The Human P\textsuperscript{k} Histo-Blood Group Antigen Provides Protection Against HIV-1 Infection

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Abstract

Several human histo-blood groups are glycosphingolipids (GSLs), including P/P1/P^k. GSLs are implicated in HIV-host-cell-fusion and several bind to HIV-gp120 in vitro. Based on our previous studies on Fabry disease, where P^k accumulates and reduces infection, and a soluble P^k-analogue that inhibits infection, we investigated cell-surface-expressed P^k in HIV infection. HIV-1 infection of peripheral blood-derived mononuclear cells (PBMCs) from otherwise healthy individuals, with blood group P_1^k, where P^k is over-expressed, or blood group p, that completely lacks P^k, were compared to draw-date-matched controls. FACS analysis and/or TLC were used to verify P^k levels. P_1^k 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^k, but not CD4 or chemokine co-receptor expression, correlated with infection. P^k-liposome fused cells and CD4^+-HeLa cells manipulated to express high or low P^k levels confirmed a protective effect of P^k. We conclude that P^k expression strongly influences susceptibility to HIV-1 infection which implicates P^k as a new endogenous cell-surface factor that may provide protection against HIV-1 infection.
Introduction

HIV-1 infection and development of AIDS varies greatly among individuals and populations and is likely, at least in part, dependent on genetic factors. Indeed, the first natural resistance factor reported for HIV infection was a polymorphism within the CCR5 HIV-1 co-receptor gene, termed CCR5-△32. 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.

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. The P/P1/Pk blood group antigens are of particular interest, with many defined pathogen interactions, and an expression profile not limited to erythrocytes. Galabiose (Galα1-4Gal) is the terminal structure of P1 and Pk, also known as globotriaosylceramide (Gb3) and a marker for germinal center B lymphocytes (CD77). Pk is the precursor for the P antigen, also known as globotetraosylceramide (globoside, Gb4), which terminates with β1-3GalNAc. P1 and P2 are the two common P/P1/Pk-related blood group phenotypes. P1 individuals (~80% of Caucasians but only ~20% of Asians) express P and P1 but normally express only low amounts of Pk on their cell surfaces. P2 individuals (~20% of Caucasians and ~80% of Asians) express only P and low amounts of Pk. However, rare phenotypes exist, having anomalies in one or more of the P/P1/Pk blood group antigens. Individuals deficient in P antigen have mutations in the B3GALNT1 gene causing lack of functional P (Gb4) synthase (β3GalNAc transferase), and consequently express increased levels of precursor, Pk. These individuals may express P1 antigen (P1k phenotype) or not (P2k), but the molecular basis for this is still unclear.

Individuals without any P/P1/Pk antigens have mutations in the A4GALT gene (α4Gal transferase or Pk (Gb3) synthase), causing lack of Pk synthesis, and the rare p blood group phenotype. (Table 1).

The P and Pk antigens are glycosphingolipids (GSLs) and GSLs play an important role in HIV-host cell interactions. HIV envelope glycoprotein gp120 targets CD4 and CCR5 or CXCR4 chemokine...
co-receptors on monocytes and T-cells, as the major HIV-host cell interaction,\textsuperscript{19-21} but HIV gp120 also binds to several GSLs \textit{in vitro}, including P\textsuperscript{k}.\textsuperscript{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.\textsuperscript{18,22} Inhibition of GSL biosynthesis can prevent HIV-host cell membrane fusion and infection.\textsuperscript{23,24} This can be overcome by re-introduction of purified GSLs, or over-expression of CD4 and CXCR4, suggesting GSLs have a facilitative role.\textsuperscript{24} P\textsuperscript{k}, and to a lesser extent GM3, has appeared to be primarily implicated in augmenting HIV-membrane fusion, at least in \textit{in vitro} reconstitution models.\textsuperscript{24}

In contrast, our recent work suggested P\textsuperscript{k}, when accumulated due to reduced activity of \( \alpha \)-galactosidase A in Fabry disease,\textsuperscript{25} is protective against R5 HIV-1.\textsuperscript{26} In addition, a soluble analogue of P\textsuperscript{k} inhibits HIV infection \textit{in vitro}.\textsuperscript{27} Moreover, HIV-infected PBMCs have increased GSL expression, including P\textsuperscript{k}, indicating a potential cellular response to HIV-1.\textsuperscript{28} Recently, pharmacological modulation of P\textsuperscript{k} expression \textit{in vitro} in HIV-1 infectable P\textsuperscript{k}-expressing non-T cells has further implicated an important role for P\textsuperscript{k} in HIV infection.\textsuperscript{29}

In light of these findings, we have now assessed HIV-1 susceptibility of PBMCs that are naturally high in P\textsuperscript{k} (P\textsubscript{1}k phenotype) or naturally devoid of P\textsuperscript{k} (p phenotype). In addition, we have genetically and biochemically manipulated P\textsuperscript{k} expression in HIV-1 infectable CD4\textsuperscript{+}-HeLa cells and Jurkat T-cells. Our findings show significant differences that reveal P\textsuperscript{k} status to be an important factor for susceptibility to HIV-1 infection.
Materials and Methods

Cells and chemicals

Waste buffy coat material from anonymous regular blood donors was from the Lund University Hospital Blood Centre (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 made anonymous for this study. The protocol was reviewed and approved by the Canadian Blood Services Institutional Review Board (IRB) Committee. The regular donor controls were matched for ABO group and date of donation. Peripheral blood mononuclear cells (PBMCs) and lymphoblastic cell lines were cultured in ‘complete medium’ consisting of RPMI1640 medium (Invitrogen Canada, Burlington, Ontario) plus 10% Fetal Bovine Serum, 2mM L-glutamine and 10 μM gentamicin antibiotics. The human T cell line, Jurkat FHCRC (Jurkat C), was a gift from Dr. Gordon Mills (MD Anderson Cancer Center, Houston, TX) and Jurkat E6.1 and MT-4 cells were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). The human cervical cancer cell line, HeLa (Clones 6C (HT4-6C) and 1022), stably transfected to express CD4 (CD4⁺-HeLa) and sorted for high expression for infection with HIV-1, were obtained from the NIH AIDS Research and Reference Reagent Program or from Dr. Alan Cochrane, University of Toronto, Ontario and cultured in RPMI plus10% fetal bovine serum (FBS), 0.01% gentamicin antibiotics and 0.1% Amphotericin B. Test and control blood were collected into acid-citrate-dextrose (ACD) anticoagulant on the same day and transported together to Canada for analyses. On arrival, PBMCs were isolated and activated using PHA/IL-2 or PHA alone as described. D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4) was purchased from Matreya LLC (Pleasant Gap, PA).
Blood group characterization

Blood samples acquired for this study were characterized extensively for categorization as control (P₁ or P₂), p or P₁^k. Standard serological techniques determined the erythrocyte phenotype and antibody specificities of blood samples. DNA was isolated from whole blood with Qiagen QIAmp Blood Extraction kit (Qiagen GmbH, Hilden, Germany). Genotypic characterization of samples was performed as reported.⁹,¹⁴ (Tables 2 and 3).

Viruses and in vitro infections

X4 HIV-1IIIB and R5 HIV-1Ba-L were from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HTLVIIIB = HIV-1IIIB from Dr. Robert Gallo, HIV-1Ba-L from Dr. Suzanne Gartner, HIV-1JR-FL from Dr. Irvin Chen, HIV-1Ada-M from Dr. Howard Gendelman, HIV-1NL4-3gp41(36G) V38E, N42S from Trimeris Inc. HIV-1IIIB viral stocks were grown in Jurkat C cells and multiplicity of infection (m.o.i.) was determined as described using MT-4 cells.³⁰ All other viral stocks were grown in PBMCs, and infectious dose calculated from total p24^gag levels³¹,³² measured by Enzyme Linked Immunosorbant Assay (ELISA: Beckman Coulter, Miami, FL OR ZeptoMetrix, Buffalo, NY). Briefly, cells were incubated with HIV-1 for 1 hour at 37°C, the cells washed extensively with PBS, and cultured in complete medium. Culture supernatant aliquots were taken two hours after initial viral infection and subsequent time points thereafter. To determine viral production, ELISA was used to measure p24^gag antigen levels.

FACS analysis

FACS analysis was performed as previously described using PHA- and PHA/IL2-activated PBMCs and 1.5μg monoclonal mouse anti-GM3 or anti-P^k (both from Seikagaku Corp., Tokyo, Japan).²⁶ Alternatively, 12.5μg/ml monoclonal mouse anti-CCR5 (clone 45549.111, NIH AIDS Research and Reference Reagent Program) was used. Secondary antibodies were either 5μg/ml APC-labeled goat
anti-mouse IgG (Invitrogen Molecular Probes) or 1μl FITC-labeled goat anti-mouse IgG (Sigma-Aldrich). For anti-GM3 labeled samples, 10μg/ml APC-labeled goat anti-mouse IgM was used (Cedarlane). FACS analysis for Pk expression of siRNA transfected CD4⁺-HeLa cells was carried out using 5μg/ml VT1B-Alexa458 (produced in the Lingwood laboratory). For tri-colour FACS analysis, an additional incubation with 20μl of 10% mouse serum in FACS Buffer for 10 minutes at 4°C in the dark was carried out prior to incubation with 10μl mouse anti-CD4-PerCP Cy5.5 (BD Biosciences) and/or 5μl of mouse anti-CXCR4-PE (Serotec). Data was collected with a calibrated (BD CaliBRITE, BD Biosciences, San Jose, CA) Becton Dickinson FACS Calibur cell cytometer and analyzed using Cell Quest software.

**Thin layer chromatography (TLC) of GSLs**

Extraction and TLC separation of GSLs including ganglioside GM3 was as previously described. GSL species were detected either by orcinol spray (Sigma) at 110 °C for 10 min. or Pk selectively detected by verotoxin-1 (VT1) TLC overlay. For VT1 overlay, the plate was blocked with 1% bovine gelatin and after incubation at 37°C, the plate was washed with 50mM TBS pH 7.4. The plate was incubated for 45 minutes at room temperature with purified VT1, 1μg per 10ml in TBS. After washing, the plate was incubated for 45 minutes with a monoclonal anti-verotoxinB subunit (diluted 1/2000), washed and incubated with HRP-conjugated goat anti-mouse IgG (diluted 1/2000; BioRad). For GM3 immunostaining, monoclonal anti-GM3 was substituted for VT1, followed by incubation with HRP-conjugated goat anti-mouse IgG. The plate was developed for 1-10 minutes with a 3mg/mL solution of 4-chloro-1-naphthol (4-CN) in methanol freshly mixed with 5 volumes of TBS and 1/2000 dilution of 30% H₂O₂. Where band intensity was calculated from TLC blots, ImageJ software (NIH), was used to quantify relative intensities of bands visualized on TLC, where background signals were subtracted.
**Liposome fusion of Jurkat E6.1 cells**

Jurkat E6.1 cells do not express Pk and are highly infectable with HIV-1. Pk- or P-liposomes were prepared by drying 400 μg Pk or P (Sigma Aldrich) with 200 μg phosphatidylethanolamine and 200 μg phosphatidylserine in chloroform/methanol (C/M; 2:1) under nitrogen. Alternatively, control phospholipids (PL)-liposomes were prepared by drying 200 μg phosphatidylethanolamine with 200 μg phosphatidylserine. Liposomes were generated by vortexing well in 400 ul of PBS and sonicating for 30 min. Liposomes or equivalent volumes of PBS were incubated with 16x10^6 Jurkat E6.1 cells (4x10^6 cells/ml) in serum-free RPMI1640 for 1 hr at 37°C on a shaker (100 rpm). Following incubation, cells were washed twice with PBS, and cultured 18-24 hrs at 37°C prior to infection with HIV-1ⅢB.

**Adenoviral vector production**

Ad5/F35 vectors were generated as previously described\(^35\) by *in vivo* recombination in *E. Coli* BJ5183 cells between pAdenoVator transfer plasmids and pAdEasy-1/F35 Adenoviral genome (a generous gift from Dr. X. Fan, Lund University)\(^36\) using the AdenoVator™ Vector system (QBiogene, Carlsbad, CA). Transfer plasmids containing the CMV promoter/enhancer with a β-globin/IgG chimeric intron (CMVi) were purchased from QBiogene. For EYFP control Ad5/F35 vectors, EYFP from pIRES-EYFP (Clontech, Palo Alto, CA) was cloned into the transfer plasmids described above. For Pk expression, Ad5/F35 vectors containing an expression cassette encoding EYFP under the control of the mouse PGK promoter\(^35\) was first cloned into the CMVi transfer plasmid, and the full-length human Pk synthase (Pk-S) cDNA, cloned from CaCo-2 cells using primers for RT-PCR that were designed based on the published sequence (Genbank database accession No.AB037883), 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 than amplified by transduction of large HEK293 cell cultures. Viruses were extracted by three 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 30000 concentrators (Millipore, Ville St- Laurent, QC, Canada) 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 manufacturer’s instructions (QBiogene). Two adenoviral vectors were made, a control vector(pAd5/F35-CMVi-EYFP) and a test vector (Ad5/F35-CMVi-Pk-S-EYFP). The titers of the viral stocks were between 3 x 10^8 and 1 x 10^9 infectious units/μl.

**P^k-synthase gene transduction**

Approximately 5x10^5 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 250μl of IMDM containing the control (pAd5/F35-CMVi-EYFP) or test (Ad5/F35-CMVi-Pk-S-EYFP) vector at a multiplicity of infection (m.o.i.) of 25. Untransduced control cells received 250μl of IMDM only. After incubation, IMDM media was added to a volume of 2ml. 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-1IIIb (m.o.i, 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 pre-treat CD4^+ HeLa cells for 5 days prior to HIV infection. FACS analysis indicated that this treatment was sufficient to greatly reduce cell-surface expression of P^k without cell toxicity. After 5 days of P4 treatment, cells were washed and then infected with X4 HIV-1IIIb (m.o.i, 0.3) and productive infection monitored over time.

**Transient siRNA Depletion of P^k synthase gene expression**

Approximately 2x10^5 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^k-S by siRNA was carried out as per manufacturer’s instructions (ThermoFisher
Scientific, Lafayette, CO) with modifications. Briefly, 2µl Dharmafect-1 was added to 198µl of serum-free RPMI (Tube 1). At the same time, 100µl of siRNA (2µM) was added to 100µl of 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⁺-HeLa cells, with demonstrated reduction in surface levels of Pk (>70%), were infected with X4 HIV-1IIIb (m.o.i. 0.3) and aliquots of culture supernatant taken over time to monitor p24[ gag ] production by ELISA.

Statistics

A two-sample Student’s t-test, assuming unequal variance with two-tailed distribution, was used to determine significance. The means of the data points for blood group phenotype were compared to their respective matched controls and represented +/-SEM, where n=4. The means of the data points for treated/manipulated cells were compared to their respective control-treated or un-manipulated cells and represented +/-SEM, where n=3 or 4. Data was considered statistically significant if p<0.05 (*), or highly significant if p<0.002 (**).
Results

**P1<sup>k</sup>-PBMCs are protected against R5 and X4 HIV-1 Infection**

We first assessed the susceptibility to HIV-1 infection of PBMCs from P1<sup>k</sup> individuals. Given the rarity of these samples (Table 1),<sup>6</sup> P1<sup>k</sup>-PBMCs from one donor (P1<sup>k</sup>-a) were used to assess R5 HIV-1 infection and a second donor (P1<sup>k</sup>-b) to assess X4 HIV-1 infection (see Tables 2 and 3 for test and control designations). Infection of PHA-activated P1<sup>k</sup>-a with R5 HIV-1<sub>Ba-L</sub> showed significantly lower productive HIV-1<sub>Ba-L</sub> infection compared to its draw-date- and ABO-matched control (Figure 1A). PHA/IL2-activated P1<sup>k</sup>-b were similarly protected against productive X4 HIV-1<sub>IIIB</sub> infection (Figure 1B) compared to the respective control. Based on comparison to draw-date-matched controls, infection levels for P1<sup>k</sup>-PBMCs for both HIV-1<sub>Ba-L</sub> and HIV-1<sub>IIIB</sub> were less than 12% (data not shown).

**CD4, co-receptor and P<sup>k</sup> expression are increased in P1<sup>k</sup>-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 P1<sup>k</sup>-a showed ~10% less CD4-expressing cells than the matched control, however CD4 expression levels (MFI) were ~1.5-fold higher (Figures 1C,D (left panel)). There were also ~11% more CCR5-expressing cells in P1<sup>k</sup>-a, and slightly higher CCR5 expression (MFI ~1.2-fold difference) (Figures 1C,D). The percentage of R5 HIV-1 susceptible target PBMCs, expressing both CD4 and CCR5, was also slightly higher in P1<sup>k</sup>-a (Figure 1C).

PHA/IL2-activated P1<sup>k</sup>-b demonstrated ~7% more CD4-expressing cells compared to control, and ~3.5-fold higher CD4 expression levels (MFI) (Figures 1C,D (right panel)). In addition, there were 27% more CXCR4-expressing cells than control, and ~3.5-fold higher CXCR4 expression in P1<sup>k</sup>-b (Figures 1C, D). Indeed, even the percentage of X4 HIV-1-susceptible target PBMCs, expressing both CD4 and CXCR4, was higher in P1<sup>k</sup>-b (Figure 1C). The percentage of cells expressing cell-surface P<sup>k</sup> on PHA- and PHA/IL2-activated P1<sup>k</sup>-PBMCs was approximately 1.5- to 2-fold higher than that of
controls (Figure 1E), with up to 4.5-fold higher density of cell-surface Pk expression as measured by MFI (Figure 1E). 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 to their respective controls (Figure 1F).

**p-PBMCs are hyper-susceptible to R5 and X4 HIV-1 Infection**

We also assessed susceptibility of PBMCs from three Pk-deficient p individuals (Table 2) to R5 and X4 HIV-1 infection. HIV-1Ba-L infection of PHA-activated p-PBMCs (denoted p1, p2 and p3; see Table 2) resulted in much higher levels of productive infection in comparison to their draw-date- 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 ~5-fold higher for p1, 12-fold higher for p2 and ~3000-fold higher for p3 (data not shown). This increased infection was consistent for R5 HIV-1 infection in general, as two other R5 strains, HIV-1Ada-M and HIV-1JR-FL also showed much higher productive HIV-1 infection in the p-PBMCs in comparison to control (Figure 2C).

As with R5 infection, HIV-1IIIB infection of PHA/IL2-activated p-PBMCs from two individuals (p1 and p3) also showed much higher levels of productive infection in comparison to their matched-controls (Figures 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-PBMCs 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 ~600- to 1000-fold higher for p3 (Figure 2B). One other X4 strain, HIV-1NL4-3 gp41, used to infect PHA/IL2-activated p3 also showed >1000-fold higher productive HIV-1 infection in comparison to control (Figure 2D), consistent with X4 HIV-1IIIB results.
**CD4 & co-receptor 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% to 40%), which also translated into higher CD4 expression levels (MFI 1.3- to 3-fold higher) (Figures 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% to 19%), and 3-fold higher CCR5 expression levels (MFI) (Figures 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 (Figures 3A, C).

PHA/IL2-activated p-PBMCs (p1, p3) demonstrated more CD4-expressing cells compared to their controls (an increase of ~21% to 42%), and up to 3-fold higher CD4 expression levels (MFI) (Figures 3B, D). 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-PBMC from both p1 and p3 samples (Figures 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 ~15% less CD4 and ~5% less CXCR4 expressing cells compared to 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 as compared to p-PBMC (Figure 4A), calculated according to band intensity on the TLC plate to be ~3-fold different (Figure 4C). Resting or PHA/IL2-activated PBMCs however showed minimal differences in total p-PBMC GM3 levels compared to the respective control (Figures 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) and 4E). Although there was a slightly higher percentage of GM3 expressing p-PBMCs in the PHA/IL2-activated population, only subtle differences were seen in cell surface GM3 expression (Figure 4D (right panel) and 4E).

**P^k-liposome fusion of Jurkat T-cells decreases susceptibility to X4 HIV-1**

Exogenous P^k was introduced into P^k-deficient Jurkat T-cell membranes by P^k-liposome fusion. After fusion, ~35% of the Jurkat cell population expressed surface P^k at high levels (MFI) (Figures 5A and 5B). Within the CD4^+ target population, ~32% expressed both CXCR4 and P^k (Figure 5C (right panel)). P^k-liposome treated cells showed no differences in CD4 or CXCR4 expression in comparison to PBS or phospholipid (PL)-liposome controls (Figures 5B,C). Increased P^k expression following P^k-liposome transfer was confirmed by TLC (Figure 5E). A significant reduction in X4 HIV-1IIIB infection was observed in the P^k supplemented Jurkat cells, being only 43% of the HIV-1IIIB infection levels of PBS, P- or PL-liposome controls (Figure 5F).

**Increase in P^k synthase shows increased expression of P^k and decreased HIV-1 infection**

To confirm that P^k expression levels influence HIV-1 infection levels, we tested whether modulating the expression of P^k in CD4^+ HeLa cells, that express P^k and are infectable, would correlate with subsequent HIV-1 infection (Figure 6). Cells transduced with adenoviral vector expressing P^k synthase (P^k-S) resulted in increased levels of total and cell surface P^k compared to non-transduced cells or cells transduced with a control adenoviral vector (Figures 6A, B). Compared to untreated cells or control adenoviral vector transduced cells, HIV-1 infection was significantly lower in the increased P^k-expressing CD4^+ HeLa cells transduced with the adenoviral vector P^k-S (Figure 6C).
Depletion of glucosyl ceramide based GSLs, including \( P^k \), shows increased HIV-1 infection

P4 was used to inhibit glucosylceramide-based GSL synthesis, thus blocking the biosynthetic pathway to \( P^k \). \(^{37} \) P4-treatment of CD4\(^+\)-HeLa cells resulted in a substantial decrease in cell populations expressing \( P^k \) (Figure 6D). A decrease in \( P^k \) expression was further 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 \( P^k \) synthase reduced \( P^k \) expression and increased HIV-1 infection**

To demonstrate that specific reduction of \( P^k \) influences the level of HIV-1 infection, siRNA was used to transiently silence the \( P^k \) synthase gene, encoding the enzyme responsible for the addition of the terminal galactose to the precursor for \( P^k \). \(^{5, 10} \) Transfection of \( P^k \) synthase-specific siRNAs into CD4\(^+\)-HeLa cells resulted in a substantial decrease in cells expressing \( P^k \) compared to siRNA controls (Figures 7A, B). The decrease in total \( P^k \) was selective without significant decrease in \( P^k \) precursors GlcCer or LacCer, monitored by TLC and VT1 overlay (Figures 7B, C). Cells transiently transfected with siRNA to \( P^k \) synthase demonstrated significantly increased HIV-1 infection levels (Figure 7D).
Discussion

Our findings indicate a new phenomenon of P^k^-mediated reduced susceptibility to HIV-1 infection. P_r1^k^-PBMCs, that highly express P^k on their cell surface, demonstrate lower levels of productive R5 and X4 HIV-1 infection. In contrast, p-PBMCs, that do not express P^k, show a higher susceptibility to R5 and X4 HIV-1 infection. Accordingly, P^k^-liposomal-transfer or P^k^-synthase gene transduction facilitated a reduction in HIV-1 infection, whereas GlcCer-based GSL (P^k) depletion or P^k^-synthase gene silencing resulted in an increase in HIV-1 infection. Thus, higher expression of P^k in vivo and in vitro correlates with decreased HIV-1 infection while a lower expression or lack of P^k expression results in increased HIV-1 infection.

Our studies indicate that susceptibility to HIV-1 infection in p-PBMCs might be influenced both by the lack of P^k antigen and by increased receptor and co-receptor expression; however, this is not the case with the P_r1^k phenotype. P_r1^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 P_r1^k-PBMCs showed increased patterns of HIV receptor and co-receptor expression, but this resulted in higher susceptibility to HIV infection only in the p-PBMCs. Thus, P^k expression was a better indicator of susceptibility to HIV-1 infection than CD4 or chemokine co-receptor expression.

While the presence (or absence) of P^k is important in blood group classification and transfusion medicine, P^k is not restricted to erythrocytes. P^k is expressed on monocyte populations, which encompass R5 HIV-1 susceptible target cells.\cite{39,40} T-lymphoblasts mostly represent X4 HIV-1 susceptible target cell populations, and have been reported to express little or no P^k;\cite{39} thus, T cells are similar to the p phenotype in their lack of P^k expression, which may promote susceptibility to HIV-1 infection. Furthermore, variations in P^k expression occur in the general population\cite{41} which could explain differences in susceptibility to HIV-1 infection seen in vitro and in vivo.
Differences in \( P^k \) expression could influence lipid raft composition of target cell membranes and affect CD4 and/or co-receptor localization. Lipid rafts are central to HIV infection\(^ {42} \) and CD4 and CCR5 are known to be associated with lipid rafts, while CXCR4 is not.\(^ {43} \) However, even CD4-HIV gp120-CXCR4 associations have been demonstrated within rafts, and are required for membrane fusion.\(^ {44} \) If \( P^k \) levels were able to influence appropriate localization of CD4 and/or co-receptors in lipid rafts, due to changes in the membrane milieu, this could affect target cell susceptibility to HIV-1.

Importantly, heightened susceptibility of cells without \( P^k \), and reduced susceptibility of cells that express increased \( P^k \), to both X4 and R5 HIV-1 infection would argue against current models suggesting \( P^k \) is important in post-CD4-binding.\(^ {22} \) Increased GM3 has been proposed to promote membrane fusion in p-CD4\(^ + \) T cells.\(^ {38} \) However, cell surface expression and total GM3 do not correlate with enhanced PHA- or PHA/IL2-activated PBMC HIV-1 infection in our study, although purified target cells remain to be assessed. It is clear, however, that \( P^k \) is not an absolute requirement for membrane fusion and infection. HIV-gp120 binds \( P^k \) via the V3 loop.\(^ {17,18,22} \) This loop also mediates chemokine receptor binding.\(^ {45,46} \) Thus, \( P^k \) (or a soluble mimic\(^ {27} \)) binding to gp120 may interfere with post-CD4 recognition of chemokine co-receptor binding to prevent fusion and infection. Indeed, the binding motif, \( \text{XXXGPGRAFXXX} \),\(^ {47} \) within the V3 loop for \( P^k \) binding overlaps with the consensus binding motif, \( \text{S/GXXXGPGXXXXXXE/D} \),\(^ {45} \) for chemokine co-receptors. It has also been shown that CD4 enhances gp120-\( P^k \) interaction\(^ {48} \), likely by a similar mechanism that allows for the interaction of chemokine co-receptor with gp120 following CD4 binding.\(^ {49} \) 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 \( P^k \) is expressed at higher levels, \( P^k \) has the potential to interfere with the appropriate interactions between gp120 and chemokine co-receptors, thus inhibiting viral internalization (see Figure 7E for a working model).

The lack of P in P\(_1^k \) cells could suggest that 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.
Also, gp120 binds P<sup>k</sup> but not P. Furthermore, the introduction of P<sup>k</sup> by liposome transfer into a cell line deficient in P<sup>k</sup> expression (providing a close representation to the p phenotype), confirmed the decrease in susceptibility to HIV-1 upon increased P<sup>k</sup> 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<sup>k</sup> closely correlate to HIV susceptibility. This is further supported by use of a cell line, HeLa, that does not express P (Figure 7B), whereby following the introduction of the P<sup>k</sup> synthase gene (α<sub>4</sub>Gal transferase), which increased the cell surface expression levels of P<sup>k</sup> was able to reduce HIV-1 infection. Also, specific gene silencing using siRNAs to the P<sup>k</sup> synthase gene resulted in increased HIV-1 infection.

In our previous study of Fabry patient samples, which present intracellular P<sup>k</sup> accumulation due to 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<sup>k</sup>, 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 co-receptors for HIV-1 infection. Indeed, Fabry PBMCs only demonstrated a reduction in R5 HIV-1 infection and CCR5 co-receptor 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<sup>k</sup>, and this is largely independent of CXCR4 or CCR5 co-receptor expression. When P<sup>k</sup> is highly expressed on the cell surface, as is the case in P<sub>1</sub><sup>k</sup> individuals’ PBMCs, infection with HIV-1 X4 and R5 viruses is largely reduced. However, when there is no P<sup>k</sup> cell-surface expression, such as in p individuals’ PBMCs, HIV-1 infection is potentially several logs greater than in cells having normal P<sup>k</sup> 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. The Δ32 polymorphism in the CCR5 chemokine cell-surface receptor that provides natural resistance to HIV
infection is due to a mutation that prevents the transport of this receptor to the cell surface. Thus, individuals with this polymorphism do not express the receptor for R5 viruses on their cell surface.\textsuperscript{2}

We now provide the first evidence of a possible role for a naturally-expressed cell-surface factor, the P\textsuperscript{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\textsubscript{1}\textsuperscript{k} phenotype in cohorts of HIV-infected, HIV-exposed but uninfected, HIV progressers and non-progressers would be desirable, the frequency of these extremely rare phenotypes, estimated for p to be 5.8 per million, and with P\textsubscript{1}\textsuperscript{k} much less frequent (~1 per million),\textsuperscript{5,6} precludes these studies. Significantly, genetic studies identified chromosome 22q12-13 to be associated with HIV resistance\textsuperscript{50} and this region contains the P\textsuperscript{k} synthase gene\textsuperscript{13} and HIV transgenic mice showed increased P\textsuperscript{k} synthesis.\textsuperscript{51} To determine whether P\textsuperscript{k} cell-surface expression may indeed represent a natural resistance factor for HIV infection, population studies are required using normal cohorts with common P\textsubscript{1}/P\textsubscript{2} phenotypes known to have differential P\textsuperscript{k} expression\textsuperscript{41} to assess HIV-1 susceptibility \textit{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{k} alone provides some protection to infection with HIV-1 and studies of modulation of P\textsuperscript{k} expression, by pharmacological\textsuperscript{29} or other intervention, may prove to be important for future HIV/AIDS treatment modalities.
Acknowledgments

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Authorship

Contributions: 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. C.A.L. and D.R.B. contributed to the design of experiments, analysis of the data, and to the writing of the manuscript.

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

**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 10⁶</td>
</tr>
<tr>
<td>P₁ₖ</td>
<td>P₁, Pₖ</td>
<td>~1 per 10⁶</td>
</tr>
<tr>
<td>P₂ₖ</td>
<td>Pₖ</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 while the GLOB blood group system includes the P antigen. The remaining related antigens (Pₖ and LKE not mentioned here) are part of the GLOB blood group collection.

**Phenotypic frequencies are for Caucasians.
Table 2. Summary of the genetic and blood group serological findings in the rare individuals 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>p1 657delG</td>
<td>-</td>
<td>-</td>
<td>P, P1, Pk</td>
<td>P</td>
<td>Hellberg et al., 2003(^{14})</td>
</tr>
<tr>
<td>p2 548T&gt;A</td>
<td>-</td>
<td>-</td>
<td>P, P1, Pk</td>
<td>P</td>
<td>Steffensen et al., 2000(^{13})</td>
</tr>
<tr>
<td>p3 548T&gt;A</td>
<td>-</td>
<td>-</td>
<td>P, P1, Pk</td>
<td>P</td>
<td>Steffensen et al., 2000(^{13})</td>
</tr>
<tr>
<td>P(_1)^k.a 811G&gt;A</td>
<td>+</td>
<td>+</td>
<td>P</td>
<td>P(_1)^k</td>
<td>Hellberg et al., 2002(^{9})</td>
</tr>
<tr>
<td>P(_1)^k-b 538insA</td>
<td>+</td>
<td>+</td>
<td>P</td>
<td>P(_1)^k</td>
<td>Hellberg et al., 2002(^{9})</td>
</tr>
</tbody>
</table>

*The P1 antigen is present in P\(_1\) and P\(_1\)^k phenotype samples, detectable with anti-P1, whilst absent in P\(_2\) and P\(_2\)^k and p.

**Anti-PP1P\(_k\) is also known as anti-T\(_3\)^a and is only found in individuals having the p phenotype.
Table 3. Summary of the P/GLOB-related blood group genetic and serological findings in the control individuals 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></td>
<td>A4GALT</td>
<td>B3GALNT1</td>
<td>P1</td>
<td>P</td>
</tr>
<tr>
<td>Control a (C-a)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control b (C-b)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control p1 (C-p1)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control p2 (C-p2)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control p3 (C-p3)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*All blood group phenotypes with the exception of p, P₁⁺ and P₂⁺ express low levels of Pk due to incomplete conversion to P. The amount of Pk expressed varies from individual to individual.
Figure Legends

Figure 1. Decreased susceptibility of P\textsubscript{1}\textsuperscript{k}-PBMCs to R5 and X4 HIV-1 infection inversely correlates with P\textsuperscript{k} and does not correlate with CD4 and/or chemokine co-receptor expression. (A) PHA-activated PBMCs were infected with HIV-1\textsubscript{Ba-L} (0.5ng of HIV p24\textsuperscript{gag}/5x10\textsuperscript{5} cells) and (B) PHA/IL2-activated PBMCs were infected with HIV-1\textsubscript{IIIb} (m.o.i. 0.3). Viral propagation was monitored by p24\textsuperscript{gag} antigen up to 12 days, and plotted as a function of time: (A) **p<0.002; (B) *p<0.05. Data are representative of the mean ±SEM, where n=4 infection data points. (C-F) Scatter plots of PBMCs labeled with anti-CD4 PerCP Cy5.5, anti-CCR5 GAM-FITC (or GAM-APC) and anti-CXCR4 PE were analyzed, and background compensated to isotype controls. Alternatively, anti-CD4 PerCP Cy5.5, and anti-P\textsuperscript{k} GAM-APC were used to label PBMCs and analyzed as described. (C) Percentage of cell populations expressing CD4, CCR5 and CXCR4, present in PHA-activated (left) or PHA/IL2 activated PBMCs (right) plotted as histograms for ease of comparison. (D) Mean fluorescence intensity (MFI) of surface expressed CD4, CCR5 and CXCR4 was measured on ungated cell populations and Fold Difference in expression levels calculated based on control MFI values for PHA-activated PBMCs (left) or PHA/IL2-activated PBMCs (right). (E) Percentage of P\textsubscript{1}\textsuperscript{k}-PBMC cell populations expressing surface P\textsuperscript{k} (left) and P\textsuperscript{k} expression levels based on MFI (right) are represented as Fold Difference based on control values. (F) Scatter plots representing CD4 and P\textsuperscript{k} expressing cell populations, Top panel: PHA-activated PBMCs, Lower panel: PHA/IL2-activated PBMCs Left: Control PBMCs, Right: P\textsubscript{1}\textsuperscript{k}-PBMCs.

(◊) or (□)= healthy PBMCs controls designated C-a or C-b (see Table 3); (■) or P\textsubscript{1}\textsuperscript{k} = P\textsubscript{1}\textsuperscript{k}-PBMCs designated P\textsubscript{1}\textsuperscript{k}-a or P\textsubscript{1}\textsuperscript{k}-b (see Table 2).
Figure 2. Increased susceptibility of p-PBMCs to R5 and X4 HIV-1 infection. PHA-activated PBMCs or PHA/IL2-activated PBMCs were infected with R5 or X4 HIV-1 strains. HIV-1 propagation was monitored by p24\textsuperscript{gag} antigen up to 25 days post-infection, and plotted as a function of time. (A) R5 HIV-1\textsubscript{Ba-L} (0.5ng of HIV p24\textsuperscript{gag}/5 x 10\textsuperscript{5} cells), (B) X4 HIV-1\textsubscript{IIIb} (m.o.i. 0.3), (C) R5 HIV-1\textsubscript{Ada-M} (13.3ng of HIV p24\textsuperscript{gag}/ 5 x 10\textsuperscript{5} cells), R5 HIV-1\textsubscript{JR-FL} (3.25ng of HIV p24\textsuperscript{gag}/ 5 x 10\textsuperscript{5} cells), (D) X4 HIV-1\textsubscript{NL4-3 gp41 36G} (11.6ng of HIV p24\textsuperscript{gag}/ 5 x 10\textsuperscript{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. Data are representative of the mean ± SEM, where n=4 infection data points, *p<0.05, **p<0.002. (◊) = healthy PBMCs controls designated C-p1, C-p2 or C-p3 (see Table 3); (■) = p-PBMCs designated p1, p2 or p3 (see Table 2).
Figure 3. FACS analysis of CD4, CCR5 and CXCR4 expression on p-PBMCs. PBMCs were either stimulated with PHA or PHA/IL2 (as per conditions for HIV infection) and tri-color FACS analysis was performed. Scatter plots of PBMCs labeled with anti-CD4 PerCP Cy5.5, anti-CCR5 GAM-FITC (or GAM-APC) and anti-CXCR4 PE were analyzed, and background compensated to isotype controls. (A-B) Percentage of cell populations expressing CD4, CCR5 and CXCR4, present in PHA-activated PBMCs (A) or PHA/IL2 activated PBMCs (B) plotted as histograms for ease of comparison. (C-D) Mean fluorescence intensity (MFI) of surface expressed CD4, CCR5 and CXCR4 was measured and Fold Difference in expression levels calculated based on control values for PHA-activated PBMCs (C) or PHA/IL2-activated PBMCs (D).

□ = healthy PBMC controls designated C-p1, C-p2 or C-p3 (see Table 3); ■ = p-PBMCs designated p1, p2 or p3 (see Table 2).
**Figure 4. FACS and TLC of GM3 expression in p-PBMCs.** PBMCs were either resting or stimulated with PHA or PHA/IL2 and analysed for total and surface expressed GM3. (A-B) TLC of total GSLs extracted from control PBMCs (C-p3: lanes 3, 5 & 7) and p-PBMCs (p3: lanes 2, 4 & 6). Lane 1: GSL standards, Lanes 2&3: resting PBMCs, Lanes 4&5: PHA-activated PBMCs, Lanes 6&7: PHA/IL2-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 (B) was measured by ImageJ software, compensated to background levels and Fold Difference in p-PBMC expression levels calculated based on control values. GlcC=glucosylceramide; GalC=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), **Lower panel:** p-PBMCs (p3). **Left:** Resting PBMCs, **Centre:** PHA-activated PBMCs, **Right:** PHA/IL2-activated PBMCs. (E) MFI of surface expressed GM3 was measured and Fold Difference calculated based on control values.
**Figure 5. Susceptibility of P^k^-liposome-fused Jurkat T-cells to X4 HIV-1 infection.** Jurkat T-cells lacking P^k^- were incubated with P^k^- or P-liposomes and cultured for 18hrs, where PBS or phospholipid (PL-) liposome controls were used. Tri-color FACS analysis was performed and scatter plots of Jurkat labeled with anti-CD4 PerCP Cy5.5, anti-CXCR4 PE and anti-P^k^- GAM-FITC (or GAM-APC) were analyzed, where background was compensated to isotype controls. (A) Histogram representing percentage of cell populations expressing P^k^- (B) Scatter plots representing cell populations expressing P^k^- and CXCR4, and gated on CD4 positive populations; **Left**: PBS-treated Jurkat; **Centre**: PL-liposome fused Jurkat; **Right**: P^k^-liposome fused Jurkat. (C) Scatter plots representing percentage of cell populations expressing CD4 and CXCR4; **Left**: PBS-treated Jurkat; **Centre**: PL-liposome fused Jurkat; **Right**: P^k^-liposome fused Jurkat. (D) Surface expression levels of CD4, CXCR4 and P^k^- are represented as Mean Fluorescence Intensity (MFI). (E) TLC of total GSLs extracted from control and liposome fused Jurkat cells; **Lane 1**: GSL standards, **Lane 2**: P^k^- expressing B-cell line control (Daudi), **Lane 3**: PBS-treated Jurkat control, **Lane 4**: PL-liposome control, **Lane 5**: P^k^-liposome fused Jurkat. (F) Infection with HIV-1_{IIIb} (m.o.i. 0.3) and p24^{gag} monitored at day 3 post-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 three independent experiments in order to show significance between PL-liposome controls and P^k^-liposomes, (*p<0.05, **p<0.002).

**PBS** = PBS control; **PL or PL-Lp** = phospholipid liposome control; **P^k^- or P^k^-Lp** = P^k^-liposomes, **P** = P-liposomes.
**Figure 6. Molecular and chemical modulation of P<sup>k</sup> expression.** CD<sup>4</sup><sup>+</sup>-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 P<sup>k</sup> synthase (P<sup>k</sup>-S) cDNA (P<sup>k</sup>S Vector). Both the Control and P<sup>k</sup>-S Vectors contained an EYFP gene to detect transduction efficiency. After 48 hours FACS analysis was performed and scatter plots of CD<sup>4</sup><sup>+</sup>-HeLa cells labeled with anti-P<sup>k</sup> GAM-FITC were analyzed, where background was compensated to isotype controls. (A) Histogram plots representing percentage of cell populations expressing EYFP (**top panel**) or P<sup>k</sup> (**lower panel**) for No Vector Control (**left**), Ctrl Vector (**centre**) and P<sup>k</sup>-S Vector (**right**). (B) VT1 overlay for P<sup>k</sup> detection was carried out on TLC of total GSLs extracted from control and transduced CD<sup>4</sup><sup>+</sup>-HeLa cells. Lane 1: GSL standards; Lane 2: Cells without adenovector (No Vector); Lane 3: Cells with control adenovector (Ctrl Vector); Lane 4: Cells with adenovector expressing P<sup>k</sup> synthase gene (P<sup>k</sup>-S Vector). (C) HIV-1<sub>IIB</sub> (m.o.i. 0.1) was used to infect CD<sup>4</sup><sup>+</sup>-HeLa cells with No Vector, Ctrl Vector or P<sup>k</sup>-S Vector. After 3 days, HIV-1 infection was measured by p24<sup>gag</sup> production. Percentage difference in HIV-1 infection was calculated based on CD<sup>4</sup><sup>+</sup>-HeLa cells without adenovector (No Vector). Data are representative of the mean ± SEM where n=3 infection data points; *p<0.05 comparing P<sup>k</sup>-S transduced cells to un-transduced cells. This figure is representative of 3 independent experiments. (D) Histogram plots representing percentage of cell populations expressing P<sup>k</sup> after CD<sup>4</sup><sup>+</sup>-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 P<sup>k</sup>. (E) VT1 overlay for P<sup>k</sup> detection was carried out on TLC of total GSLs extracted from untreated and P4-treated CD<sup>4</sup><sup>+</sup>-HeLa cells. Lane 1: Control (untreated) cells; Lane 2: P4-treated cells; Lane 3-5: GSL standards. (F) HIV-1<sub>IIB</sub> (m.o.i. 0.1) infection of untreated or P4-treated CD<sup>4</sup><sup>+</sup>-HeLa cells was measured by p24<sup>gag</sup> 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 ± SEM where n=3 infection data points; *p<0.05. This figure is representative of 3 independent experiments.
Figure 7. Specific depletion of \( P^k \) correlates with increased HIV-1 infection. CD4\(^+\)-HeLa cells (clone 6C) were transfected daily with either Control siRNA or \( P^k \) synthase (\( P^k\)-S) siRNA, and cultured for 72 hours to deplete \( P^k\)-S, and subsequently \( P^k \). FACS analysis was performed and scatter plots of CD4\(^+\)-HeLa cells labeled with VT1B-Alexa\(_{458}\) were analyzed, where background was compensated to unstained controls. (A) Histogram plots representing percentage of cell populations expressing \( P^k \). (B) TLC of total GSLs extracted from control and \( P^k\)-S siRNA transfected CD4\(^+\)-HeLa cells. Lane 1: GSL standards; Lane 2: Control siRNA-transfected cells; Lane 3: \( P^k\)-S siRNA-transfected cells. (C) VT1 overlay for \( P^k \) detection was carried out on TLC of total GSLs Lane 1: GSL standards; Lane 2: Control siRNA-transfected cells; Lane 3: \( P^k\)-S siRNA-transfected cells. (D) HIV-1\(_{\text{HIV}}\) (m.o.i. 0.3) infection of Control(control siRNAs)- or \( P^k\)-S siRNA-transfected CD4\(^+\)-HeLa cells was measured by p24\(_{\text{Gag}}\) production after 5 days of culture. Data are representative of the mean ± SEM where n=3 infection data points; \(*p<0.05\). This figure is representative of 3 independent experiments. (E) A schematic working model for \( P^k \)-induced protection against HIV-1 infection. HIV first binds to CD4 which exposes the GSL and chemokine co-receptor binding site within the V3 loop of HIV gp120. When \( P^k \) is highly expressed, it can successfully compete with chemokine co-receptor for the exposed sites within the V3 loop and, thus, \( P^k \) interferes with the process of membrane fusion.
Figure 2

A

HIV-1 Ba-L Infection (p24 pg/ml)

Days post-infection

C-p1
p1

C-p2
p2

C-p3
p3

B

HIV-1 IIIB Infection (p24 pg/ml)

Days post-infection

C-p1
p1

C-p2
p2

C-p3
p3

C

Fold difference in R5 HIV-1 Infection

Ada-M
JR-FL

Fold difference in X4 HIV-1 Infection

NL4-3
gp41 (36G)

p3

* p < 0.05
** p < 0.01
The human \( P^k \) histo-blood group antigen provides protection against HIV-1 infection

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