The human P<sup>k</sup> histo-blood group antigen provides protection against HIV-1 infection

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Several human histo-blood groups are glycosphingolipids, including P/P1/P<sup>k</sup>. 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 P<sup>k</sup> accumulates and reduces infection, and a soluble P<sup>k</sup> analog that inhibits infection, we investigated cell surface–expressed P<sup>k</sup> in HIV infection. HIV-1 infection of peripheral blood–derived mononuclear cells (PBMCs) from otherwise healthy persons, with blood group P<sub>1</sub><sup>k</sup>, where P<sup>k</sup> is overexpressed, or blood group p, that completely lacks P<sup>k</sup>, were compared with draw date–matched controls. Fluorescence-activated cell sorter analysis and/or thin layer chromatography were used to verify P<sup>k</sup> levels. P<sub>1</sub><sup>k</sup> 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 P<sup>k</sup>, but not CD4 or chemokine coreceptor expression, correlated with infection. P<sup>k</sup> liposome–fused cells and CD4<sup>+</sup> HeLa cells manipulated to express high or low P<sup>k</sup> levels confirmed a protective effect of P<sup>k</sup>. We conclude that P<sup>k</sup> expression strongly influences susceptibility to HIV-1 infection, which implicates P<sup>k</sup> 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/P<sup>k</sup> blood group antigens are of particular interest, with many defined pathogen interactions,4,5 and an expression profile not limited to erythrocytes. Galabiose (Galect-1-4Gal) is the terminal structure of P1 and P<sup>k</sup>, also known as globotriaosylceramide (Gb<sub>3</sub>) and a marker for germinal center B lymphocytes (CD77).8 P<sup>k</sup> is the precursor for the P antigen, also known as globotetraosylceramide (globoside, Gb<sub>4</sub>), which terminates with β1,3GalNAc.9 P1 and P<sub>2</sub> are the 2 common P/P1/P<sup>k</sup>-related blood group genotypes. P<sub>1</sub> persons (~80% of whites but only ~20% of Asians)6,10 express P and P1 but normally express moderate to low amounts of P<sup>k</sup> on their cell surfaces. P<sub>2</sub> persons (~20% of whites and ~80% of Asians)6,10 express only P and low amounts of P<sup>k</sup>. However, rare phenotypes exist, having anomalies in one or more of the P/P1/P<sup>k</sup> blood group antigens. Individuals deficient in P antigen have mutations in the B3GALNT1 gene causing lack of functional P (Gb<sub>3</sub>) synthase (β3GalNAc transferase)9,11 and consequently express increased levels of precursor, P<sup>k</sup>. These persons may express P<sub>1</sub> antigen (P<sub>1</sub><sup>k</sup> phenotype) or not (P<sub>2</sub><sup>k</sup>), but the molecular basis for this is still unclear.10 Persons without any P/P1/P<sup>k</sup> antigens have mutations in the A4GALT gene (α4Gal transferase or P<sup>k</sup> (Gb<sub>3</sub>) synthase), causing lack of P<sup>k</sup> synthesis, and the rare P blood group phenotype.11-14 (Table 1).

The P and P<sup>k</sup> 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,19-21 but HIV gp120 also binds to several GSLs in vitro, including P<sup>k</sup>.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.24 P<sup>k</sup>, and to a lesser extent GM3, has appeared to be primarily implicated in augmenting HIV-membrane fusion, at least in in vitro reconstitution models.24

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P2k Pk, P 20%–25% genetically and biochemically manipulated Pk expression in HIV-1–infectable CD4+ HeLa cells and Jurkat T cells. Our findings show significant differences that reveal Pk status to be an important factor for susceptibility to HIV-1 infection.

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 P1 and Pk phenotype blood and made anonymous for this study. The protocol was reviewed and approved by the Canadian Blood Services Institutional Review Board Committee. The regular donor controls were matched for ABO group and date of arrival, PBMCs were isolated and activated using phytohemagglutinin (PHA)/interleukin-2 (IL-2) or PHA alone as described.36 P-threo-1-phenyl-2-palmitoylaminono-3-pyrrolidino-1-propanol (P4) was purchased from Matreya (Pleasant Gap, PA).

Blood group characterization

Blood samples acquired for this study were characterized extensively for categorization as control (P1 or P2), p, or Pk.37 Standard serologic techniques determined the erythrocyte phenotype and antibody specificities of blood samples. DNA was isolated from whole blood with the Qiagen QiAmp Blood Extraction kit (QIAGEN, Hilden, Germany). Genotypic characterization of samples was performed as reported.38,39 (Tables 2, 3).

Viruses and in vitro infections

X4 HIV-1NL4-3gp41(36G) V38E, N42S from Trimeris (Durham, NC). HIV-1IIIB viral stocks were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH: HTLVIIIB from Dr Robert Gallo, HIV-1RFL from Dr Irvin Chen, HIV-1Ada-M from Dr Howard Gendelman, and HIV-1NL4-3gp41(36G) V38E, N42S from Trimeris (Durham, NC). HIV-1IIIB viral stocks were grown in Jurkat C cells, and infectious dose calculated from total p24 gag grown in PBMCs, and infectious dose calculated from total p24 gag levels30,32 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 p24 gag 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, Tokyo, Japan).26 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 IgG was used (Cedarlane Laboratories, Burlington, ON).

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, Pk, P</td>
<td>75%–80%</td>
</tr>
<tr>
<td>P2</td>
<td>Pk, P</td>
<td>20%–25%</td>
</tr>
<tr>
<td>p</td>
<td>None</td>
<td>~5 per 10^4</td>
</tr>
<tr>
<td>P1p</td>
<td>P1, P</td>
<td>~1 per 10^4</td>
</tr>
<tr>
<td>Pkp</td>
<td>Pk</td>
<td>~1 per 10^4</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 (Pp and LKE, not mentioned here) are part of the GLOB blood group collection.

†Phenotypic frequencies are for whites.

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&gt;A</td>
<td>No change</td>
<td>–</td>
<td>P, P1, Pk</td>
<td>Steffensen et al13 (2000)</td>
</tr>
<tr>
<td>p3</td>
<td>548T&gt;G&gt;A</td>
<td>No change</td>
<td>–</td>
<td>P, P1, Pk</td>
<td>Steffensen et al13 (2000)</td>
</tr>
<tr>
<td>P1p,a</td>
<td>No change</td>
<td>811G&gt;A</td>
<td>+</td>
<td>P</td>
<td>Hellberg et al15 (2002)</td>
</tr>
<tr>
<td>P1p,b</td>
<td>No change</td>
<td>538InsA</td>
<td>+</td>
<td>P</td>
<td>Hellberg et al15 (2002)</td>
</tr>
</tbody>
</table>

*The P1 antigen is present in P1 and Pk phenotype samples, detectable with anti-P1 but absent in P2 and Pp and p.
†Anti-PP1P is also known as anti-Tja and is only found in individuals having the p phenotype.
FACS analysis for P expression of small interfering (si)RNA-transfected CD4+ HeLa cells was carried out using 5 μg/mL VT1B-Alexa458 (produced in the Linding 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–CXCR4-phycocerythrin (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.33 GSL species were detected either by orcinol spray (Sigma-Aldrich) at 110°C for 10 minutes, or P selectively detected by verotoxin-1 (VT1) TLC overlay.4 For VT1 overlay, the plate was blocked with 1% bovine gelatin; and after incubation at 37°C, the plate was washed with 50 mL Tris-buffered saline (TBS), pH 7.4. The plate was incubated for 45 minutes at room temperature with purified VT1, 1 μg per 10 mL in TBS. After washing, the plate was incubated for 45 minutes with a monoclonal antiserotonin B subunit (diluted 1/2000), washed and incubated with horseradish peroxidase-conjugated goat antimouse IgG (diluted 1/2000; Bio-Rad, Hercules, CA). For GM3 immunostaining, monoclonal anti-GM3 was substituted for VT1, followed by incubation with horseradish peroxidase-conjugated goat antimouse IgG. The plate was developed for 1 to 10 minutes with a 3 mg/mL solution of 4-chloro-1-naphthol in methanol freshly mixed with 5 volumes of TBS and 1/2000 dilution of 30% H2O2. Where band intensity indicated that this treatment was sufficient to greatly reduce cell-surface P expression without cell toxicity.29 After 5 days of P4 treatment, cells indicated that this treatment was sufficient to greatly reduce cell-surface P expression without cell toxicity.

### Liposome fusion of Jurkat E6.1 cells

Jurkat E6.1 cells do not express P or P, and are highly infectable with HIV-1. P or P liposomes were prepared by drying 400 μg P or P (Sigma-Aldrich) with 200 μg phosphatidylethanolamine and 200 μg phosphatidylserine in chloroform/methanol (2:1) under nitrogen. Alternatively, control phospholipid (PL) liposomes were prepared by drying 200 μg phosphatidylethanolamine with 200 μg phosphatidylserine. Liposomes were generated by vortexing well in 400 μL PBS and sonicing for 30 minutes. Liposomes or equivalent volumes of PBS were incubated with 16 × 10⁶ Jurkat E6.1 cells (4 × 10⁶ cells/mL) in serum-free RPMI 1640 for 1 hour at 37°C on a shaker (100 rpm). After incubation, cells were washed twice with PBS and cultured 18 to 24 hours at 37°C before infection with HIV-1Env.

### Adenoviral vector production

Ad5/F35 vectors were generated as previously described by in vivo recombination in Escherichia coli B15183 cells between pAdenoVator transfer plasmids and pAdEasy-1/F35 adenoviral genome (a generous gift from Dr X. Fan, Lund University, Lund, Sweden).36 The Ad5/F35 vector system (Qbiogene, Irvine, CA). Transfer plasmids containing the cytomegalovirus (CMV) promoter/enhancer with a β-globin/IgG chimeric intron (CMVI) were purchased from Qbiogene. For enhanced yellow fluorescent protein (EYPF) control Ad5F35 vectors, EYPF from pRES-EYPF (Clontech, Mountain View, CA) was cloned into the transfer plasmids. For P expression, Ad5/F35 vectors containing an expression cassette encoding EYPF under the control of the mouse PGK promoter was first cloned into the CMVI transfer plasmid, and the full-length human P synthase (P-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),37 was then cloned into the CMVI 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 3 consecutive freeze-thaw cycles and purified by a discontinuous CsCl gradient followed by a continuous CsCl gradient to completely separate infectious from defective viral particles. The viral preparations were dialysed against Tris 20 mM, pH 8, 2.5% glycerol, and 25 mM NaCl, concentrated using Amicon Ultra-4 MWCO 30 000 concentrators (Millipore, Ville St Laurent, QC) and sterilized by filtration through 0.22 μm Millex-GV filters (Millipore). The viral titers were determined by the tissue-culture infectious dose (TCID50) method following the manufacturer’s instructions (Qbiogene). Two adenoviral vectors were made: a control vector (Ad5/F35-CMVI-EYPF) and a test vector (Ad5/F35-CMVI-P-S-EYPF). The titers of the viral stocks were between 3 × 10⁸ and 1 × 10⁹ infectious units/mL.

### P-synthase gene transduction

Approximately 5 × 10⁶ CD4+ HeLa cells were plated in triplicate in 6-well plates and incubated overnight. Cells were then incubated for 1 hour at 37°C and 5% CO₂ in 250 μL Iscove modified Dulbecco medium (IMDM) containing the control (Ad5/F35-CMVI-EYPF) or test (Ad5/F35-CMVI-P-S-EYPF) vector at a MOI of 25. Untransduced control cells received 250 μL of IMDM only. After incubation, IMDM media was added to a volume of 2 mL Plates were incubated at 37°C and 10% CO₂ for 48 hours; and after transduction, cells were recovered and subjected to FACS analysis and infection with X4 HIV-1m (MOI, 0.3) where productive infection was monitored over time.

### Depletion of glucosyl ceramide-based GSLs

The glucosyl ceramide synthase inhibitor, P₄, was used at 2 μM to pretreat CD4+ HeLa cells for 5 days before HIV infection. FACS analysis indicated that this treatment was sufficient to greatly reduce cell-surface expression of P without cell toxicity. After 5 days of P4 treatment, cells were washed and then infected with X4 HIV-1m (MOI, 0.3) and productive infection monitored over time.

### Transient siRNA depletion of P-synthase gene expression

Approximately 2 × 10⁶ CD4+ HeLa cells were plated in triplicate in 6-well plates and incubated overnight in antibiotic-free RPMI media. Media was then replaced with serum-free, antibiotic-free RPMI. Depletion of P-S by siRNA was carried out as per the manufacturer’s instructions (Thermo Electron, Waltham, MA) with modifications. Briefly, 2 μL Dharmafect-1 was added to 198 μL serum-free RPMI (tube 1). At the same time, 100 μL siRNA (2μM) was added to 100 μL serum-free RPMI (tube 2). Both tubes were mixed separately by pipetting and incubated for 5 minutes. Tube 1 was then added to tube 2, mixed
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. The means of the data points for treated/manipulated cells were compared with their respective control-treated or unmanipulated cells and represented plus or minus SEM, where n = 3 or 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-1 Ba-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-1 Ba-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, although CD4 expression levels (mean fluorescence intensity [MFI]) were approximately 1.5-fold higher than control values (Figure 1C,D left panels). There were also approximately 11% more
CCR5-expressing cells in P1k-a and slightly higher CCR5 expression (MFI \( \sim 1.2 \)-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 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 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 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 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).

**Figure 1.** CCR5 expression in PHA- and PHA/IL-2–activated P1k PBMCs. (A) CCR5 expression in PHA-activated P1k-a and P1k-b PBMCs. (B) CCR5 expression in PHA/IL-2–activated P1k-a and P1k-b PBMCs. (C) Percentage of R5 HIV-1–susceptible target PBMCs, expressing both CD4 and CCR5, in PHA- and PHA/IL-2-activated P1k PBMCs. (D) Percentage of CXCR4-expressing cells in PHA- and PHA/IL-2-activated P1k PBMCs.

**Figure 2.** Increased susceptibility of p PBMCs to R5 and X4 HIV-1 infection. PHA-activated P1k PBMCs or PHA/IL-2–activated P1k PBMCs were infected with R5 or X4 HIV-1 strains. HIV-1 propagation was monitored by p24 antigen up to 25 days after infection, and plotted as a function of time. (A) R5 HIV-1Ba-L (0.5 ng HIV p24 antigen/10^5 cells). (B) X4 HIV-1IIIB (MOI, 0.3). (C) R5 HIV-1Ada-M (13.3 ng HIV p24 antigen/10^5 cells), R5 HIV-1 JR-FL (3.25 ng HIV p24 antigen/10^5 cells). (D) X4 HIV-1NL4-3 gp41 (11.6 ng HIV p24 antigen/10^5 cells). (C,D) Fold change was calculated for each p donor (p1, p2, and p3) based on control infection levels of samples taken at the last time point.

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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 P1k PBMCs (denoted p1, p2, and p3; Table 2) resulted in much higher levels of productive infection compared with their 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-1 JR-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 2D). One other X4 strain, HIV-1NL4-3 gp41, 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.

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

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,C). These differences were once again most evident in p3, which demonstrated the highest increase in X4 HIV-1 infection. The p3 sample showed more CXCR4-expressing cells than control (>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. Although GM3 is reported less fusogenic than Pk, 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-PBMC (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).
**P**<sup>+</sup>-liposome fusion of Jurkat T cells decreases susceptibility to X4 HIV-1

Exogenous **P**<sup>+</sup> was introduced into **P**<sup>-</sup>-deficient Jurkat T-cell membranes by **P**<sup>+</sup>-liposome fusion. After fusion, approximately 35% of the Jurkat cell population expressed surface **P** at high levels (MFI; Figure 5A,B). Within the CD4<sup>+</sup>/H11001 target population, approximately 32% expressed both CXCR4 and **P** (Figure 5C right panel). **P**<sup>-</sup>-liposome–treated cells showed no differences in CD4 or CXCR4 expression compared with PBS or PL-liposome controls (Figure 5B,C). Increased **P** expression after **P**<sup>-</sup>-liposome transfer was confirmed by TLC (Figure 5E). A significant reduction in X4 HIV-1<sub>IIIB</sub> infection was observed in the **P**<sup>-</sup>-supplemented Jurkat cells, being only 43% of the HIV-1<sub>IIIB</sub> infection levels of PBS, **P**-, or PL-liposome controls (Figure 5F).

**P**<sup>+</sup>-synthase shows increased expression of **P**<sup>+</sup> and decreased HIV-1 infection

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

**Depletion of glucosyl ceramide–based GSLs, including **P**<sup>+</sup>, shows increased HIV-1 infection**

P4 was used to inhibit glucosylceramide-based GSL synthesis, thus blocking the biosynthetic pathway to **P**<sup>+</sup>. P4 treatment of CD4<sup>+</sup>/H11001 cells resulted in a substantial decrease in cell populations expressing **P** (Figure 6D). A decrease in **P** 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 **P**<sup>+</sup> synthase reduced **P**<sup>-</sup> expression and increased HIV-1 infection**

To demonstrate that specific reduction of **P**<sup>-</sup> influences the level of HIV-1 infection, siRNA was used to transiently silence the **P**<sup>+</sup> synthase gene, encoding the enzyme responsible for the addition of the terminal galactose to the precursor for **P**<sup>-</sup>.<sup>3,4,10</sup> Transfection of **P**<sup>-</sup>-specific siRNAs into CD4<sup>+</sup> HeLa cells resulted in a substantial decrease in cell populations expressing **P**<sup>-</sup> (Figure 6D). A decrease in **P**<sup>-</sup> expression was also shown in the total GSL profile (Figure 6E). P<sup>-</sup>-treated cells further demonstrated significantly increased HIV-1 infection levels (Figure 6F).
Discussion

Our findings indicate a new phenomenon of Pk-mediated reduced susceptibility to HIV-1 infection. Pk BMCs, which highly express Pk on their cell surface, demonstrate lower levels of productive R5 and X4 HIV-1 infection. In contrast, p BMCs, 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.

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 p24gag 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; Pk or Pk-Lp, Pk liposomes; P, P-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 P1k phenotype. P1k PBMCs demonstrated reduced susceptibility to R5 and X4 HIV-1 infection despite having increased expression of HIV-1 receptors. Thus, both rare p and P1k 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 expression was a better indicator of susceptibility to HIV-1 infection than CD4 or chemokine coreceptor expression.

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

Differences in Pk expression could influence lipid raft composition of target cell membranes and affect CD4 and/or coreceptor localization. Lipid rafts are central to HIV infection, and CD4 and CCR5 are known to be associated with lipid rafts, whereas CXCR4 is not. However, even CD4-HIVgp120-CXCR4 associations have been demonstrated within rafts and are required for membrane fusion. 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, and reduced susceptibility of cells that express increased Pk, to both X4 and R5 HIV-1 infection would argue against current models, suggesting that Pk is important in post-CD4-binding. Increased GM3 has been proposed to promote membrane fusion in p-CD4+ T cells. 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 is not an absolute requirement for membrane fusion and infection. HIV-gp120 binds Pk via the V3 loop. This loop also mediates chemokine coreceptor binding; thus, Pk (or a soluble mimic) binding to gp120 may interfere with post-CD4 recognition of chemokine coreceptor binding to prevent fusion and infection. Indeed, the binding motif, XXXGPGRAFXXX, within the V3 loop for Pk binding overlaps with the consensus binding motif, S/GXXXG-PGXXXXXXX/D, for chemokine coreceptors. It has also been shown that CD4 enhances gp120-Pk interaction, probably by a similar mechanism that allows for the interaction of chemokine
coreceptor with gp120 after CD4 binding.\textsuperscript{50} Perhaps, under conditions of chemokine receptor deficiency (or the absence of CD4), P\textsuperscript{k} may thus (less efficiently) mediate viral internalization. However, when receptor levels are normal, and P\textsuperscript{0} is expressed at higher levels, P\textsuperscript{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\textsubscript{1k} cells could suggest that P can facilitate, rather than P\textsuperscript{0}, inhibit infection. However, the high susceptibility of the \textit{p} phenotype, which lack both P and P\textsuperscript{0}, makes this unlikely. In addition, gp120 binds P\textsuperscript{0} but not P\textsuperscript{1k}.\textsuperscript{5} Furthermore, the introduction of P\textsuperscript{0} by liposome transfer into a cell line deficient in P\textsuperscript{0} expression (providing a close representation to the \textit{p} phenotype), confirmed the decrease in susceptibility to HIV-1 on increased P\textsuperscript{0} 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\textsuperscript{0} 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\textsuperscript{k} synthase gene (α4Gal transferase), which increased the cell-surface expression levels of P\textsuperscript{k} was able to reduce HIV-1 infection. In addition, specific gene silencing using siRNAs to the P\textsuperscript{k} synthase gene resulted in increased HIV-1 infection.

In our previous study of Fabry patient samples,\textsuperscript{26} which present intracellular P\textsuperscript{0} 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\textsuperscript{0}, 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.\textsuperscript{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\textsuperscript{0}, and this is largely independent of CXCR4 or CCR5 coreceptor expression. When P\textsuperscript{0} is highly expressed on the cell surface, as is the case in P\textsubscript{1k} persons’ PBMCs, infection with HIV-1 \( \times 4 \) and R5 viruses is largely reduced. However, when there is no P\textsuperscript{k} cell-surface expression, such as in \( p \) persons’ PBMCs, HIV-1 infection is potentially several logs greater than in cells having normal P\textsuperscript{0} 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.\textsuperscript{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.\textsuperscript{3} We now provide the first evidence of a possible role for a naturally expressed cell-surface factor, the P\textsuperscript{0} 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\textsubscript{1k} phenotype in cohorts of HIV-infected, HIV-exposed but uninfected, HIV progressors and non-progressors would be desirable, the frequency of these extremely rare phenotypes, estimated for \( p \) to be 5.8 per million, and with P\textsubscript{1k} much less frequent (\( \sim 1 \) per million),\textsuperscript{5,6} precludes these studies. Significantly, genetic studies identified chromosome 22q12-13 to be associated with HIV resistance,\textsuperscript{51} and this region contains the P\textsuperscript{k} synthase gene\textsuperscript{13} and HIV transgenic mice showed increased P\textsuperscript{k} synthesis.\textsuperscript{52} To determine whether P\textsuperscript{0} cell-surface expression may indeed represent a natural resistance factor for HIV infection, population studies are required using normal cohorts with common P\textsuperscript{p}/P\textsuperscript{1k} phenotypes known to have differential P\textsuperscript{0} expression\textsuperscript{53} 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\textsuperscript{0} alone provides some protection to infection with HIV-1 and studies of modulation of P\textsuperscript{0} expression, by pharmacologic\textsuperscript{29} or other intervention, may prove to be important for future HIV/AIDS treatment modalities.

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### 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.

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### References


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

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