The human Pk histo-blood group antigen provides protection against HIV-1 infection

Nicole Lund,1,2 Martin L. Olsson,3 Stephanie Ramkumar,1 Darinka Sakac,2 Vered Yahalom,4 Cyril Levene,4 Åsa Hellberg,3 Xue-Zhong Ma,5,6 Beth Binnington,7 Daniel Jung,8,9 Clifford A. Lingwood,1,7,10 and Donald R. Branch,1,2,5,6

1Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON; 2Canadian Blood Services, Toronto, ON; 3Division of Hematology and Transfusion Medicine, Department of Laboratory Medicine, Lund University & University Hospital Blood Centre, Lund, Sweden; 4Magen David Adom National Blood Services, Ramat Gan, Israel; 5Department of Medicine, University of Toronto, Toronto, ON; 6Division of Cell and Molecular Biology, Toronto General Research Institute of the University Health Network, Toronto, ON; 7Research Institute, Hospital for Sick Children, Toronto, ON; 8Héma-Québec Research & Development, Québec, QC; 9Department of Microbiology and Biochemistry, Laval University, Québec, QC; and 10Department of Biochemistry, University of Toronto, Toronto, ON

Several human histo-blood groups are glycosphingolipids, including P/P1/Pk. Glycosphingolipids are implicated in HIV-host-cell-fusion and some bind to HIV-gp120 in vitro. Based on our previous studies on Fabry disease, where Pk accumulates and reduces infection, and a soluble Pk analog that inhibits infection, we investigated cell surface–expressed Pk in HIV infection. HIV-1 infection of peripheral blood–derived mononuclear cells (PBMCs) from otherwise healthy persons, with blood group P1k, where Pk is overexpressed, or blood group p, that completely lacks Pk, were compared with draw date–matched controls. Fluorescence-activated cell sorter analysis and/or thin layer chromatography were used to verify Pk levels. P1k PBMCs were highly resistant to R5 and X4 HIV-1 infection. In contrast, p PBMCs showed 10- to 1000-fold increased susceptibility to HIV-1 infection. Surface and total cell expression of Pk, but not CD4 or chemokine coreceptor expression, correlated with infection. Pk liposome–fused cells and CD4+ HeLa cells manipulated to express high or low Pk levels confirmed a protective effect of Pk. We conclude that Pk expression strongly influences susceptibility to HIV-1 infection, which implicates Pk as a new endogenous cell-surface factor that may provide protection against HIV-1 infection. (Blood. 2009;113:4980-4991)

Introduction

HIV-1 infection and development of AIDS vary greatly among persons and populations and are probably, at least in part, dependent on genetic factors.1 Indeed, the first natural resistance factor reported for HIV infection was a polymorphism within the CCR5 HIV-1 coreceptor gene, termed CCR5-Δ32.1,2

However, no genetic factors thus far have been able to adequately explain the variability in both in vitro and in vivo susceptibility to HIV-1 infection.2,3

There is a longstanding association between pathogens and histo-blood groups, both in protection conferred by a specific blood type and in pathogen interactions with blood group antigens.4 The P/P1/Pk blood group antigens are of particular interest, with many defined pathogen interactions,4,7 and an expression profile not limited to erythrocytes. Galabiose (Galβ1-3GalNAcβ1) is the terminal structure of P1 and Pk, also known as globotriaosylceramide (Gb3) and a marker for germinal center B lymphocytes (CD77).8 Pk is the precursor for the P antigen, also known as globotetraosylceramide (globoside, Gb4), which terminates with 1-4GalNAc transferase.9,11 and consequently ex- pressed precursor, Pk. These persons may express P1 antigen (P1k phenotype) or not (P2k), but the molecular basis for this is still unclear.10 Persons without any P/P1/Pk antigens have mutations in the A4GALT gene (α4Gal transferase)9,11 and consequently expressed levels of precursor, Pk. These persons may express P1 antigen (P1k phenotype) or not (P2k), but the molecular basis for this is still unclear.10 Persons without any P/P1/Pk antigens have mutations in the A4GALT gene (α4Gal transferase)9,11 and consequently expressed levels of precursor, Pk. These persons may express P1 antigen (P1k phenotype) or not (P2k), but the molecular basis for this is still unclear.10

The P and Pk antigens are glycosphingolipids (GSLs), and GSLs play an important role in HIV-host cell interactions.15-18 HIV envelope glycoprotein gp120 targets CD4 and CCR5 or CXCR4 chemokine coreceptors on monocytes and T cells, as the major HIV-host cell interaction,19 but HIV gp120 also binds to several GSLs in vitro, including Pk.15-17,22 GSL interactions are mediated by a sphingolipid recognition motif on the gp120-V3 loop, thought to facilitate post-CD4 binding and membrane fusion.18,22 Inhibition of GSL biosynthesis can prevent HIV-host cell membrane fusion and infection.23,24 This can be overcome by reintroduction of purified GSLs, or overexpression of CD4 and CXCR4, suggesting that GSLs have a facilitative role.23,24,25,26 and to a lesser extent GM3, has appeared to be primarily implicated in augmenting HIV-membrane fusion, at least in vitro reconstitution models.24

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

© 2009 by The American Society of Hematology
### Table 1. P/GLOB-related blood group phenotypes and frequencies

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Antigen present on red blood cells</th>
<th>Frequency of red blood cell phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₁⁺</td>
<td>P₁, P, P₁⁺</td>
<td>75%–80%</td>
</tr>
<tr>
<td>P₂⁺</td>
<td>P₁⁺, P</td>
<td>20%–25%</td>
</tr>
<tr>
<td>p⁻</td>
<td>None</td>
<td>~ 5 per 10⁶</td>
</tr>
<tr>
<td>P₁⁺⁺</td>
<td>P₁⁺, P</td>
<td>~ 1 per 10⁶</td>
</tr>
<tr>
<td>P₂⁺⁺</td>
<td>P⁺</td>
<td>~ 1 per 10⁶</td>
</tr>
</tbody>
</table>

*According to the International Society of Blood Transfusion working party on terminology of red cell-surface antigens, the P blood group system only contains the P₁ antigen, whereas the GLOB blood group system includes the P antigen. The remaining related antigens (P⁰ and LKE, not mentioned here) are part of the GLOB blood group collection.

†Phenotypic frequencies are for whites.

In contrast, our recent work suggested P⁺, when accumulated because of a lack of activity of α-galactosidase A in Fabry disease, is protective against R5 HIV-1. In addition, a soluble analog of Pk is important role for Pk in HIV infection.29

remaining related antigens (Pk and LKE, not mentioned here) are part of the GLOB blood group system, whereas the GLOB blood group system includes the P antigen. The significant differences that reveal Pk status to be an important factor in HIV infection.29

In light of these findings, we have now assessed HIV-1 susceptibility of PBMCs that are naturally high in Pk (P₁k phenotype) or naturally devoid of Pk (p phenotype). In addition, we have genetically and biochemically manipulated Pk expression in HIV-1–infectable Pk-expressing non-T cells has further implicated an important role for Pk in HIV infection.29

### Methods

#### Cells and chemicals

Waste buffy coat material from anonymous regular blood donors was from the Lund University Hospital Blood Center (Lund, Sweden). This provision complies with current national regulation regarding the use of superfluous material from blood donations where the donor origin cannot be traced. Consent was obtained at the time of donation. Waste buffy coat material was provided from various centers with informed consent according to the Declaration of Helsinki from the donors of P₁k and p phenotype blood and was made anonymous for this study. The protocol was reviewed and approved by the Swedish National Board of Health and Welfare (National Board of Health and Welfare). Waste buffy coat material from anonymous regular blood donors was from the Lund University Hospital Blood Center (Lund, Sweden). This provision complies with current national regulation regarding the use of superfluous material from blood donations where the donor origin cannot be traced. Consent was obtained at the time of donation. Waste buffy coat material was from various centers with informed consent according to the Declaration of Helsinki from the donors of P₁k and p phenotype blood and was made anonymous for this study. The protocol was reviewed and approved by the Swedish National Board of Health and Welfare (National Board of Health and Welfare). In light of these findings, we have now assessed HIV-1 susceptibility of PBMCs that are naturally high in Pk (P₁k phenotype) or naturally devoid of Pk (p phenotype). In addition, we have genetically and biochemically manipulated Pk expression in HIV-1–infectable Pk-expressing non-T cells has further implicated an important role for Pk in HIV infection.29

#### Blood group characterization

Blood samples acquired for this study were characterized extensively for categorization as control (P₁ or P₂), p, or P₁k. Standard serologic techniques determined the erythrocyte phenotype and antibody specificities of blood samples. DNA was isolated from whole blood with the Qiagen QIAamp Blood Extraction kit (QIAGEN, Hilden, Germany). Genotypic characterization of samples was performed as reported.55 (Tables 2, 3).

#### Viruses and in vitro infections

X4 HIV-1ⅢM and R5 HIV-1ⅢB, were from the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH: HTLVⅢM = HIV-1ⅢM from Dr Robert Gallo, HIV-1ⅢB, from Dr Suwanee Gartner, HIV-1ⅢB from Dr Irvin Chen, HIV-1ⅢA-M from Dr Howard Gendelman, and HIV-1ⅢA-L from Trimeris (Durham, NC). HIV-1ⅢB viral stocks were grown in Jurkat C cells, and multiplicity of infection (MOI) was determined as described using MT-4 cells.30 All other viral stocks were grown in PBMCs, and infectious dose calculated from total p24ag levels measured by enzyme-linked immunosorbant assay (ELISA; Beckman Coulter, Fullerton, CA or ZeptoMetrix, Buffalo, NY). Briefly, cells were incubated with HIV-1 for 1 hour at 37°C, the cells washed extensively with phosphate-buffered saline (PBS), and cultured in complete medium. Culture supernatant aliquots were taken 2 hours after initial viral infection and subsequent time points. To determine viral production, ELISA was used to measure p24ag antigen levels.

#### FACS analysis

Fluorescence-activated cell sorter (FACS) analysis was performed as previously described using PHA- and PHA/IL-2–activated PBMCs and 1.5 µg monoclonal mouse anti-GM3 or anti-Pk (both from Seikagaku, Japan, Japan).30 Alternatively, 12.5 µg/mL monoclonal mouse anti-CCR5 (clone 45549.111, NIH AIDS Research and Reference Reagent Program) was used. Secondary antibodies were either 5 µg/mL allophycocyanin (APC)–labeled goat antimouse IgG (Invitrogen) or 1 µL fluorescein isothiocyanate (FITC)–labeled goat antimouse IgG (Sigma-Aldrich, St Louis, MO). For anti-GM3–labeled samples, 10 µg/mL APC-labeled goat anti-mouse IgM was used (Cedarlane Laboratories, Burlington, ON).

### Table 2. Summary of the P/GLOB-related blood group genetic and serologic findings in the rare persons whose cells were used in this study

<table>
<thead>
<tr>
<th>Sample ID in this study</th>
<th>Genetic change</th>
<th>Cellular antigens</th>
<th>Antibodies in serum†</th>
<th>Phenotype</th>
<th>Original description of the allele causing phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>p₂</td>
<td>548T&gt;G</td>
<td>A</td>
<td>No change</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>p₃</td>
<td>548T&gt;G</td>
<td>A</td>
<td>No change</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

† indicates present; and –, absent.

*The P₁ antigen is present in P₁ and P₁⁺ phenotype samples, detectable with anti-P₁ but absent in P₂ and P₂⁺ and p.

†Anti-PP1Pk is also known as anti-Tja and is only found in individuals having the p phenotype.
FACS analysis for Pκ expression of small interfering (si)RNA-transfected CD4+ HeLa cells was carried out using 5 μg/mL VT1B-Alexa 488 (produced in the Lingwood laboratory). For tricolor FACS analysis, an additional incubation with 20 μL 10% mouse serum in FACS buffer for 10 minutes at 4°C in the dark was carried out before incubation with 10 μL mouse anti-CD4-peridinin chlorophyll protein (PerCP) Cy5.5 (BD Biosciences, San Jose, CA) and/or 5 μL mouse anti–CXCRC4-phycocerythin (PE; Serotec, Oxford, United Kingdom). Data were collected with a calibrated BD (CalibRITE; BD Biosciences) Becton Dickinson FACSCalibur cell cytometer and analyzed using CellQuest software.

**TLC of GSLs**

Extraction and thin layer chromatography (TLC) separation of GSLs, including ganglioside GM3, was as previously described. Sialic acid residues were detected either by orcinol spray (Sigma-Aldrich) at 110°C for 15 minutes. Liposome fusion of Jurkat E6.1 cells was assessed by 100% co-incubation with 200 μg phosphatidylserine in 4°C in the dark was carried out before incubation with 10 μL mouse anti-CXCR4-phycocerythin (PE; Serotec, Oxford, United Kingdom). Data were collected with a calibrated BD (CalibRITE; BD Biosciences) Becton Dickinson FACSCalibur cell cytometer and analyzed using CellQuest software.

**Adenovector production**

Ad5/F35 vectors were generated as previously described by in vivo recombination in Escherichia coli BJS183 cells between pAdenoVator transfer plasmids and pAdEasy-1/F35 adenoviral genome (a generous gift from Dr X. Fan, Lund University, Lund, Sweden) using the AdenoVator Vector system (Qbiogene, Irvine, CA). Transfer plasmids containing the cytomegalovirus (CMV) promoter/enhancer with a β-globin/IgG chimeric intron (CMVI) were purchased from Qiagen. For enhanced yellow fluorescent protein (EYFP) control Ad5/F35 vectors, EYFP from pIRESEYFP (Clontech, Mountain View, CA) was cloned into the transfer plasmids. For Pκ expression, Ad5/F35 vectors containing an expression cassette encoding EYFP under the control of the mouse PGK promoter was first cloned into the CMVII transfer plasmid, and the full-length human Pκ synthase (Pκ-S) cDNA, cloned from CaCo-2 cells using primers for reverse-transcribed polymerase chain reaction that were designed based on the published sequence (GenBank database accession no. AB037883), was then cloned into the CMVII expression cassette.

Recombinant Ad5/F35 vectors were transfected into QBI-293A cells using a standard calcium phosphate transfection procedure, and recombinant viruses were plaque-purified. Viruses were then amplified by transduction of large HEK293 cell cultures. Viruses were extracted by 100% of sucrose from disrupted cells, and the virus preparations were dialyzed against Tris buffer, pH 8.5, containing 2.5% glycerol, 25 mM NaCl, and 0.5 mM EDTA. After 5 days of Pκ treatment, cells expressing or not expressing Pκ were infected with X4 HIV-1IIIB (MOI, 0.3) and subjected to FACS analysis and infection with X4 HIV-1IIIB (MOI, 0.3) where productive infection was monitored over time.

### Table 3. Summary of the P/GLOB-related blood group genetic and serologic findings in the control persons whose cells were used in this study

<table>
<thead>
<tr>
<th>Sample ID in this study</th>
<th>Genetic change</th>
<th>Cellular antigens</th>
<th>Antibodies in serum</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control a (C-a)</td>
<td>A4GALT</td>
<td>Pκ</td>
<td>None</td>
<td>Pκ</td>
</tr>
<tr>
<td>Control b (C-b)</td>
<td>B3GALNT1</td>
<td>Pκ</td>
<td>None</td>
<td>Pκ</td>
</tr>
<tr>
<td>Control p1 (C-p1)</td>
<td></td>
<td>Pκ</td>
<td>None</td>
<td>Pκ</td>
</tr>
<tr>
<td>Control p2 (C-p2)</td>
<td></td>
<td>Pκ</td>
<td>None</td>
<td>Pκ</td>
</tr>
<tr>
<td>Control p3 (C-p3)</td>
<td></td>
<td>Pκ</td>
<td>None</td>
<td>Pκ</td>
</tr>
</tbody>
</table>

*All blood group phenotypes with the exception of P, Pκ, and PκE express low levels of Pκ because of incomplete conversion to P. The amount of Pκ expressed varies from person to person.
carefully, and incubated for 20 minutes at room temperature. This mixture was added to cells, which were subsequently cultured for 24 hours. This procedure was repeated after 24 and 48 hours. The siRNA depletion of Pk-S was monitored by FACS analysis of Pk expression. CD4/H11001 HeLa cells, with demonstrated reduction in surface levels of Pk (70%), were infected with X4 HIV-1IIIB (MOI, 0.3) and aliquots of culture supernatant taken over time to monitor p24 gag production by ELISA.

Statistics

A 2-sample Student $t$-test, assuming unequal variance with 2-tailed distribution, was used to determine significance. The means of the data points for blood group phenotype were compared with their respective matched controls and represented plus or minus SEM, where $n = 4$. Data were considered statistically significant if $P$ was less than .05 or highly significant if $P$ was less than .002.

Results

P1k PBMCs are protected against R5 and X4 HIV-1 infection

We first assessed the susceptibility to HIV-1 infection of PBMCs from P1k persons. Given the rarity of these samples (Table 1), P1k PBMCs from one donor (P1k-a) were used to assess R5 HIV-1 infection and a second donor (P1k-b) to assess X4 HIV-1 infection (Tables 2 and 3 for test and control designations, respectively). Infection of PHA-activated P1k-a with R5 HIV-1 Ba-L showed significantly lower productive HIV-1Ba-L infection compared with its draw-date- and ABO-matched control (Figure 1A). PHA/IL-2–activated P1k-b were similarly protected against productive X4 HIV-1IIIB infection (Figure 1B) compared with the respective control. Based on comparison with draw-date–matched controls, infection levels for P1k PBMCs for both HIV-1Ba-L and HIV-1IIIB were less than 12% (data not shown).

CD4, coreceptor, and Pk expression are increased in P1k PBMCs

To determine whether expression levels of HIV receptors may have influenced the reduced infection levels, cell-surface CD4, CCR5, and CXCR4 levels on the same cell populations used for infection studies were analyzed by flow cytometry. PHA-activated P1k-a showed approximately 10% less CD4-expressing cells than the matched control; however, CD4 expression levels (mean fluorescence intensity [MFI]) were approximately 1.5-fold higher (Figure 1C,D left panels). There were also approximately 11% more
CCR5-expressing cells in P1k-a and slightly higher CCR5 expression (MFI/H11011 1.2-fold difference; Figure 1C,D). The percentage of R5 HIV-1-susceptible target PBMCs, expressing both CD4 and CCR5, was also slightly higher in P1k-a (Figure 1C).

PHA/IL-2-activated P1k-b demonstrated approximately 7% more CD4-expressing cells compared with control and approximately 3.5-fold higher CD4 expression levels (MFI) (Figure 1C,D right panel). In addition, there were 27% more CXCR4-expressing cells than control, and approximately 3.5-fold higher CXCR4 expression in P1k-b (Figure 1C,D). Indeed, even the percentage of cells expressing cell-surface Pk on PHA- and PHA/IL-2-activated P1k PBMCs was approximately 1.5- to 2-fold higher than that of controls (Figure 1E). The percentage of cells expressing both CD4 and Pk, which encompass HIV-1-susceptible target cells, were also found to be twice as frequent in P1k PBMCs compared with their respective controls (Figure 1F).

**p PBMCs are hypersusceptible to R5 and X4 HIV-1 infection**

We also assessed susceptibility of PBMCs from 3 Pk-deficient p persons (Table 2) to R5 and X4 HIV-1 infection. HIV-1Ba-L infection of PHA-activated PBMCs (denoted p1, p2, and p3; Table 2) resulted in much higher levels of productive infection compared with their drawdate- and ABO-matched control (Figure 2A). Following infection over time depicts exponential kinetics in HIV-1 production for p PBMCs. The difference in infection levels between p PBMCs and control showed a change of approximately 5-fold higher for p1, 12-fold higher for p2, and approximately 3000-fold higher for p3 (data not shown). This increased infection was consistent for R5 HIV-1 infection in general, as 2 other R5 strains, HIV-1Ada-M and HIV-1JR-FL, also showed much higher productive HIV-1 infection in the p PBMCs compared with control (Figure 2C).

As with R5 infection, HIV-1IIIB infection of PHA/IL-2-activated p PBMCs from 2 persons (p1 and p3) also showed much higher levels of productive infection compared with their matched controls (Figure 2B,D). However, the p2 sample showed a 2-fold lower infection level (Figure 2B center panel), but overall infection in this experiment (C-p2 and p2) was much less than for the other p PBMC experiments. For the last p1 and p3 samples analyzed, the difference in infection levels between p PBMCs and their respective controls showed a change of 3-fold higher for p1 and approximately 600- to 1000-fold higher for p3 (Figure 2B). One other X4 strain, HIV-1XL4-3 gp122, used to infect PHA/IL-2-activated p3 also showed more than 1000-fold higher productive HIV-1 infection compared with control (Figure 2D), consistent with X4 HIV-1IIIB results.

**CD4 and coreceptor expression are increased in p PBMCs**

To determine whether expression levels of HIV receptors influenced the observed susceptibility to infection, the same cell
population used for infection was subjected to flow cytometry to determine cell-surface CD4, CCR5, and CXCR4. In general, PHA-activated p PBMCs (p1, p3) presented more CD4-expressing cells than their controls (an increase of ~14%-40%), which also translated into higher CD4 expression levels (MFI, 1.3- to 3-fold higher; Figure 3A,C). This was most evident for p3, which showed the highest susceptibility to R5 HIV-1. There were also more CCR5-expressing cells in p PBMCs (p1, p2; an increase of ~12%-19%), and 3-fold higher CCR5 expression levels (MFI; Figure 3A,C). Furthermore, the percentage of R5 HIV-1–susceptible target PBMCs, expressing both CD4 and CCR5, was greater in p PBMCs (p1, p2, and slightly in p3; Figure 3A). However, p3 PBMCs showed reduced CCR5 levels (Figure 3A,C).

PHA/IL-2–activated p PBMCs (p1, p3) demonstrated more CD4-expressing cells compared with their controls (an increase of ~21%-42%), and up to 3-fold higher CD4 expression levels (MFI; Figure 3B,D). These differences were once again most evident in p3, which demonstrated the highest susceptibility to R5 HIV-1 infection. There were also more CXCR4-expressing cells in p PBMCs (p1, p2; an increase of ~10%), and overall there was 1.3- to 2.3-fold higher CXCR4 expression in p-PBMCs from both p1 and p3 samples (Figure 3B,D). The percentage of X4 HIV-1–susceptible target PBMCs, expressing both CD4 and CXCR4, was noticeably higher in p PBMCs from p3 (Figure 3B). In contrast, p2 showed a somewhat opposite expression profile, exhibiting approximately 15% less CD4 and approximately 5% less CXCR4 expressing cells compared with the matched control (Figure 3B center panel), as well as lower receptor expression levels (MFI; Figure 3D center panel).

**GM3 expression in p PBMCs does not account for increased infection**

Increased HIV-1–induced T-cell fusion has been reported in p-CD4+ T cells, ascribed to higher total levels of GM3.39 Although GM3 is reported less fusogenic than Pk,24, we investigated the possibility that GM3 levels influenced p-PBMC (p3) susceptibility to infection in our system. Total GSLs isolated from PHA-activated PBMCs revealed loss of GM3 in control compared with p-PBMCs (Figure 4A), calculated according to band intensity on the TLC plate to be approximately 3-fold different (Figure 4C). Resting or PHA/IL-2–activated PBMCs, however, showed minimal differences in total p-PBMC GM3 levels compared with the respective control (Figure 4A-C). Higher total GM3 expression in PHA-activated p PBMCs did not translate to higher percentage of cells or cell-surface GM3 expression as measured by FACS analysis (Figure 4D center panel; Figure 4E). Although there was a slightly higher percentage of GM3-expressing p PBMCs in the PHA/IL-2–activated population, only subtle differences were seen in cell-surface GM3 expression (Figure 4D right panel; Figure 4E).
Plk-liposome fusion of Jurkat T cells decreases susceptibility to X4 HIV-1

Exogenous Plk was introduced into Plk-deficient Jurkat T-cell membranes by Plk-liposome fusion. After fusion, approximately 35% of the Jurkat cell population expressed surface Plk at high levels (MFI; Figure 5A,B). Within the CD4+/H11001 target population, approximately 32% expressed both CXCR4 and Plk (Figure 5C right panel). Plk-liposome–treated cells showed no differences in CD4 or CXCR4 expression compared with PBS or PL-liposome controls (Figure 5B,C). Increased Plk expression after Plk-liposome transfer was confirmed by TLC (Figure 5E).

A significant reduction in X4 HIV-1IIIB infection was observed in the Plk supplemented Jurkat cells, being only 43% of the HIV-1IIIB infection levels of PBS, P-, or PL-liposome controls (Figure 5F).

Increase in Plk synthase shows increased expression of Plk and decreased HIV-1 infection

To confirm that Plk expression levels influence HIV-1 infection levels, we tested whether modulating the expression of Plk in CD4+ HeLa cells, which express Plk and are infectable, would correlate with subsequent HIV-1 infection (Figure 6). Cells transduced with adeno viral vector expressing Plk synthase (P-S) resulted in increased levels of total and cell surface Plk compared with nontransduced cells or cells transduced with a control adeno viral vector (Figure 6A,B). Compared with untreated cells or control adeno viral vector transduced cells, HIV-1 infection was significantly lower in the increased Plk-expressing CD4+ HeLa cells transduced with the adeno viral vector P-S (Figure 6C).

Depletion of glucosyl ceramide–based GSLs, including Plk, shows increased HIV-1 infection

P4 was used to inhibit glucosylceramide-based GSL synthesis, thus blocking the biosynthetic pathway to Plk.38 P4 treatment of CD4+/H11001 cells resulted in a substantial decrease in cell populations expressing Plk (Figure 6D). A decrease in Plk expression was also shown in the total GSL profile (Figure 6E). P4-treated cells further demonstrated significantly increased HIV-1 infection levels (Figure 6F).

Transient siRNA depletion of Plk synthase reduced Plk expression and increased HIV-1 infection

To demonstrate that specific reduction of Plk influences the level of HIV-1 infection, siRNA was used to transiently silence the Plk synthase gene, encoding the enzyme responsible for the addition of the terminal galactose to the precursor for Plk.5,10 Transfection of Plk synthase-specific siRNAs into CD4+ HeLa cells resulted in a substantial decrease in cell populations expressing Plk (Figure 6D). A decrease in Plk expression was also shown in the total GSL profile (Figure 6E). P4-treated cells further demonstrated significantly increased HIV-1 infection levels (Figure 6F).

Figure 4. FACS and TLC of GM3 expression in p PBMCs. PBMCs were either resting or stimulated with PHA or PHA/IL-2 and analyzed for total and surface expressed GM3. (A,B) TLC of total GSLs extracted from control PBMCs (C; p3: lanes 3, 5, and 7) and p PBMCs (p3: lanes 2, 4, and 6). Lane 1: GSL standards. Lanes 2 and 3: Resting PBMCs. Lanes 4 and 5: PHA-activated PBMCs. Lanes 6 and 7: PHA/IL-2-activated PBMCs. (A) TLC of total GSLs. (B) TLC overlay to confirm the position of GM3. (C) Band intensity of GSLs represented on the TLC plate in panel B was measured by ImageJ software, compensated to background levels and fold difference in p-PBMC expression levels calculated based on control values. GlcC indicates glucosylceramide; GaC, galactosylceramide; LC, lactosylceramide; Pk, globotriosylceramide; P, globoside or globotetraosylceramide; Gb5, globopentaosylceramide; GM3, ganglioside. (D) Histogram plots representing percentage of PBMCs labeled with anti-GM3 GAM-APC were analyzed, and background compensated to isotype controls. (Top panel) Control PBMCs (C-p3). (Bottom panel) p PBMCs (p3). (Left) Resting PBMCs. (Center) PHA-activated PBMCs. (Right) PHA/IL-2-activated PBMCs. (E) MFI of surface expressed GM3 was measured and fold difference calculated based on control values.
Discussion

Our findings indicate a new phenomenon of Pk-mediated reduced susceptibility to HIV-1 infection. Pk+ PBMCs, which highly express Pk on their cell surface, demonstrate lower levels of productive R5 and X4 HIV-1 infection. In contrast, p PBMCs, which do not express Pk, show a higher susceptibility to R5 and X4 HIV-1 infection. Accordingly, Pk-liposomal transfer or Pk-synthase gene transduction facilitated a reduction in HIV-1 infection, whereas GlcCer-based GSL (Pk) depletion or Pk-synthase gene silencing resulted in an increase in HIV-1 infection. Thus, higher expression of Pk in vivo and in vitro correlates with decreased HIV-1 infection, whereas a lower expression or lack of Pk expression results in increased HIV-1 infection.

Figure 5. Susceptibility of Pk-liposome–fused Jurkat T cells to X4 HIV-1 infection. Jurkat T cells lacking Pk were incubated with Pk- or P-liposomes and cultured for 18 hours, where PBS or PL-liposome controls were used. Tricolor FACS analysis was performed and scatter plots of Jurkat labeled with anti–CD4 PerCP Cy5.5, anti–CXCR4-PE, and anti–Pk GAM-FITC (or GAM-APC) were analyzed, where background was compensated to isotype controls. (A) Histogram representing percentage of cell populations expressing Pk. (B) Scatter plots representing cell populations expressing Pk and CXCR4, and gated on CD4-positive populations. (Left) PBS-treated Jurkat. (Center) PL-liposome–fused Jurkat. (Right) Pk-liposome–fused Jurkat. (C) Scatter plots representing percentage of cell populations expressing CD4 and CXCR4. (Left) PBS-treated Jurkat. (Center) PL-liposome–fused Jurkat. (Right) Pk-liposome–fused Jurkat. (D) Surface expression levels of CD4, CXCR4, and Pk are represented as MFI. (E) TLC of total GSLs extracted from control and liposome fused Jurkat cells. Lane 1: GSL standards. Lane 2: Pk-expressing B-cell line control (Daudi). Lane 3: PBS-treated Jurkat control. Lane 4: PL-liposome control. Lane 5: Pk-liposome–fused Jurkat. (F) Infection with HIV-1IIIB (MOI, 0.3) and p24 gag monitored at day 3 after infection (n = 3 or 4 infection data points). Percentage difference in infection was calculated based on PBS control infection levels, and data were pooled from 3 independent experiments to show significance between PL-liposome controls and Pk-liposomes (*P < .05, **P < .002). PBS indicates PBS control; PL or PL-Lp, phospholipid liposome control; P or P-Lp, Pk liposomes; P, Pk liposomes.
Figure 6. Molecular and chemical modulation of Pk expression. CD4⁺ HeLa cells (clone 1022) were either untreated (no vector) or transduced with control adenoviral vector alone (control [Ctrl] vector) or adenoviral vector containing full-length human Pk synthase (Pk-S) cDNA (Pk-S vector). Both the control and Pk-S vectors contained an EYFP gene to detect transduction efficiency. After 48 hours, FACS analysis was performed and scatter plots of CD4⁺ HeLa cells labeled with anti-Pk GAM-FITC were analyzed, where background was compensated to isotype controls. (A) Histogram plots representing percentage of cell populations expressing EYFP (top panel) or Pk (lower panel) for no vector control (left), control vector (center), and Pk-S vector (right). (B) VT1 overlay for Pk detection was carried out on TLC of total GSLs extracted from control and transduced CD4⁺ HeLa cells. Lane 1: GSL standards. Lane 2: Cells without adenovector (no vector). Lane 3: Cells with control adenovector (control vector). Lane 4: Cells with adenovector-expressing Pk synthase gene (Pk-S vector). (C) HIV-1 IIIB (MOI, 0.1) was used to infect CD4⁺ HeLa cells with no vector, control vector, or Pk-S vector. After 3 days, HIV-1 infection was measured by p24 gag production. Percentage difference in HIV-1 infection was calculated based on CD4⁺ HeLa cells without adenovector (no vector). Data are representative of the mean plus or minus SEM where n = 3 infection data points; *P < .05 comparing Pk-S–transduced cells to untransduced cells. This figure is representative of 3 independent experiments. (D) Histogram plots representing percentage of cell populations expressing Pk after CD4⁺ HeLa cells (clone 6C) were either untreated (control) or treated with a GSL biosynthesis inhibitor (P4-treated, 2 μM) for 5 days to deplete glucosyl ceramide based GSLs, which includes Pk. (E) VT1 overlay for Pk detection was carried out on TLC of total GSLs extracted from untreated and P4-treated CD4⁺ HeLa cells. Lane 1: Control (untreated) cells. Lane 2: P4-treated cells. Lane 3–5: GSL standards. (F) HIV-1 IIIB (MOI, 0.1) infection of untreated or P4-treated CD4⁺ HeLa cells was measured by p24 gag production after 3 days of culture. Percentage difference in HIV-1 infection of P4-treated cells was calculated based on untreated control representing 100% infection. Data are representative of the mean plus or minus SEM where n = 3 infection data points; *P < .05. This figure is representative of 3 independent experiments.
Our studies indicate that susceptibility to HIV-1 infection in p PBMCs might be influenced both by the lack of Pk antigen and by increased receptor and coreceptor expression; however, this is not the case with the Pk\textsuperscript{k} phenotype. Pk\textsuperscript{k} PBMCs demonstrated reduced susceptibility to R5 and X4 HIV-1 infection despite having increased expression of HIV-1 receptors. Thus, both rare p and Pk\textsuperscript{k} PBMCs showed increased patterns of HIV receptor and coreceptor expression, but this resulted in higher susceptibility to HIV infection only in the p PBMCs. Thus, Pk\textsuperscript{k} expression was a better indicator of susceptibility to HIV-1 infection than CD4 or chemokine coreceptor expression.

Although the presence (or absence) of Pk\textsuperscript{k} is important in blood group classification and transfusion medicine, Pk\textsuperscript{k} is not restricted to erythrocytes. Pk\textsuperscript{k} is expressed on monocyte populations, which encompass R5 HIV-1-susceptible target cells,\textsuperscript{30,41} T-lymphoblasts mostly represent X4 HIV-1-susceptible target cell populations and have been reported to express little or no Pk\textsuperscript{k}; thus, T cells are similar to the p phenotype in their lack of Pk\textsuperscript{k} expression, which may promote susceptibility to HIV-1 infection. Furthermore, variations in Pk\textsuperscript{k} expression occur in the general population,\textsuperscript{42} which could explain differences in susceptibility to HIV-1 infection seen in vitro and in vivo.

Differences in Pk\textsuperscript{k} expression could influence lipid raft composition of target cell membranes and affect CD4 and/or coreceptor localization. Lipid rafts are central to HIV infection,\textsuperscript{43} and CD4 and CCR5 are known to be associated with lipid rafts, whereas CXCR4 is not.\textsuperscript{44} However, even CD4-HIVgp120-CXCR4 associations have been demonstrated within rafts and are required for membrane fusion.\textsuperscript{45} If Pk levels were able to influence appropriate localization of CD4 and/or coreceptors in lipid rafts, because of changes in the membrane milieu, this could affect target cell susceptibility to HIV-1.

Importantly, heightened susceptibility of cells without Pk\textsuperscript{k}, and reduced susceptibility of cells that express increased Pk\textsuperscript{k}, to both X4 and R5 HIV-1 infection would argue against current models, suggesting that Pk\textsuperscript{k} is important in post-CD4-binding.\textsuperscript{22} Increased GM3 has been proposed to promote membrane fusion in p-CD4\textsuperscript{T} cells.\textsuperscript{39} However, cell-surface expression and total GM3 do not correlate with enhanced PHA- or PHA/IL-2-activated PBMC HIV-1 infection in our study, although purified target cells remain to be assessed. It is clear, however, that Pk\textsuperscript{k} is not an absolute requirement for membrane fusion and infection. HIV-gp120 binds Pk\textsuperscript{k} via the V3 loop.\textsuperscript{17,18,22} This loop also mediates chemokine coreceptor binding.\textsuperscript{46,47} Thus, Pk\textsuperscript{k} (or a soluble mimic\textsuperscript{27}) binding to gp120 may interfere with post-CD4 recognition of chemokine coreceptor binding to prevent fusion and infection. Indeed, the binding motif, XXXGPGRAFXXX,\textsuperscript{48} within the V3 loop for Pk\textsuperscript{k} binding overlaps with the consensus binding motif, S/GXXXG-PGXXXXXXXE/D,\textsuperscript{49} for chemokine coreceptors. It has also been shown that CD4 enhances gp120-Pk\textsuperscript{k} interaction,\textsuperscript{49} probably by a similar mechanism that allows for the interaction of chemokine...
coreceptor with gp120 after CD4 binding.50 Perhaps, under conditions of chemokine receptor deficiency (or the absence of CD4), P^k may thus (less efficiently) mediate viral internalization. However, when receptor levels are normal, and P^p is expressed at higher levels, P^k has the potential to interfere with the appropriate interactions between gp120 and chemokine coreceptors, thus inhibiting viral internalization (see Figure 7E for a working model).

The lack of P in P^1 cells could suggest that P^p can facilitate, rather than P^k inhibit, infection. However, the high susceptibility of the p phenotype, which lack both P and P^k, makes this unlikely. In addition, gp120 binds P^p but not P^k.51 Furthermore, the introduction of P^k by liposome transfer into a cell line deficient in P^k expression (providing a close representation to the p phenotype), confirmed the decrease in susceptibility to HIV-1 on increased P^p levels. The fact that introduction of P into this cell line does not affect HIV infection would argue against any ability to facilitate infection. Only the levels of P^p closely correlate to HIV susceptibility. This is further supported by use of a cell line, HeLa, which does not express P (Figure 7B), whereby after the introduction of the P^k synthase gene (α4Gal transferase), which increased the cell-surface expression levels of P^k was able to reduce HIV-1 infection. In addition, specific gene silencing using siRNAs to the P^k synthase gene resulted in increased HIV-1 infection.

In our previous study of Fabry patient samples,26 which present intracellular P^k accumulation because of the lack of α-galactosidase A activity, we demonstrated a reduced susceptibility to HIV-1 infection. However, because we could only detect low levels of cell-surface expressed P^k, the mechanism of reduced HIV infection was unclear. This could have involved aspects of the abnormal pathology as a result of Fabry disease and/or abnormal trafficking of necessary coreceptors for HIV-1 infection.26 Indeed, Fabry PBMCs only demonstrated a reduction in R5 HIV-1 infection, and CCR5 coreceptor was greatly decreased on the cell-surface of these patient samples. In contrast, in the current study, we show that HIV-1 infection directly correlates to increased or decreased cell-surface expression of P^k, and this is largely independent of CXCR4 or CCR5 coreceptor expression. When P^k is highly expressed on the cell surface, as is the case in P^k persons’ PBMCs, infection with HIV-1 X 4 and R5 viruses is largely reduced. However, when there is no P^k cell-surface expression, such as in p persons’ PBMCs, HIV-1 infection is potentially several logs greater than in cells having normal P^k cell-surface expression.

Although natural resistance factors to HIV infection have been actively sought, there have been no reports as yet of a cell-surface receptor that can provide a natural barrier to HIV infection.1,4 The Δ32 polymorphism in the CCR5 chemokine cell-surface receptor that provides natural resistance to HIV infection is the result of a mutation that prevents the transport of this receptor to the cell surface. Thus, persons with this polymorphism do not express the receptor for R5 viruses on their cell surface.3 We now provide the first evidence of a possible role for a naturally expressed cell-surface factor, the P^k GSL, as potentially providing some protection to both R5 and X4 strains of HIV-1. Although studies examining the incidence of the p and P^1 phenotype in cohorts of HIV-infected, HIV-exposed but uninfected, HIV progressors and nonprogressors would be desirable, the frequency of these extremely rare phenotypes, estimated for p to be 5.8 per million, and with P^k much less frequent (~1 per million)5,6 precludes these studies. Significantly, genetic studies identified chromosome 22q13-12 to be associated with HIV resistance,51 and this region contains the P^k synthase gene13 and HIV transgenic mice showed increased P^k synthesis.52 To determine whether P^k cell-surface expression may indeed represent a natural resistance factor for HIV infection, population studies are required using normal cohorts with common P^p/P^k phenotypes known to have differential P^k expression52 to assess HIV-1 susceptibility in vitro. Furthermore, analyses of HIV-1-infected and HIV-1-resistant cohorts, using genetic and serologic/flow cytometric techniques are necessary. Nonetheless, based on our findings, P^k alone provides some protection to infection with HIV-1 and studies of modulation of P^k expression, by pharmacologic29 or other intervention, may prove to be important for future HIV/AIDS treatment modalities.

Acknowledgments

The authors thank those donors whose PBMCs were used in this study, Mathieu Drouin for preparation of the P^k synthase–containing adenovector, and Dr Xiaolong Fan from Lund University for providing the Ads5/F35 adenovirus backbone.

This work was supported by the Canadian Blood Services through a graduate fellowship award (N.L.), the Canadian Institutes for Health Research via a Doctoral Research Award (N.L.) and operating grants, Canadian Institutes for Health Research (C.A.L., D.R.B.), the Ontario HIV Treatment Network (C.A.L., D.R.B.), the Canadian Association for HIV Research (C.A.L., D.R.B.), the Swedish Research Council (project no. K2005/2008-14251), the Medical Faculty of Lund University, governmental grants for clinical research to Lund University Hospital, and Region Skåne, Sweden (Å.H., M.L.O.).

Authorship

Contribution: N.L. performed experiments, analyzed data, and contributed to the writing of the manuscript; M.L.O. provided essential samples, analyzed data, and contributed to design of experiments and to the writing of the manuscript; Å.H. provided and characterized essential samples; S.R., D.S., and B.B. performed experiments; V.Y. and C.L. provided essential samples; X.-Z.M. and D.J. provided essential reagents and contributed to the writing of the manuscript; and C.A.L. and D.R.B. contributed to the design of experiments, analysis of the data, and the writing of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Donald R. Branch, Canadian Blood Services, Toronto General Research Institute, 67 College Street, Toronto, ON M5G 2M1 Canada; e-mail: don.branch@utoronto.ca.

References


