HIV-1 infected individuals to bacterial and fungal infections.^{1, 2} Indeed, functional deficits^{3–6} as well as an accelerated rate of apoptosis^{5, 7} have been

observed with neutrophils of HIV-1 infected individuals. The relationship between neutrophil function and HIV-1 disease, however, is expanding and becoming more complex. Neutrophils contain α -defensins and lactoferrin,⁸ both of which have anti-HIV-1 activity^{9–11} and, thus, suggest a protective role for neutrophils in HIV-1 infection. Conversely, other neutrophil-derived mediators including reactive oxygen species,^{12, 13} TNF- α ,¹² and IL-8¹⁴ can increase HIV-1 infection and/or replication in infected cells,^{12–17} although IL-8 has also been reported recently to inhibit HIV-1 replication.¹⁸

The finding that HIV-1 binds to human neutrophils and that neutrophil-bound HIV-1 efficiently infects activated peripheral blood mononuclear cells (PBMC)^{19, 20} identified a third potential role for neutrophils in HIV-1 disease. Neutrophils infiltrate mucosal tissue in association with local inflammation or infection,^{21, 22} which are established risk factors for HIV-1 transmission²³ and which also contribute to immune activation during chronic HIV-1 infection.²⁴ Neutrophils have also been observed in the draining lymph nodes of HIV-1-infected individuals,^{25, 26} and studies in mice have shown that neutrophils can transport antigen or live microorganisms to draining lymph nodes.^{27, 28} Thus, the ability of HIV-1 to bind to neutrophils and the subsequent ability of neutrophil-bound HIV-1 to infect PBMC may provide an additional mechanism by which mucosal inflammation increases HIV-1 transmission. Indeed, HIV-1 bound to neutrophils is approximately ten times more efficient, as compared to free HIV-1, in its ability to infect PBMC¹⁹, and activation of the neutrophils by TNF- α , as may occur at sites of mucosal inflammation,^{29, 30} increases both the amount of bound HIV-1 and the rate of PBMC infection.²⁰

Neutrophils rapidly undergo programmed cell death,³¹ but the accelerated infection of PBMC by neutrophil-bound HIV-1 is dependent on intact neutrophils.²⁰ Thus, the ability of neutrophil-bound HIV-1 to infect PBMC will be augmented by a cellular milieu that promotes neutrophil survival as well as facilitates neutrophil-CD4 cell interaction. GM-CSF is prominent among cytokines that can prolong neutrophil survival,^{31–33} and GM-CSF also induces expression of HLA-DR molecules by neutrophils.³⁴ The present study was performed to assess the effect of the local milieu created by activated PBMC alone and after HIV-1 infection on neutrophil survival and on HLA-DR expression, with emphasis placed specifically on assessing a role for GM-CSF. As a corollary to these studies, we also evaluated the HLA-DR response of neutrophils isolated from HIV-1 infected subjects at different stages of clinical disease to GM-CSF.

METHODS

Reagents and antibodies

The following monoclonal antibodies were purchased from BD Biosciences (San Jose, CA): FITC-conjugated anti-human HLA-DR (IgG2a; clone L243); PE-Cy5-conjugated anti-human CD80 (IgG1; clone L307.4); APC-conjugated anti-human CD86 (IgG1; clone 2331); PE-conjugated anti-human CD40 (IgG1; clone 5C3); anti-human CD3 (IgG2a; clone HIT3a); anti-human CD28 IgG1; clone CD28.8); APC-conjugated anti-human CD69 (IgG1; clone FN50); FITC-conjugated anti-human CD3 (IgG1; clone 3K7); PE-conjugated rat anti-human GM-CSF; and isotype control antibodies (FITC-conjugated mouse IgG2a, PE-Cy5-conjugated mouse IgG1, and APC-conjugated mouse IgG1). Neutralizing monoclonal antibody (mAb) specific for GM-CSF (IgG1, clone MAB215) and isotype matched control mAb were purchased from R&D Systems (Minneapolis, MN). FITC-conjugated annexin V and propidium iodide were purchased from BD Biosciences. Recombinant human GM-CSF was purchased from PeproTech, Inc. (Rocky Hill, NJ).

Neutrophil isolation and culture conditions

Neutrophils were isolated from freshly drawn venous blood of healthy adult individuals or HIV-1⁺ individuals by density gradient centrifugation as described previously^{20, 35} and according to a study protocol approved by the Institutional Review Board of Rush University Medical Center. The HIV-1⁺ individuals were recruited from outpatients visiting the Mark Weiss Memorial Infectious Disease Clinic at Rush University Medical Center for routine medical care. Viral loads and CD4 cell counts were obtained from their clinical records at the time of their outpatient visit. Neutrophils (10⁶) of the healthy adult individuals were cultured without or with the indicated dilutions of conditioned medium in RPMI 1640 containing 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, and 10 % heat-inactivated autologous serum for 48 hours at 37° C in a 5% CO₂ atmosphere. Recombinant GM-CSF, neutralizing anti-GM-CSF mAb, or isotype control mAb were added to the cultures as indicated. Neutrophils of the HIV-1⁺ individuals were cultured under the same conditions without or with recombinant GM-CSF. Cultures were performed in a total incubation volume of 0.5 ml in 48-well tissue culture plates. In all cases, the cultures were stopped by centrifugation at 300 g for 10 minutes at 4° C. Neutrophil viability and detection of surface molecule expression were determined by flow cytometry as described below.

Preparation of HIV-1⁺ and HIV-1⁻ conditioned media

Peripheral blood mononuclear cells (PBMC) were isolated from the venous blood of healthy adult individuals by density gradient centrifugation. Briefly, blood was diluted 1:3 with sterile saline (Baxter, Inc.; Deerfield, IL) containing 4 mM EDTA, was overlaid onto a 10-ml cushion of lymphocyte separation medium (Lonza, Walkersville, MD), and was centrifuged at 300 g for 30 minutes at 25° C. The PBMC were recovered at the boundary of the plasma and lymphocyte separation medium, were diluted 1:3 with sterile saline, and were collected by centrifugation at 250 g for 10 minutes at 4° C. After washing once in RPMI 1640, the PBMC (3 × 10⁶ cells/ml) were activated by culture with 3 µg/ml phytohemagglutinin (PHA) in RPMI 1640 containing 2 mM L-glutamine, 50 µg/ml gentamicin, and 10 % fetal bovine serum (lymphocyte culture medium) for 72 hours in a 5% CO₂ atmosphere.^{19, 20} The PHA-activated PBMC (3 × 10⁶ cells/ml) were cultured in lymphocyte culture medium (supplemented with 20 units/ml of recombinant IL-2) alone, in culture medium containing 3,000 TCID₅₀/ml HIV-1_{BaL}, or in culture medium containing 3,000 TCID₅₀/ml HIV-1_{BaL} plus 1 µM AZT in a T 25 cm² tissue culture flask (Corning Inc., Corning, NY). The HIV-1_{BaL} was obtained from the Molecular Infectious Disease Core Facility (Rush University Medical Center). Culture medium was replenished on day 3, and culture supernatant (conditioned medium) was harvested on day 6 by centrifugation at 300 g for 10 minutes at 4° C. The amounts of HIV-1 in the conditioned media were quantified as p24 antigen content, which was measured using a p24 ELISA (AIDS Vaccine Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) after addition of 0.2 % Triton X-100 to the samples. The conditioned media were stored frozen in aliquots at -70° C and were subjected to a single freeze-thaw cycle.

Neutrophil viability

Neutrophil viability was determined in most cases by reactivity with FITC-annexin V and uptake of propidium iodide (PI). The neutrophils (10⁶) were incubated with manufacturer recommended concentrations of FITC-conjugated annexin V and PI in apoptosis binding buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4) for 15 minutes at room temperature. The fluorescence intensity of 10,000 cells was measured ungated using a FACScan flow cytometer (BD Biosciences). In some experiments as indicated, neutrophil viability was determined by the percentage of cells exhibiting a forward-scatter and side-scatter light profile for viable neutrophils.

Neutrophil HLA-DR expression

Neutrophils (10^6) were incubated with surface marker-specific mAbs (FITC-conjugated anti-HLA-DR, PE-Cy5-conjugated anti-CD80, APC-conjugated anti-CD86, PE-conjugated anti-CD40) or with isotype controls at manufacturer recommended concentrations in PBS containing 1% mouse serum for 30 minutes on ice. Cells were collected by centrifugation at $300 \times g$ for 10 minutes, were washed twice in ice-cold PBS, and were suspended in 1% formaldehyde in PBS for analysis. The fluorescence intensity of 10,000 cells in each sample was analyzed by flow cytometry. Non-viable neutrophils were eliminated from the analysis by electronic gating on the viable cells as determined by the forward light-scatter and side light-scatter profile. Results are expressed as the percentage of neutrophils positive for the surface marker of interest after subtraction of the percentage of cells reacting non-specifically (usually $< 3\%$) with isotype control mAb.

Culture of activated PBMC with HIV-1

PBMC were isolated from the venous blood of healthy adult individuals as described above and were activated by culture (2×10^6 cells/ml) with the indicated concentrations of PHA or $1 \mu\text{g/ml}$ each of soluble anti-CD3 mAb and anti-CD28 mAb in lymphocyte culture medium for 48 hours. The activated PBMC were washed once with RPMI 1640 and then were cultured at 10^6 cells/ml in IL-2-supplemented (20 units/ml) lymphocyte culture medium alone or in medium containing 3,000 TCID₅₀/ml HIV-1_{BaL} for six days. Cultures were performed in a 24-well tissue culture plate (Corning), and total culture volume was 1 ml. Culture medium (800 μl) was carefully harvested by aspiration at day 3 of culture and was replaced by the same volume of fresh IL-2-supplemented lymphocyte culture medium. Culture media harvested at day 3 and day 6 were centrifuged at $300 g$ for 10 minutes at 4°C , and the cell-free supernatants were stored at -70°C until measurement of GM-CSF content and p24 levels. In some experiments as indicated, a portion of the activated PBMC were incubated with APC-conjugated anti-CD69 mAb and analyzed by flow cytometry as described above to confirm lymphocyte activation.

Detection of intracellular GM-CSF

PBMC of healthy adult individuals were activated by culture with $3 \mu\text{g/ml}$ of PHA or $1 \mu\text{g/ml}$ of soluble anti-CD3 mAb and anti-CD28 mAb as described above. The activated PBMC were washed once with RPMI 1640 and then were cultured at 10^6 cells/ml in IL-2-supplemented lymphocyte culture medium alone or in medium containing 3,000 TCID₅₀/ml HIV-1_{BaL} in a 24-well tissue culture plate for three days in a $5\% \text{CO}_2$ atmosphere. Phorbol myristate acetate (50 ng/ml) and 0.25 $\mu\text{g/ml}$ of A23187 plus GolgiStop (BD Biosciences) were added for the last 5 hours of culture. The cells were collected by centrifugation at $300 g$ for 10 minutes at 4°C , were washed twice in PBS containing 5% fetal bovine serum and 0.1% sodium azide, and then were incubated with FITC-conjugated anti-CD3 mAb according to manufacturer instructions for 30 minutes at 4°C . The cells were washed twice, were fixed in 1% formaldehyde in PBS, and were permeabilized using BD Cytotfix/Cytoperm according to manufacturer instructions. The cells were incubated with 0.2 μg of PE-conjugated rat anti-human GM-CSF for 45 min at 4°C . Non-specific staining was determined using PE-conjugated rat anti-human GM-CSF that had been preincubated with 0.1 μg of recombinant GM-CSF according to the manufacturer's instructions prior to addition to the cells. The fluorescence intensity of 100,000 cells in each sample was analyzed by flow cytometry. Non-viable cells were eliminated from the analysis by electronic gating on the viable cells as determined by the forward light-scatter and side light-scatter profile.

Measurement of GM-CSF

GM-CSF levels were quantified using a commercial ELISA assay (Biosource/Invitrogen, Carlsbad, CA).

Statistical analysis

Statistical analysis for HLA-DR expression by HIV-1⁺ subjects was performed using Kruskal-Wallis and Dunn's multiple comparisons test. Other statistical analyses were performed using Student's paired *t* test or one-way ANOVA and Tukey's multiple comparisons test as appropriate. A *p* value < 0.05 was accepted as statistically significant.

RESULTS

HIV-1⁻ and HIV-1⁺ conditioned medium promote GM-CSF-mediated neutrophil survival

The effect of conditioned medium (CM) collected from PHA-activated PBMC that had been cultured in the absence of HIV-1 (HIV-1⁻ CM), with HIV-1 (HIV-1⁺ CM), or with HIV-1 plus 1 μ M AZT (HIV-1⁺/AZT CM) on neutrophil survival was evaluated. HIV-1 levels in the HIV-1⁺ CM and HIV-1⁺/AZT CM were 367,895 pg/ml and 37,443 pg p24/ml, respectively. The HIV-1⁻ CM, HIV-1⁺ CM, and HIV-1⁺/AZT CM each increased the percentage of viable neutrophils present after 48 hours of culture up to approximately 80 % (Fig. 1A), which was similar to the percentage of viable neutrophils (78 ± 11 %; mean $\pm$ SE) after culture with 1 ng/ml of recombinant GM-CSF in the same experiments (results not shown). Results of a representative experiment are shown in Fig. 1B. The HIV-1⁺ CM was approximately 10-fold more potent than HIV-1⁻ CM or HIV-1⁺/AZT CM (Fig. 1A), and this difference approximated the 6-fold to 10-fold higher concentration of GM-CSF in HIV-1⁺ CM (2,420 pg/ml) compared to the GM-CSF concentration in HIV-1⁺/AZT CM (418 pg GM-CSF/ml) or in HIV-1⁻ CM (260 pg GM-CSF/ml), respectively (results not shown). In three additional experiments, addition of 2 μ g/ml of neutralizing anti-GM-CSF mAb to the neutrophil cultures completely blocked the increase in neutrophil viability caused by a 1:170 dilution (corresponding to 1.5 pg GM-CSF/ml) of HIV-1⁻ CM and inhibited by 69 ± 11 % (mean $\pm$ SEM) the larger increase in neutrophil viability caused by a 1:170 dilution (corresponding to 15 pg GM-CSF/ml) of HIV-1⁺ CM (Fig. 1C).

HIV-1⁺ conditioned medium induces GM-CSF-mediated expression of HLA-DR by neutrophils

The abilities of HIV-1⁻ and HIV-1⁺ CM to induce HLA-DR expression by neutrophils were examined in similar manner. In three experiments, each using neutrophils of a different donor, HIV-1⁻ CM at dilutions of 1:500 to 1:50 (corresponding to 0.5 to 5 pg GM-CSF/ml) did not induce HLA-DR expression (Fig. 2A), whereas the same dilutions of HIV-1⁺ CM (corresponding to 5 to 50 pg GM-CSF/ml) induced HLA-DR expression by up to approximately 60 % of the neutrophils in a concentration-dependent manner (Fig. 2A). Recombinant GM-CSF (1 ng/ml) induced HLA-DR expression by 64 ± 10 % (mean $\pm$ SE) of the neutrophils in the same experiments. Results of a representative experiment are shown in Fig. 1B. The results in Fig. 2C show that addition of 2 μ g/ml of neutralizing anti-GM-CSF mAb to the neutrophil cultures inhibited HLA-DR expression induced by HIV-1⁺ CM (1:170 dilution; 15 pg GM-CSF/ml) by 68 ± 3 % (mean $\pm$ SE). The neutralizing anti-GM-CSF mAb also inhibited HLA-DR expression induced by 1 ng/ml of GM-CSF by 65 ± 10 % in the same experiments (Fig. 2C). The results in Fig. 2D demonstrated that the ability of HIV-1⁺ CM (1:170 dilution; 15 pg GM-CSF/ml) to induce HLA-DR expression paralleled that of 1 ng/ml of recombinant GM-CSF. The HIV-1⁺ CM induced HLA-DR expression by neutrophils of four healthy individuals (subjects 1 to 4) previously established to express HLA-DR in response to GM-CSF but not by neutrophils of an individual (subject 5)

previously established to express minimal HLA-DR in response to GM-CSF (Fig. 2D). In results not shown, neither HIV-1⁺ CM nor recombinant GM-CSF induced expression of CD80, CD86, or CD40 by neutrophils of subjects 1 to 4 in the same experiments.

HIV-1 infection variably increases GM-CSF production by PHA-activated PBMC

The ability of HIV-1 infection to enhance GM-CSF production by activated PBMC was evaluated further using two experimental protocols. Initially, HIV-1⁻ CM, HIV-1⁺ CM, and HIV-1⁺/AZT CM were again generated in bulk in tissue culture flasks using PHA-activated PBMC (3×10^6 cells/ml) of two additional individuals. The HIV-1 levels in HIV-1⁺ CM and HIV-1⁺/AZT CM were $218,210 \pm 61,107$ (mean $\pm$ range) pg p24/ml and $4,022 \pm 329$ pg p24/ml, respectively. In the two experiments, HIV-1 infection caused a 1.4 to 2-fold increase in GM-CSF production by PHA-activated PBMC, with GM-CSF levels of $1,362 \pm 261$ pg/ml (mean $\pm$ range) in the HIV-1⁻ CM, $1,966 \pm 350$ pg/ml in HIV-1⁺ CM, and $1,004 \pm 313$ pg/ml in HIV-1⁺/AZT CM (results not shown). Given the higher amounts of GM-CSF produced by the PHA-activated PBMC alone in the two experiments, the effect of HIV-1 infection on GM-CSF production was then evaluated in a non-bulk format (24-well tissue culture plate) using PBMC (10^6 cells/ml) activated by a range (0.3 to 3 μ g/ml) of PHA concentrations or by 1 μ g/ml of soluble anti-CD3/anti-CD28 mAbs. In six experiments, each using PBMC of a different individual, PHA alone stimulated minimal or no GM-CSF production after either three days (Fig. 3A) or six days (Fig. 3B) of culture. Infection by HIV-1 caused a small, but not statistically significant, increase in GM-CSF production stimulated by 3 μ g/ml of PHA at day 3 (Fig. 3A). The HIV-1 did, however, significantly enhance GM-CSF production stimulated by 3 μ g/ml of PHA at day 6 (Fig. 3B). Interestingly, HIV-1 infection caused pronounced increases in PHA-stimulated GM-CSF production by PBMC of three of the six individuals tested and smaller, though statistically significant, increases by PBMC of the other three individuals (Fig. 3C). In contrast, soluble anti-CD3/anti-CD28 stimulated significant levels of GM-CSF production at both the day 3 and day 6 time points, but HIV-1 infection did not increase the level of GM-CSF production at either time point (Figs. 3A and B). Instead, HIV-1 infection was associated with a diminished level of GM-CSF production by anti-CD3/anti-CD28-activated PBMC at day 6, although the decline was not statistically significant (Fig. 3B). The levels of HIV-1 infection in the PHA-activated PBMC and the CD3/CD28-activated PBMC were $38,290 \pm 18,691$ pg p24/ml and $30,743 \pm 13,761$ pg p24/ml, respectively, at day 6 in the six experiments (results not shown). Measurement of CD69 expression in four of the six experiments confirmed that PHA and anti-CD3/anti-CD28 mAbs each caused marked lymphocyte activation, with activation by PHA occurring in a concentration-dependent manner (Fig. 3D).

CD3⁺ lymphocytes are a cellular source of GM-CSF in the PBMC cultures

PHA (3 μ g/ml)-activated PBMC and anti-CD3/CD28-activated PBMC were cultured for three days in the presence and absence of HIV-1 and then stained for surface CD3 and intracellular GM-CSF to identify the cellular source of GM-CSF production in the PBMC. In two experiments, each using PBMC of a different individual, 3 % and 1 % of the PHA-activated PBMC and 7 % and 21 % of the anti-CD3/CD28-activated PBMC were positive for CD3 and GM-CSF (Fig. 4). The percentage of CD3⁺GM-CSF⁺ cells in CD3/CD28-activated PBMC cultures diminished to 4 % and 5 %, respectively, in the two experiments in the presence of HIV-1. In contrast, HIV-1 either had no effect or slightly increased the percentage of CD3⁺GM-CSF⁺ cells in the PHA-activated PBMC cultures. None of the CD3-negative cells stained positive for GM-CSF in the absence or presence of HIV-1.

Neutrophils of HIV-1⁺ elite controllers express a minimal HLA-DR response to GM-CSF

As reported previously³⁴ and illustrated above, (c.f. Fig. 2D), the ability of GM-CSF to induce HLA-DR expression varies among neutrophils of individual donors. To see how this

variability extended to neutrophils of HIV infected individuals, we evaluated the effect of GM-CSF on HLA-DR expression by neutrophils of 12 HIV-1⁺ subjects and, for comparison, by neutrophils of 17 randomly selected healthy adults. The 12 HIV-1⁺ individuals were comprised of four individuals with a low viral load (LVL) on anti-retroviral therapy, four individuals with a high viral load (HVL), and four individuals who met the criteria of elite controllers (> 7 years) (EC).³⁶ The viral loads, CD4 cell counts, percentages of CD4 cells, and neutrophil yields for the HIV-1⁺ individuals are summarized in Table 1. Neutrophils of the HIV-1⁺ individuals did not express HLA-DR ex vivo and were greater than 90 % viable ex vivo, as determined by reactivity with FITC-annexin V (results not shown). Recombinant GM-CSF, as expected,³⁴ induced a range of HLA-DR expression by neutrophils of the 17 healthy individuals (Fig. 5A), which was accompanied by GM-CSF-induced increases in neutrophil viability, as determined by the forward-scatter and side-scatter light profiles (Fig. 5B). The GM-CSF also induced a range of levels of HLA-DR expression by viable neutrophils of the four LVL individuals and the four HVL individuals (Fig. 5A). In contrast, GM-CSF induced minimal HLA-DR expression by neutrophils of the four EC individuals (Fig. 5A), such that the mean level of HLA-DR⁺ neutrophils (4 ± 2 %; mean $\pm$ SE) was significantly less than that for neutrophils of the four LVL individuals (27 ± 6 %) after culture with GM-CSF. GM-CSF induced an intermediate level of HLA-DR expression (15 ± 6 % HLA-DR⁺ cells) by neutrophils of the four HVL subjects. GM-CSF did, however, significantly increase the percentage of viable EC neutrophils detected after culture (Fig. 5B). GM-CSF also increased the percentage of viable HVL and LVL neutrophils, although the increases were not statistically significant. The somewhat higher percentage of viable HVL and LVL neutrophils after culture in the absence of GM-CSF (Fig. 5B) reflects, in each case, an elevated percentage of viable neutrophils for one of the four subjects (results not shown).

DISCUSSION

The results presented here demonstrate that conditioned medium collected from PHA-activated PBMC (HIV-1⁻ CM) and from PHA-activated PBMC after infection with HIV-1_{BaL} (HIV-1⁺ CM) prolonged neutrophil survival in culture and that HIV-1⁺ CM also induced expression of class II HLA-DR molecules by neutrophils. The ability of GM-CSF to prolong neutrophil survival^{31–33} and induce HLA-DR expression by neutrophils³⁴ is well established, and four findings demonstrate that GM-CSF largely mediated the effects of HIV-1⁻ CM and HIV-1⁺ CM on neutrophil survival and HLA-DR expression. First, the relative abilities of HIV-1⁻ CM and HIV-1⁺ CM to prolong neutrophil survival or induce HLA-DR expression by neutrophils paralleled their GM-CSF content. Second, addition of neutralizing anti-GM-CSF mAb to the neutrophil cultures completely blocked the pro-survival activity of HIV-1⁻ CM at a dilution equivalent to 1.5 pg GM-CSF/ml and inhibited the pro-survival activity of the HIV-1⁺ CM at a dilution equivalent to 15 pg GM-CSF/ml by approximately 70 %. Third, neutralizing anti-GM-CSF mAb inhibited by approximately 70 % the ability of HIV-1⁺ CM (at the dilution equivalent to 15 pg GM-CSF/ml) to induce neutrophil HLA-DR expression. Fourth, the ability of HIV-1⁺ CM to induce HLA-DR expression paralleled the same activity of recombinant GM-CSF. Interferon- γ and IL-3 have also been reported to have a more modest effect on HLA-DR expression by neutrophils,³⁴ but several other cytokines, including G-CSF, IL- β , TNF- α , and TGF- β , do not induce neutrophil HLA-DR expression, at least over the time frame (24–48 hours) in which GM-CSF induces HLA-DR expression.³⁴

The effect of HIV-1 infection on GM-CSF production by PHA-activated PBMC was evaluated in bulk culture and in non-bulk culture. The level of GM-CSF production by PHA-activated PBMC varied in the two systems, with PHA alone stimulating a large amount of GM-CSF production in two of the three bulk culture experiments but stimulating

little or no GM-CSF production in the six experiments using the non-bulk format. The lower level of GM-CSF production by the PHA-activated PBMC alone and after HIV-1 infection in the non-bulk format likely reflects the lower concentration of PBMC as well as the lower level of HIV-1 infection, as measured by the p24 antigen levels. Importantly, HIV-1 infection enhanced GM-CSF production by PHA-activated PBMC in all nine experiments, each of which used PBMC of a different individual. The effect of HIV-1 infection required PBMC activation by the highest concentration (3 $\mu\text{g/ml}$) of PHA tested in the six non-bulk culture experiments and required six days of culture to become statistically significant. The effect of HIV-1 infection on PHA-activated PBMC, however, was not uniform, as HIV-1 infection caused a markedly higher level of GM-CSF production by PHA-activated PBMC in three of the six experiments. In contrast to the results obtained with PHA-activated PBMC, anti-CD3/CD28-activated PBMC produced significant amounts of GM-CSF after both three and six days of culture in the same six experiments, but infection by HIV-1 did not cause a further increase in the level of GM-CSF production. Rather, HIV-1 infection diminished somewhat the level of GM-CSF production by the anti-CD3/CD28-activated PBMC. Importantly, the levels of GM-CSF produced by activated PBMC alone or after infection by HIV-1 in both the bulk cultures and the non-bulk cultures are in the range of GM-CSF concentrations (5 to 50 pg/ml) that promote significant increases in neutrophil survival and induce significant levels of HLA-DR expression, as determined by the results obtained with HIV-1⁻ CM and HIV-1⁺ CM (c.f. Figs. 1A and 2A).

It is unlikely that the failure of HIV-1 infection to enhance GM-CSF production by anti-CD3/CD28-activated PBMC reflected differences in the level of HIV-1 infection or of lymphocyte activation. The level of HIV-1 infection, as measured by p24 antigen level, was comparable for both PHA-activated and anti-CD3/CD28-activated PBMC, and activation of the PBMC by anti-CD3/CD28 produced a higher percentage of activated lymphocytes, as measured by the level of CD69 expression, than did activation by the highest concentration (3 $\mu\text{g/ml}$) of PHA tested. Instead, the failure of HIV-1 infection to enhance GM-CSF production by anti-CD3/CD28 activated PBMC may reflect loss of CD4 lymphocytes as a consequence of HIV-1 infection. Intracellular staining for GM-CSF showed that GM-CSF production by both PHA-activated PBMC and anti-CD3/CD28-activated PBMC was limited to CD3⁺ cells and that HIV-1 infection reduced the percentage of CD3⁺GM-CSF⁺ cells in the anti-CD3/CD28-activated PBMC cultures. Although CD8 T cells produce GM-CSF,³⁷ we presume that CD4 cells constitute the CD3⁺GM-CSF⁺ cells.

As reported previously³⁴ and as illustrated here (Figs. 2D and 5A), the ability of GM-CSF to induce HLA-DR expression varies among neutrophils of different individuals, with neutrophils of some individuals minimally expressing HLA-DR in response to GM-CSF. Neutrophils of the 12 HIV-1⁺ individuals also exhibited a range of HLA-DR responses, although the distribution was not uniform across all three classifications of HIV-1 disease. Neutrophils of HVL and LVL subjects each displayed a range of HLA-DR responses, whereas neutrophils of the four individuals classified as elite controllers (EC)³⁶ expressed minimum levels of HLA-DR in response to GM-CSF. Consequently, the mean percentage of HLA-DR⁺ neutrophils after culture of the EC neutrophils with GM-CSF was significantly less than that for LVL neutrophils after culture with GM-CSF. The four EC individuals did not differ, however, in viral load, CD4 cell count, or CD4 cell percentage from the four LVL individuals. The failure of GM-CSF to induce greater levels of HLA-DR expression by neutrophils of EC individuals also cannot be attributed to increased loss of neutrophil viability or general unresponsiveness to GM-CSF. In agreement with previous reports that neutrophils of HIV-1⁺ individuals are viable upon isolation,^{5, 7} the viability of EC neutrophils as well as HVL and LVL neutrophils exceeded 90 % ex vivo and, importantly, GM-CSF significantly prolonged survival of the EC neutrophils. Other have also reported previously that differences in GM-CSF-induced HLA-DR expression occur in the absence of

differences in the pro-survival effect of GM-CSF.³⁴ Together, these results suggest that EC individuals belong, as a group, to the category of individuals whose neutrophils express minimal HLA-DR in response to GM-CSF.

The ability of GM-CSF to prolong neutrophil survival and induce HLA-DR expression by neutrophils has direct implications for HIV-1 infection. Infiltrating neutrophils encounter CD4 T cells present in the mucosa,³⁸ and neutrophils have also been identified in the lymph nodes of HIV-1-infected individuals.^{25, 26} GM-CSF is produced by a number of cells^{39, 40}, including cells in the mucosa^{30, 41} and in lymph nodes⁴² and in concentrations shown here to increase neutrophil survival and induce HLA-DR expression.⁴² Together, these findings provide a mechanism for sustained contact between viable neutrophils and CD4 T lymphocytes in the mucosa or lymph nodes that, in turn, may facilitate infection of the CD4 cells by neutrophil-bound HIV-1.^{19, 20} That neutrophils may transport the bound HIV-1 to the lymph nodes is supported indirectly by studies in mice showing that neutrophils carry antigen²⁷ as well as live organisms²⁸ from peripheral tissues to the draining lymph nodes. Additionally, HLA-DR⁺ neutrophils support CD4 lymphocyte activation by bacterial superantigens.^{43, 44} and, thus, may facilitate immune activation associated with increased permeability within the gastrointestinal tract.^{21, 22, 24, 45} Together, these findings indicate that GM-CSF-induced increases in neutrophil viability and in the percentage of HLA-DR⁺ neutrophils may serve to promote both acute and chronic HIV-1 infection. The finding here that neutrophils of EC individuals minimally express HLA-DR in response to GM-CSF provides indirect support for this conclusion.

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Figure 1.

HIV-1⁻ and HIV-1⁺ CM prolong neutrophil survival in culture in a GM-CSF-dependent manner. (A) Neutrophils were cultured with the indicated dilutions of HIV-1⁻ CM, HIV-1⁺/AZT CM, or HIV-1⁺ CM for 48 hours. The corresponding GM-CSF concentration at each dilution of CM is indicated. The percentage of viable neutrophils (negative for FITC-annexin V and PI) is expressed as the mean $\pm$ SE for three experiments, each using neutrophils of a different donor. * $p < 0.05$ compared to the HIV-1⁻ CM. (B) Results of one of the experiments in panel A are shown for culture medium alone (Spont), a 1:170 dilution of HIV-1⁻ CM, HIV-1⁺ CM, or HIV-1⁺/AZT CM, or 1 ng/ml of recombinant GM-CSF. (C) Neutrophils were cultured in culture medium alone (Spont) or with a 1:170 dilution of HIV-1⁻ CM or HIV-1⁺ CM in the absence or presence of 2 μ g/ml of mouse IgG1 or neutralizing anti-GM-CSF mAb for 48 hours. The percentage of viable neutrophils is expressed as the mean $\pm$ SE for three experiments, each using neutrophils of a different donor. * $p < 0.05$ compared to the corresponding spontaneous value.

Figure 2.

HIV-1⁺ CM induces GM-CSF-mediated expression of HLA-DR by neutrophils. (A) Neutrophils were cultured with the indicated dilutions of HIV-1⁻ CM or HIV-1⁺ CM for 48 hours. The GM-CSF concentration for each dilution of CM is indicated. The percentage of HLA-DR⁺ neutrophils is expressed as the mean $\pm$ SE for three experiments, each using neutrophils of a different donor. * $p < 0.05$ compared to the HIV-1⁻ CM. (B) Results of one of the experiments in panel A are shown for culture medium alone (Spont), a 1:170 dilution of HIV-1⁻ CM, HIV-1⁺ CM, or HIV-1⁺/AZT CM, or 1 ng/ml of recombinant GM-CSF. (C) Neutrophils were cultured in culture medium alone (Spont), with 1 ng/ml GM-CSF, or with HIV-1⁺ CM (15 pg GM-CSF/ml) in the absence or presence of 2 μ g/ml of mouse IgG1 or neutralizing anti-GM-CSF mAb for 48 hours. The percentage of HLA-DR⁺ neutrophils is expressed as the mean $\pm$ SE for five experiments, each using neutrophils of a different donor. * $p < 0.05$ compared to the percentage of HLA-DR⁺ neutrophils incubated in the absence of mAb. (D) Neutrophils of five individuals were cultured for 48 hours in medium alone (Spont), with 1 ng/ml GM-CSF, or with a 1:170 dilution of HIV-1⁻ CM or HIV-1⁺ CM. The percentage of HLA-DR⁺ neutrophils for each individual is shown.

Figure 3.

HIV-1 infection increases GM-CSF production by PHA-activated PBMC. (A, B) PBMC were activated by the indicated concentrations of PHA or by soluble anti-CD3/anti-CD28 mAbs and then were cultured alone or in the presence of HIV-1 in a 24-well tissue culture plate. GM-CSF content in the culture medium was quantified after (A) three days and (B) six days of culture and is expressed as the mean $\pm$ SE for six experiments, each using PBMC of a different donor. (C) Results obtained for PHA (3 μ g/ml)-activated PBMC in the six experiments in panel B are grouped according to the level of GM-CSF production in the presence of HIV-1. (D) Expression of CD69 by the PHA-activated PBMC and the anti-CD3/CD28-activated PBMC was assessed prior to the cultures in four of the six experiments depicted in panels A-C. The percentage of CD69⁺ cells was quantified by flow cytometry, and the results are expressed as the mean $\pm$ SE for the four experiments. * $p < 0.05$ compared to the corresponding value for PHA alone

Figure 4.

CD3⁺ lymphocytes are a cellular source of GM-CSF in the PBMC cultures. PHA (3 μ g/ml)-activated PBMC and anti-CD3/CD28-activated PBMC were cultured for three days in the presence and absence of HIV-1. Phorbol myristate acetate and A23187 plus GolgiStop were added for the final 5 hours of culture, and expression of surface CD3 and intracellular GM-CSF were quantified by flow cytometry. PE-conjugated anti-GM-CSF that had been preincubated with 0.1 μ g of recombinant GM-CSF was used to set non-specific background reactivity at 0 % GM-CSF⁺ cells. Results of two experiments, each using PBMC of a different individual, are presented.

Figure 5.

GM-CSF induces minimal HLA-DR expression by neutrophils of HIV-1⁺ EC individuals. (A) Neutrophils of 17 healthy individuals and 12 HIV-1⁺ individuals (four HVL, four LVL, and four EC) were cultured in the absence or presence of 1 ng/ml of GM-CSF for 48 hours. The percentage of HLA-DR⁺ viable neutrophils for each individual is shown after subtraction of non-specific staining, which was routinely < 3 % of the cells. The horizontal bar denotes the mean percentage of HLA-DR⁺ neutrophils. (B) The viability of the neutrophils in Panel A was determined as the percentage of neutrophils with a forward-scatter and side-scatter light profile of viable neutrophils. The percentage of viable neutrophils after culture in the absence (Spont) and presence of GM-CSF is expressed as the mean $\pm$ SE. * $p < 0.05$ compared to the corresponding spontaneous value

Table 1Characteristics of HIV-1⁺ Subjects

Subjects	Viral Load (RNA copies/ml)	CD4 Cells/ μ l	% CD4 Cells	Neutrophil Yield (cells/ml blood)
LVL (n=4)	143 $\pm$ 85	565 $\pm$ 70	26 $\pm$ 1	2.2 $\pm$ 0.9 $\times 10^6$
HVL (n=4)	252,109 $\pm$ 128,453 *	69 $\pm$ 22 *	7 $\pm$ 3 *	1.3 $\pm$ 0.9 $\times 10^6$
EC (n=4)	83 $\pm$ 12	815 $\pm$ 129	26 $\pm$ 4	3.5 $\pm$ 1.1 $\times 10^6$

Values are the mean $\pm$ SE.

*
p < 0.05 compared to values for LVL subjects