Inosine triphosphate pyrophosphohydrolase expression: decreased in leukocytes of HIV-infected patients using combination antiretroviral therapy.

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ABSTRACT

Objective

In HIV-infected patients, the enzyme Inosine triphosphate pyrophosphohydrolase (ITPase), involved in purine nucleotide homeostasis, was found to be decreased in erythrocytes. Since purine analogues are pivotal in the HIV treatment, a better understanding of ITPase expression in $CD4^+$ lymphocytes may lead to better understanding of nucleotide metabolism and (adverse) effects.

Design

Cross-sectional, cohort, observational study.

Methods

HIV-infected and control patients above 18 years were included. All DNA samples were genotyped for the 2 functional *ITPA* SNPs; c.94C>A (rs1127354) and c.124+21A>C (rs7270101). ITPase expression was determined by flow cytometry in all leukocyte subsets.

Results

Fifty-nine HIV-infected patients and 50 controls were included. Leukocyte subtype distribution showed no difference in monocytes and granulocytes, but lymphocytes were higher in HIV-infected patients ($p<0.001$). ITPase expression was highest in activated monocytes and lowest in lymphocytes. In HIV-infected patients, the percentage of ITPase positive cells was less in all leukocyte and lymphocyte subsets compared with controls ($p<0.01$). In HIV-infected patients, 97.4% of $CD4^+$ lymphocytes were ITPase positive versus 99.9% in controls ($p=0.002$) and 85.9% versus 99.6% of $CD8^+$ lymphocytes ($p<0.0001$), respectively. Stratification according to genotype revealed no significant differences in ITPase expression in leukocytes in HIV-infected and control patients.

Conclusions

HIV-infection seems to be interfering with the nucleotide metabolism in leukocytes, including $CD4^+$ lymphocytes, by decreasing ITPase expression, independently of *ITPA* genotype. Given that active metabolites of purine-analogue reverse transcriptase inhibitors are potential substrates for ITPase, these results warrant further research towards effectiveness and adverse events of purine analogues and ITPase activity.

INTRODUCTION

The Human Immunodeficiency Virus (HIV) is a retrovirus that copies its single-stranded RNA into double-stranded DNA, which is incorporated into the DNA of the host. Conversely, new viral RNA is transcribed from the infected host DNA and packaged into new virus particles. These processes are entirely dependent on human nucleotide metabolism in the host lymphocytes. This makes nucleotide metabolism both a target and a vehicle for anti-viral therapy, reflected by the numerous anti-retroviral nucleoside analogues that have been created. Interestingly, there are indications that nucleotide metabolism is impaired in lymphocytes and erythrocytes of HIV-infected individuals. HIV-1 positive lymphocytes were demonstrated to be unable to expand their ribonucleotide pool (both purines and pyrimidines) to the same extent as HIV-1 negative lymphocytes after a strong mitogenic stimulation, prohibiting DNA-replication and leading to cell death.[1] Furthermore, the activities of thymidine kinase 1 and thymidylate kinase (TMP-kinase), both enzymes in the pyrimidine nucleotide biosynthetic pathway, were significantly decreased in lymphocytes of HIV-infected patients compared with controls.[2,3] Purine nucleotide homeostasis may be compromised, as the enzyme Inosine 5'-triphosphate pyrophosphohydrolase (Inosine triphosphatase; ITPase) was found to be significantly decreased in erythrocytes of HIV-infected patients compared with a control population.[4] Since antiviral purine analogues are pivotal in the treatment of HIV-infection, a better understanding of ITPase expression in lymphocytes may lead to a better understanding of nucleotide metabolism and the effects of HIV treatment.

ITPase, encoded by the polymorphic gene *ITPA* (OMIM #147520), is one of the scavenger enzymes eliminating the potentially cyto- or genotoxic noncanonical nucleoside triphosphates from the nucleotide pool.[5] Multiple single nucleotide polymorphisms (SNPs) of *ITPA* have been described,[6] among which 2 SNPs are proven to decrease ITPase activity: c.94C>A (p.Pro32Thr, NCBI rs1127354) and c.124+21A>C (NCBI rs7270101). The SNP c.94C>A is found in most world populations, albeit with varying frequency,[7] whereas the SNP c.124+21A>C has not been found or was extremely rare among Asian populations.[8–10] *ITPA* genotype[6,11–13] and decreased ITPase activity[14] have been associated with protection against anemia in the treatment of hepatitis C with the purine analogue ribavirin. Furthermore, although conflicting reports exist,[15,16] SNPs in the *ITPA* genotype have been associated with adverse drug events during thiopurine therapy with azathioprine and 6-mercaptopurine.[17,18] Also, the SNP c.94C>A led to higher concentrations of methyl-mercaptopurine nucleotides in patients treated for acute lymphoblastic leukemia and a higher probability of severe febrile neutropenia in these patients, when mercaptopurine dose had been adjusted for *TPMT* (thiopurine S-methyltransferase) genotype.[19] Rare truncating mutations in the *ITPA* gene have recently been associated with a severe early-infantile encephalopathy.[20] In an *ITPA*

knockout mouse model, ITPase deficient mice showed features of growth retardation and cardiac myofiber disarray and died soon after birth.[21]

In HIV-infected patients, the decreased erythrocyte ITPase activity was independent of the presence of SNPs in the *ITPA* gene, as it was seen in both wild-type and SNP carriers.[4] This finding raised the question whether in leukocytes as well, ITPase activity is decreased, and thus purine metabolism is altered, independent of *ITPA* genotype in HIV infected patients on cART. We therefore analyzed the presence of the ITPase protein in leukocytes and leukocyte subpopulations, in association with *ITPA* genotype in a HIV-infected and a control population.

METHODS

Patients

HIV-seropositive patients, visiting the HIV outpatient clinic of the Maastricht University Medical Centre (MUMC+) aged 18 years and older, were eligible for inclusion in the study. Consecutive patients were asked for participation and included after written informed consent was obtained. Data regarding age, gender at birth, country of birth, race, CD4 nadir, and date of start combination Antiretroviral therapy (cART), were obtained from the Dutch HIV monitoring foundation (Stichting HIV Monitoring; SHM). The control population consisted of anonymous samples from the general hospital population of the Zuyderland Medical Center in Heerlen. All control samples were used according to the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies. The study was approved by the Medical Ethics Committee of the MUMC+ and all patients signed written informed consent.

Flow cytometric ITPase expression analysis

ITPase expression was determined by flow cytometry as described earlier.[22] In short, EDTA anticoagulated peripheral blood samples were processed and analyzed within 24 hours after collection. One hundred microliters of whole blood was incubated with a cocktail of directly conjugated antibodies.[22] Cell suspensions were incubated for 15 minutes in the dark at room temperature (RT). A Fix&Perm strategy (GAS004; Caltag, Invitrogen, Carlsbad, CA) was performed after washing with phosphate-buffered saline. The fixation step consisted of the addition of 100 mL fixation reagent for 15 minutes at RT and 2 wash steps (phosphate-buffered saline). This was followed by the addition of the permeabilization medium and anti-ITPase MoAb (clone 2H8, isotype IgG2a, catalog number H00003704-M01, Abnova, Taipei City, Taiwan). Except some isoforms of ITPase protein, no other human proteins have a similar sequence, thus the specificity of the antibodies used to detect the ITPase protein is considered

to be high and the chance we measured other protein products than the ITPase protein seems negligible. After incubation for 15 minutes at RT and one wash step, 5 mL PE-conjugated anti-IgG2a (Southern Biotech, Birmingham, AL) was added. Finally, after incubation (15 minutes at RT) and washing, 500 mL CellWash (BD) was added. Samples were analyzed with a BD FACSCanto (BD Biosciences, San Jose, CA). Cell-bound fluorescent labels were excited with a 488 nm, air-cooled, solid-state argon ion laser, followed by a 17 mW 633 nm, Helium–Neon laser. The flow cytometer was set up daily using 7-color beads (BD) with automatic compensation settings using the FACSCanto software to achieve optimal instrument performance. Sample analysis was completed when 5000 lymphocytes were collected. All measured events were stored and analyzed. The fluorescence signal data were recorded with logarithmical amplification. FACSDiva software (BD) was used for the analysis of acquired data. A negative control (Fluorescence-minus-one: no anti-ITPase but with addition of PE-conjugated anti-IgG2A) was used to set a threshold at a maximum of 5% positivity to exclude auto-fluorescence and to confirm ITPase-specific labeling. The expression of ITPase was determined by determining the median fluorescent intensity of a certain leukocyte population.

Gating strategy

Parental gating, based on scatter signals and immuno-fluorescence staining as described earlier,[22] resulted in a step-wise separation of subpopulations of leukocytes with accumulated complexity of phenotypical characteristics.

DNA isolation and *ITPA* genotyping

Total DNA was extracted from blood using the Blood L Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and the Hamilton Microlab STAR Line (Hamilton, Bonaduz, Switzerland). Specific intronic primers with an additional M13-tag were used to amplify exon 2 of the *ITPA* gene and flanking intronic regions (*ITPA*_ex2F-CTTTAGGAGATGGGCAGCAG; *ITPA*_ex2R-CACAGAAAGTCAGGTCACAGG). Amplification was performed in a 10 mL reaction volume using Amplitaq Gold 360 Master Mix (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) with 5% 360 GC Enhancer, 2 pmol of each primer, and 10 ng DNA. The cycle conditions were 96°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds with a final elongation step of 72°C for 10 minutes. The resulting 245-bp PCR product was bi-directionally sequenced using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit and the ABI3730XL genetic analyzer (Applied Biosystems, Foster City, CA). The 2 common functional polymorphisms c.94C>A (p.Pro32Thr; NCBI rs1127354) and c.124+21A>C (NCBI rs7270101) were determined using Mutation Surveyor DNA variant analysis software (SoftGenetics, State College, PA) with genomic NCBI reference sequence NC_000020. All sequences were evaluated independently by 2 laboratory experts.

Statistical analysis

Results were analyzed using GraphPad Prism 5.01 (Graphpad Software for Windows, San Diego, CA), Microsoft Excel (Microsoft, Redmond, WA) software, and IBM SPSS Statistics 20 (IBM Corporation, New York, NY) software. Independent samples 2-tailed T-test was used to determine significant differences. P-values <0.05 were considered to be statistically significant.

RESULTS

Patient characteristics

A total of 59 HIV-infected patients and 50 controls were included (Table 1). The mean age did not differ significantly between the groups. There was a male predominance in the HIV-infected patients. All but 1 patient used cART and the mean CD4 nadir of this group was 206 $CD4^+$ lymphocytes per liter. Although all mean values of the hematological parameters assessed were within the reference values, significant differences between the HIV-infected patients and the control patients were found (Table 1). Mean corpuscular volume and mean corpuscular hemoglobin levels were higher in the HIV-infected population, whereas hematocrit and mean corpuscular hemoglobin concentration was lower. The HIV-infected patients had a significantly lower white blood cell and platelet count compared with the control population.

Leukocytes and leukocyte subsets

The leukocyte subtype distribution showed no difference in percentages of monocytes (6.7% versus 6.7%; p=0.9, respectively) and granulocytes (56.7% versus 61.1%; p=0.08, respectively) between HIV-infected and control patients (data not shown). The HIV-infected patients had a statistically significant higher percentage of lymphocytes (29.4% versus 23.1%; p<0.001, respectively). No difference was found in the size of the fraction of B-lymphocytes between HIV-infected patients and controls (9.80% versus 10.03%; p=0.80, respectively). The percentage of T-lymphocytes was significantly higher in HIV-infected patients (80.0% versus 74.0%; p=0.001, respectively), with decreased T-helper cells ($CD4^+$ T-cells) (37.6% versus 61.6%; p<0.0001) and increased cytotoxic T-cells ($CD8^+$ T-cells) (57.9% versus 32.6%; p<0.0001) compared with the control population. The percentage of Natural killer cells was significantly lower in HIV-infected patients (10.2% versus 16.6%; p< 0.0001).

Presence of ITPase in leukocyte subsets

High percentages of ITPase positive cells could be demonstrated in the control population; lymphocytes 99.5%, monocytes 98.8%, and granulocytes 95.3% were ITPase positive. Less lymphocytes (90.9%) and granulocytes (77.1%) were ITPase positive in HIV-infected

Table 1. Demographic, clinical and hematological characteristics of patients and controls.

Characteristic	HIV-infected (N=59)	Controls (N=50)	Reference values, range	p-value
Age, Median years (range)	49.6 (28.3-68.2)	56.8 (15.9-90.2)		
Male gender, n (%)	45 (76.3)	50 (50.0)		
***ITPA* genotype**, n (%)				
Wt/wt	40 (67.8)	26 (52.0)		
Wt/c.124+21A>C	7 (11.9)	12 (24.0)		
Wt/c.94C>A	7 (11.9)	9 (18.0)		
c.124+21A>C/c.124+21A>C	1 (1.7)	3 (6.0)		
c.94C>A/c.124+21A>C	2 (3.4)	-		
Unknown	2 (3.4)	-		
Mean CD4 nadir ± SD	205.8 ± 152.4			
cART	58 (98.3)			
Race, n (%)				
Caucasian	47 (79.7)			
Hispanic	1 (1.7)			
African	4 (6.8)			
Asian	5 (8.5)			
Mix	2 (3.4)			
Mean WBC, $x10^9$/L ± SD	6.33 ± 2.05	8.69 ± 2.82	4.00–10.00	<0.001
Mean RBC, $x10^{12}$/L ± SD	4.67 ± 0.66	4.77 ± 0.34	4.20–6.20	0.31
Mean Hb, mmol/L ± SD	10.67 ± 9.40	8.94 ± 0.70	7.50–11.00	0.20
Mean Ht, L/L ± SD	0.48 ± 0.06	0.43 ± 0.03	0.36–0.51	<0.001
Mean MCV, fL ± SD	102.76 ± 9.69	90.24 ± 3.66	80.00–100.00	<0.001
Mean MCH, ± SD	2.03 ± 0.22	1.87 ± 0.09	1.70–2.10	<0.001
Mean MCHC, g/dl ± SD	19.80 ± 1.02	20.78 ± 0.61	19.30–22.50	<0.001
Mean Platelets, $x10^{12}$/L ± SD	222.07 ± 68.47	248.48 ± 60.62	130.00–400.00	0.04

cART, combined Antiretroviral therapy; WBC, White blood cells; RBC, Red blood cells; Hb, Hemoglobin; Ht, Hematocrit; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration

patients (Figure 1A) compared with the control population (p<0.0001). Furthermore, all lymphocyte subtypes showed statistically significant lower percentages of ITPase positive cells in HIV-infected patients (Figure 1B). In the controls, all lymphocyte subtypes had a mean percentages of ITPase positive cells of 99% or more, whereas in HIV-infected patients 91% of T-lymphocytes, 97% of B-lymphocytes, and 87% of natural killer cells were ITPase positive (p<0.0001 for all). Although the CD4/CD8 ratio of T-lymphocytes is decreased in HIV-infected patients, both CD4 lymphocytes and CD8 lymphocytes had less ITPase positive cells compared with the control patients; 97.4% versus 99.9% for $CD4^+$ lymphocytes (p=0.002) cells and 85.9% versus 99.6% for $CD8^+$ lymphocytes (p<0.0001).

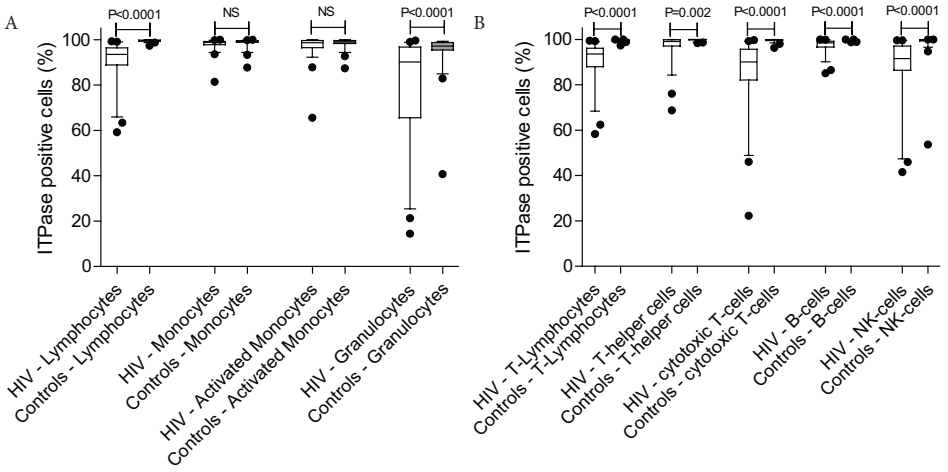

Figure 1: Comparison of percentages of ITPase positive cells in (A) white blood cell subtypes and (B) lymphocyte subsets between HIV-infected patients (white bars) and control patients (gray bars). Displayed are: the median, the 25-75% percentiles (bars), upper and lower 1,5 times interquartile range (whiskers) and outliers (dots). Except for (activated) monocytes, all the compared cell types showed significant decreases of ITPase positive cells in HIV-infected patients (p=0.002 in T-helper cells, p<0.0001 in all others).

Quantitation of ITPase expression

ITPase expression was determined in the ITPase positive cell fraction. ITPase expression (expressed as median fluorescent intensity) was highest in activated monocytes and lowest in lymphocytes, in both HIV-infected patients and controls regardless of HIV-infection (Figure 2A). In HIV-infected patients, ITPase expression was lower in (activated) monocytes, granulocytes, and lymphocytes compared with controls (Figure 2A, p<0.0001 for all). In all lymphocyte subsets, ITPase expression was significantly lower in HIV-infected patients compared with controls (Figure 2B, p-values <0.0001 for all).

Association of ITPase expression in leukocytes with *ITPA* genotype

ITPA allele frequencies did not differ significantly between HIV-infected patients and the control group (Table 1). Wt/wt was the most prominent *ITPA* genotype (67.8% in HIV-infected and 52% in controls). The ITPase expression was not associated with *ITPA* genotype (comparison between wt/wt, wt/c.124+21A>C and wt/c.94C>A + other) in monocytes, lymphocytes, and granulocytes (Fig. 3).

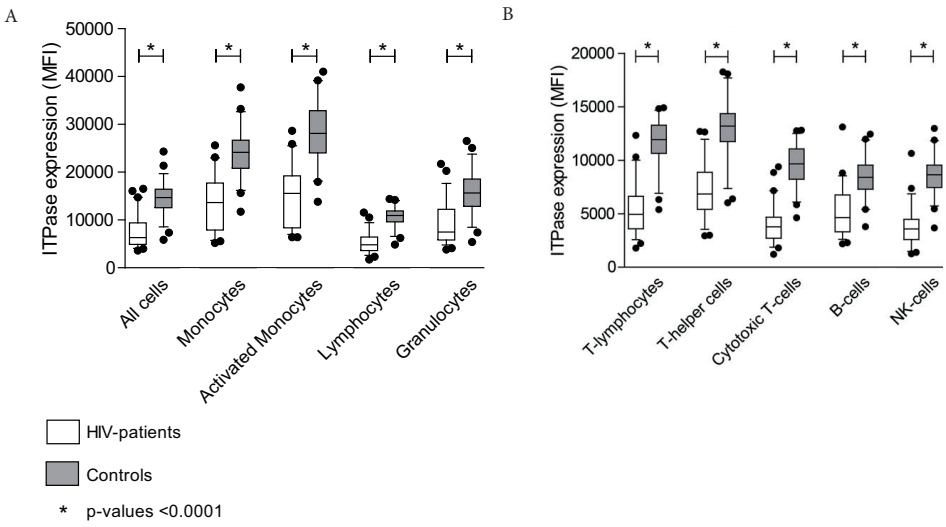

Figure 2: Comparison of ITPase expression (expressed as Median fluorescent intensity; MFI) in white blood cell subtypes (A) and lymphocyte subsets (B) between HIV-infected patients (white bars) and control patients (gray bars). Displayed are: the median, the 25-75% percentiles (bars), upper and lower 1,5 times interquartile range (whiskers), outliers (dots). All the compared cell types showed significant decreases of MFI in HIV-infected patients ($p<0.0001$ for all).

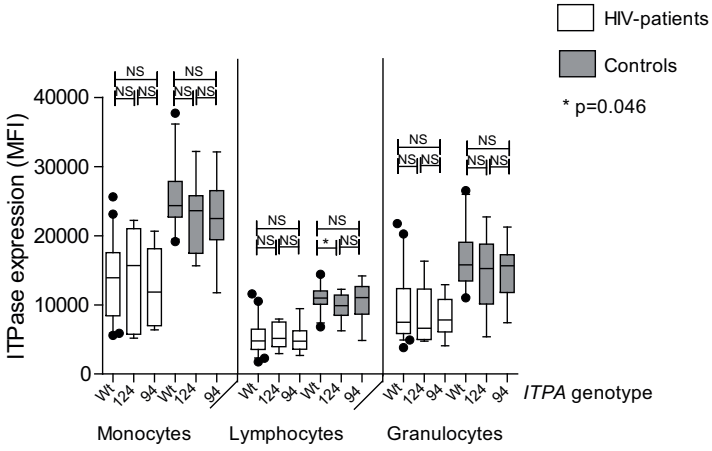

Figure 3. Stratification of different leukocyte subtypes by *ITPA* genotype and the corresponding ITPase expression (expressed as Median fluorescent intensity, MFI). No association was found between *ITPA* genotype (wt=wt/wt; 124=wt/c.124+21A>C; 94=wt/c.94C>A) and ITPase expression in HIV-infected patients (white bars) and control patients (gray bars) in monocytes, lymphocytes and granulocytes (no significant differences except for the comparison between wt/wt and wt/c.124+21A>C in lymphocytes of control patients; p=0.046).

DISCUSSION

Here, we report a striking significant decrease in ITPase expression in leukocyte subtypes and lymphocyte subsets in HIV-infected patients compared with controls. We observed an overall decrease in ITPase expression in white blood cells of treated HIV-positive patients, regardless of genotype suggesting that the decrease in ITPase expression in leukocytes was because of HIV-infection or cART, albeit that the influence of nucleoside analogues is likely to be limited.[4]

Regardless of HIV status, ITPase protein expression was highest in monocytes, being especially high in activated monocytes. This may reflect the phagocytic activity, as the destruction of pathogens and infected cells either by oxygen dependent and independent pathways releases nucleotides and very likely also noncanonical nucleotides, such as Inosine triphosphate (ITP).

Competition for the intracellular nucleotide pools between the host white blood cells and HIV appears to be a major contributor to HIV pathogenesis. Earlier reports showed that pyrimidine nucleotide metabolism (both de novo and salvage) is severely impaired in HIV-infected lymphocytes.[1–3] After mitogenic stimulation, HIV-infected lymphocytes were unable to extend both their purine and pyrimidine nucleotide pools to allow proliferation. A major difference between HIV-infected lymphocytes and controls was that the former rely predominantly on purine salvage metabolism to meet the cellular requirement opposed to de novo synthesis of purine nucleotides being the predominant biosynthetic route in the latter. Sterile alpha motif and histidine-aspartic domain-containing protein 1 (SAMHD1) is a human defense protein that inhibits the HIV-1 replication by depleting intracellular deoxynucleotide pools.[23] In HIV-2 infection, this action is counteracted by the virion-packaged accessory protein.[23] These findings suggest that the infected lymphocyte's ultimate defense mechanism is to cause a fatal imbalance in the intracellular nucleotide pool to prevent HIV-integration and replication. Our observation of reduced ITPase expression in HIV-infected lymphocytes is in line with this hypothesis. Decreased activity of ITPase leads to decreased scavenging of noncanonical nucleotides, including ITP and deoxy-ITP which contributes to the intracellular nucleotide imbalance in HIV-infected lymphocytes by increasing the availability of non-canonical nucleotides for DNA and RNA replication, causing erroneous incorporation into nucleic acids, potentially leading to programmed cell death and mutagenesis.[24]

The decreased ITPase expression and thus possibly the decreased ITPase activity in the blood compartment[4] may have implications for the efficacy of purine analogue reverse transcriptase inhibitors. It can be expected that the efficacy of nucleos(t)ide reverse tran-

scriptase inhibitors (NRTIs) of which the nucleotide triphosphate form exerts the virostatic effect is enhanced, whereas the efficacy of NRTIs of which the nucleotide monophosphate exerts the virostatic effect is reduced. This implies treatment with purine NRTIs tenofovir, didanosine, and abacavir, which are active in their nucleotide triphosphate form, benefit from the observed decreased ITPase expression. This is in contrast to the earlier reported decreased phosphorylation of ziduvodine in HIV-infected lymphocytes due to decreased thymidine kinase 2 activity.[2]

ITPase protein and *ITPA* mRNA are likely to be ubiquitously expressed in all nucleated cells.[25–27] With respect to erythrocytes, a clear-cut genotype–phenotype correlation exists, both in HIV-infected patients and in controls.[28,29] According to our present findings this seems not to be the case in white blood cells, neither in HIV-infected patients and controls. Our previous results in erythrocytes may suggest decreased stability of the P32T ITPase protein and the wild type protein in the erythrocytes of HIV-infected individuals. Because no protein synthesis takes place in erythrocytes, a HIV-induced instability or reduced biosynthesis of the ITPase protein is readily detected. A possible explanation for the fact that this is not observed in our present work may be that in white blood cells *ITPA* is transcribed and the ITPase protein is synthesized continuously, despite the amount of mRNA encoding the entire protein being reduced due to altered splicing of the pre-mRNA in the *ITPA* 94C>A and 121+21A>C.[30] Although ITPase expression appears to be generally decreased in blood, there may still exist pharmaco-genetic phenomena associated with *ITPA* genotype, apart from the present observation.

In conclusion, this study is the first to report that the expression of ITPase is significantly decreased in lymphocytes and granulocytes of treated HIV-infected patients, compared with a control population. Given that purine NRTIs are active in their triphosphate form, the present results underline their importance in the backbone of cART. Our findings corroborate previous findings that control over intracellular nucleotide pools is vital for both HIV and its host for survival.

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