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Polymorphism in HIV-1 dependency factor PDE8A affects mRNA level and HIV-1 replication in primary macrophages

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ABSTRACT

Four genome-wide RNAi screens have recently identified hundreds of HIV-1 dependency factors (HDFs). Previously, we reported a large variation in the ability of HIV-1 to replicate in monocyte-derived macrophages (MDM) derived from >400 healthy seronegative blood donors. Here we determined whether SNPs in genes encoding newly identified HDFs were associated with this variation in HIV-1 replication. We found a significant association between the minor allele of SNP rs2304418 in phosphodiesterase 8A (*PDE8A*) and lower HIV-1 replication ($p = 2.4 \times 10^{-6}$). The minor allele of SNP rs2304418 was also significantly associated with lower *PDE8A* mRNA levels in MDM ($p = 8.3 \times 10^{-5}$). In accordance with this, overexpression of *PDE8A* in HEK293T cells resulted in increased HIV-1 replication, while subsequent knock-down of *PDE8A* decreased replication. This study links host genetic variation in a newly identified HDF to variation in HIV-1 replication in a relevant primary target cell for HIV-1 and may provide new leads for treatment of this infection.

Introduction

Viruses depend on the host cellular machinery for their replication. In the case of human immunodeficiency virus (HIV) the host proteins needed for its replication have been named HIV dependency factors (HDFs). Recently, four genome-scale RNA interference (RNAi) screens for novel HDFs were published (Brass et al., 2008; König et al., 2008; Yeung et al., 2009; Zhou et al., 2008). Each study identified between 200 and 300 new HDFs (n = 281, 295, 232 and 252 respectively) with only a limited overlap between the sets. As the conditions and readouts of the screens varied between the studies, each screen may have studied host proteins involved in different stages of the viral life cycle or with different protein half-lives. Moreover, the studies used different RNAi libraries, which may influence both the level of knock-down for each gene and the off-target effects. Therefore, the screens are in many ways complementary and have thus generated a large body of data on putative new HIV–host interactions. Additional studies are however required to learn more about the precise role of each of these HDFs in the viral life cycle.

In the past, polymorphisms in HDF genes, such as *CCR5*, have been shown to affect HIV disease progression (De Roda Husman et al., 1997; Dean et al., 1996), which has led to genome-wide screens to identify additional polymorphisms and gene regions that play a role in HIV-1 pathogenesis. Recent genome-wide association studies have indeed identified single nucleotide polymorphisms (SNPs) that influence viral load at set-point and disease progression once an individual has become infected with HIV-1 (Dalmasso et al., 2008; Fellay et al., 2007, 2009; Herbeck et al., 2010; Le Clerc et al., 2009; Limou et al., 2009, 2010; Pelak et al., 2010; Petrovski et al., 2011). However, many of the SNPs identified in these studies are not in HDFs as host antiviral and immune factors also contribute to the outcome of HIV-1 infection *in vivo*.

Previously we reported on the large variation between monocyte-derived macrophages (MDM) from different donors in their ability to support *in vitro* HIV-1 replication, and that viral replication could be restricted both at entry and post-entry levels of the viral replication cycle (Fouchier et al., 1994). More recently, we have shown that the large inter-donor variation in *in vitro* HIV-1 replication in MDM could only in part be explained by the *CCR5* Δ32 genotype (Bol et al., 2009). Experiments with VSV-G pseudotyped HIV-1, in which CD4 and co-receptor are bypassed for entry, confirmed the existence of other *CCR5*

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independent host genetic factors that enhance or restrict HIV-1 replication in MDM at the post entry level. Indeed, in a subsequent GWAS, we identified a SNP in *DYRK1A* to be associated with *in vitro* HIV-1 replication in macrophages, an effect that was independent of the *CCR5* Δ32 genotype, as well as with HIV-1 disease progression *in vivo* in two independent cohort studies (Bol et al., 2011b).

In our present study we selected 19,487 SNPs in the newly identified HDFs (Brass et al., 2008; König et al., 2008; Yeung et al., 2009; Zhou et al., 2008) and tested each SNP for association with HIV-1 replication in MDM, to see if host genetic variation in HDFs could be responsible for the variation in HIV-1 replication in primary MDM from different donors.

Results

A SNP in the gene coding for phosphodiesterase 8A (PDE8A) is associated with HIV-1 replication in monocyte-derived macrophages

In a previous study we determined the *in vitro* HIV-1 replication in monocyte-derived macrophages (MDM) from 393 healthy blood donors, as measured by the amount of Gag p24 production in the culture supernatant on day 14 after inoculation (Bol et al., 2009). The variation in Gag p24 production could only in part be explained by the *CCR5* Δ32 genotype. Genome-wide SNP analysis was then performed on DNA from the donors for whom MDM ranked in the top quartile (n = 96 donors) or in the bottom quartile (n = 96 donors) with respect to HIV-1 replication (Bol et al., 2011b). In our present study, we determined associations between SNPs in the nearly 1000 HIV-1 dependency factors (HDF) that were recently identified by four genome-scale RNAi screens (Brass et al., 2008; König et al., 2008; Yeung et al., 2009; Zhou et al., 2008). Linear regression was used to test for associations between 19,487 SNPs in 997 HDF genes and the levels of *in vitro* HIV-1 replication in MDM of these two groups of donors. Table 1 shows the 26 SNPs with strongest association between SNP genotype and HIV-1 replication in MDM (cut-off p value $= 1\times10^{-3}$). One SNP (rs2304418) in the gene encoding phosphodiesterase 8A (PDE8A) was significantly associated with the replication level of HIV-1 in MDM (Fig. 1A). The observed p value was 2.4×10^{-6}, and the association remained significant after applying the conservative Bonferroni correction for multiple testing, $p = 0.047$. Furthermore, the empirical p value for linear regression using 10^7 permutations of the genotypes confirmed the significance of the association ($p = 2.3\times10^{-6}$). After genotyping SNP rs2304418 in the 202 donors whose MDM ranked in the middle for Gag p24 production (Bol et al., 2009), the p value for the association between this genotype and *in vitro* HIV-1 replication in MDM in the total group of 393 donors increased from 2.4×10^{-6} to 1.3×10^{-5} (Fig. 1B). With 180

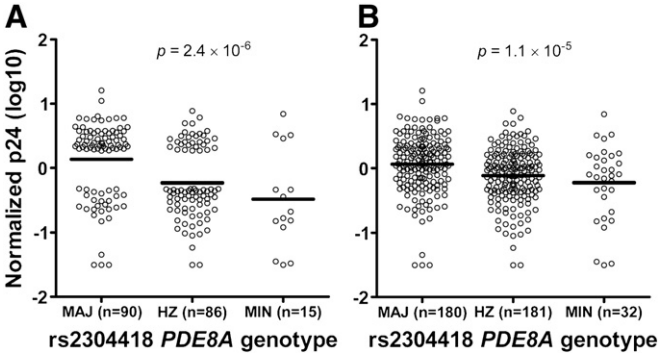

Fig. 1. Association between SNP rs2304418 genotype and normalized Gag p24 levels produced by HIV-1 infected monocyte-derived macrophages (MDM) *in vitro* for (A) 191 donors whose MDM ranked in the top quartile (n = 96) or in the bottom quartile (n = 95) and (B) the total group of donors (n = 393). MAJ, homozygous for the major allele; HZ, heterozygous; MIN, homozygous for the minor allele.

Table 1
26 SNPs in genes identified in RNAi screen studies with unadjusted p values $<1\times10^{-3}$ for association with HIV-1 replication in monocyte-derived macrophages.

SNP	Location	Gene symbol	Gene ID	RNAi study	Chr.	Position	# MIN	LD (r^2)	p value
rs2304418	Intron	*PDE8A*	5151	Zhou et al. (2008)	15	83441987	15	0.96[a]	2.4×10^{-6}*
rs12909130	Intron	*PDE8A*	5151	Zhou et al. (2008)	15	83391505	15	0.96[a]	8.3×10^{-6}
rs12469968	Intron	*DPP4*	1803	Zhou et al. (2008)	2	162632163	46		1.6×10^{-4}
rs760114	Intron	*CLPTM1*	1209	Yeung et al. (2009)	19	50156803	3		2.6×10^{-4}
rs11723566	Flanking 3′ UTR	*ANXA5*	308	Zhou et al. (2008)	4	122789490	26		2.7×10^{-4}
rs2776932	Intron	*NRP1*	8829	Yeung et al. (2009)	10	33632412	5		4.1×10^{-4}
rs227778	Intron	*DYSF*	8291	Brass et al. (2008)	2	71725741	9		4.3×10^{-4}
rs17499015	Intron	*TSPAN5*	10098	Yeung et al. (2009)	4	99660372	10	0.97[b]	4.9×10^{-4}
rs6532740	Intron	*TSPAN5*	10098	Yeung et al. (2009)	4	99662961	10	0.97[b]	4.9×10^{-4}
rs7792945	Intron	*AAA1*	404744	König et al. (2008)	7	34604797	13		5.0×10^{-4}
rs2559081	Intron	*DYSF*	8291	Brass et al. (2008)	2	71754794	3		5.5×10^{-4}
rs999466	Flanking 3′ UTR	*CA2*	760	Zhou et al. (2008)	8	86582896	7		5.5×10^{-4}
rs7593348	Flanking 5′ UTR	*DPP4*	1803	Zhou et al. (2008)	2	162645233	36		6.9×10^{-4}
rs11688740	Flanking 3′ UTR	*BCL11A*	53,335	König et al. (2008)	2	60391494	7		7.1×10^{-4}
rs2304416	Intron	*PDE8A*	5151	Zhou et al. (2008)	15	83419886	8	1.00[c]	7.1×10^{-4}
rs2304415	Intron	*PDE8A*	5151	Zhou et al. (2008)	15	83419845	8	1.00[c]	7.1×10^{-4}
rs10846681	Flanking 5′ UTR	*NCOR2*	9612	Brass et al. (2008)	12	123569658	3		7.3×10^{-4}
rs17139235	Flanking 5′ UTR	*RPP40*	10799	Yeung et al. (2009)	6	4975522	2		7.6×10^{-4}
rs2288820	Flanking 3′ UTR	*NDFIP1*	80762	König et al. (2008)	5	141528208	4		7.7×10^{-4}
rs1863812	Intron	*DYSF*	8291	Brass et al. (2008)	2	71759679	3		8.0×10^{-4}
rs10187598	Intron	*DYSF*	8291	Brass et al. (2008)	2	71760657	3		8.0×10^{-4}
rs220250	Flanking 5′ UTR	*UMODL1*	89766	König et al. (2008)	21	42345236	7		8.2×10^{-4}
rs630994	Flanking 5′ UTR	*HEATR1*	55127	Brass et al. (2008)	1	234839234	1		8.5×10^{-4}
rs737787	Intron	*NF2*	4771	Brass et al. (2008)	22	28402759	7		9.0×10^{-4}
rs4720510	Flanking 5′ UTR	*ADCY1*	107	Yeung et al. (2009)	7	45492698	2		9.4×10^{-4}
rs4641448	Flanking 3′ UTR	*PARVA*	55742	Zhou et al. (2008)	11	12518710	27		9.8×10^{-4}

Chr., chromosome.
MIN, individuals homozygous for the minor allele.
LD, linkage disequilibrium.
Information about LD is only provided when $r^2 > 0.8$.
* Statistically significant after correction for multiple testing (Bonferroni).
[a,b and c] Numbers represent the magnitude of LD for that pair of SNPs (indicated with the letter a, b or c) as measured by r^2 in our study population.

individuals homozygous for the major allele (MAJ), 181 heterozygous individuals (HZ) and 32 individuals homozygous for the minor allele (MIN) for *PDE8A* SNP rs2304418, the genotype distribution in our total study population (n = 393) did not deviate from Hardy–Weinberg equilibrium ($p > 0.05$), and was not significantly different from the distribution in the 120 HapMap CEU individuals (International HapMap Consortium, 2003; Thorisson et al., 2005) (50 MAJ, 64 HZ and 6 MIN genotypes) ($p = 0.3$, Fisher exact test).

SNP rs2304418 is located in close proximity (less than 200 base pairs) to exon 10 of the *PDE8A* gene. Three other SNPs in the *PDE8A* region (present on the Illumina 610Q SNP BeadChip) were also found to be associated with HIV-1 replication, although not significant after Bonferroni correction (Table 1). Of these three SNPs, rs12909130 is in high linkage disequilibrium (LD) with rs2304418 ($r^2 = 0.96$ in the population studied; $r^2 = 1.00$ in the 1000 Genomes Pilot 1) and is located more than 50 kb upstream of rs2304418 in the 82 kb large first intron of *PDE8A* (Figs. 2 and S1). The two other SNPs (rs2304415 and rs2304416) are only in moderate LD with rs2304418 (both with an r^2 of 0.61 in our studied population) and lie 200–250 bp upstream of exon 4.

Association of SNP rs2304418 in PDE8A with HIV-1 replication is independent of the CCR5 Δ32 and DYRK1A SNP rs12483205 genotype

The *CCR5* Δ32 genotype is associated with markedly reduced levels of CCR5 on lymphocytes and monocytes/macrophages (Hladik et al., 2005; Wu et al., 1997), which greatly affects HIV-1 replication in both cell types (Bol et al., 2009; Liu et al., 1996). MDM from donors heterozygous for the 32 base pair deletion (n = 79) had significantly lower Gag p24 production than MDM from donors without the deletion (n = 314) ($p = 0.001$, unpaired t-test, data not shown). Furthermore, in our previous study (Bol et al., 2011b) we reported the association between *DYRK1A* SNP rs12483205 and HIV-1 replication in MDM ($p = 2.1 \times 10^{-5}$, n = 393). To verify if the observed effect of *PDE8A* SNP rs2304418 on HIV-1 replication was independent of the *CCR5* Δ32 and *DYRK1A* SNP rs12483205 genotypes, multiple regression analysis was performed. After using the *CCR5* Δ32 and *DYRK1A* SNP rs12483205 genotype as additional covariates in the analysis, the association between the SNP rs2304418 genotype and the level of Gag p24 production in MDM *in vitro* remained statistically significant (6.3×10^{-6}, n = 393) (Table S1). Indeed, the group of *CCR5* Δ32 HZ donors was not overrepresented in the *PDE8A* rs2304418 SNP HZ or MIN group ($p = 0.22$, Fisher exact test, n = 393) (Table S1). Similarly, *DYRK1A* rs12483205 and *PDE8A* rs2304418 genotype distributions were not significantly associated ($p = 0.66$, Fisher exact test, n = 393).

PDE8A transcripts and protein isoforms are expressed in macrophages

To confirm the expression of *PDE8A* in MDM, we compared *PDE8A1* transcript levels in MDM with those in monocytes, unstimulated peripheral blood lymphocytes and eight different cell lines (HEK293T, C8166, Hep G2, HL-60, MT-2, TZM-bl, U87 and U937). Quantitative PCR results using either *GAPDH* or *ACTB* as reference gene showed that *PDE8A1* mRNA in monocytes and unstimulated peripheral blood lymphocytes was almost absent (data not shown). *PDE8A1* transcript levels in MDM were comparable to, or even higher than in the cell lines tested (data not shown).

To determine which *PDE8A* transcript variants are present in MDM in addition to *PDE8A1*, PCRs were performed on cDNA derived from MDM using transcript specific primers (Fig. S2 and Table S2). All described *PDE8A* variants (NCBI: transcript 1 NM_002605.2, transcript 2 NM_173454.1, transcript 3 NM_173455.1, transcript 4 NM_173456.1, and transcript 5 NM_173457.1) could be detected in MDM (Fig. 3). In addition, endogenous PDE8A protein expression in MDM was detected by immunoblotting (Fig. 4). Although isoforms 1 and 2 seem to be predominantly expressed, the other three PDE8A isoforms were also detected in MDM.

Since we showed that multiple transcript variants exist in MDM we determined whether the SNP in *PDE8A* had an effect on splicing of the *PDE8A* pre-mRNA. We tested for the presence of all transcript variants using cDNA from 6 donors, with different genotypes for SNP rs2304418 in *PDE8A* (2 MAJ, 2 HZ and 2 MIN donors). We found similar transcripts in macrophages independent of the genotype for rs2304418 (Fig. 3). In addition, no quantitative differences in *PDE8A* transcripts were observed between the 3 different *PDE8A* SNP rs12909130 (perfect proxy for rs2304418) genotypes (MAJ, HZ and MIN) after comparing all ratios between the 5 different *PDE8A* transcript levels (data not shown). Furthermore, neither *PDE8A* SNPs rs2304418/rs12909130 nor any SNPs in moderate to high LD ($r^2 > 0.6$) with these were located in close proximity to an intron–exon boundary (data not shown). These results suggest that neither rs2304418 nor any SNP in high LD has an effect on splicing of *PDE8A* pre-mRNA.

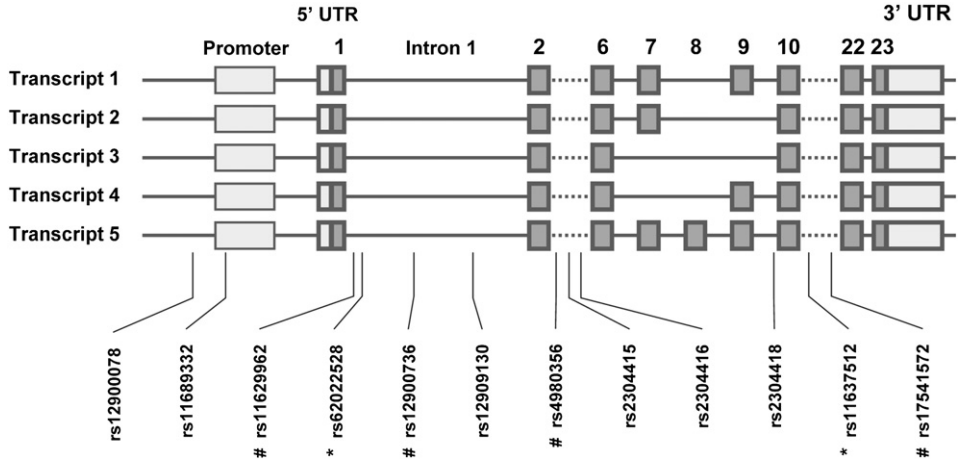

Fig. 2. Schematic overview of the five different *PDE8A* transcript variants, including 3′ UTR and a 2 kb region upstream of the ATG start codon containing the promoter and 5′ UTR. Exons are depicted as gray boxes, UTR (thick border) and promoter (thin border) as white boxes. SNPs in moderate to high LD ($r^2 > 0.6$) with rs2304418 or rs12909130 that affect possible human transcription factor binding sites (#) or microsatellites (*) are shown, as well as the SNP identified in the promoter region of *PDE8A*. SNPs rs12909130 and rs12900078 were used for genotyping since they are perfect proxies for SNP rs2304418 and the promoter SNP rs11689332 respectively. All other SNPs in LD ($r^2 > 0.6$) with rs12909130 or rs2304418 can be found in Table S3. LD, linkage disequilibrium.

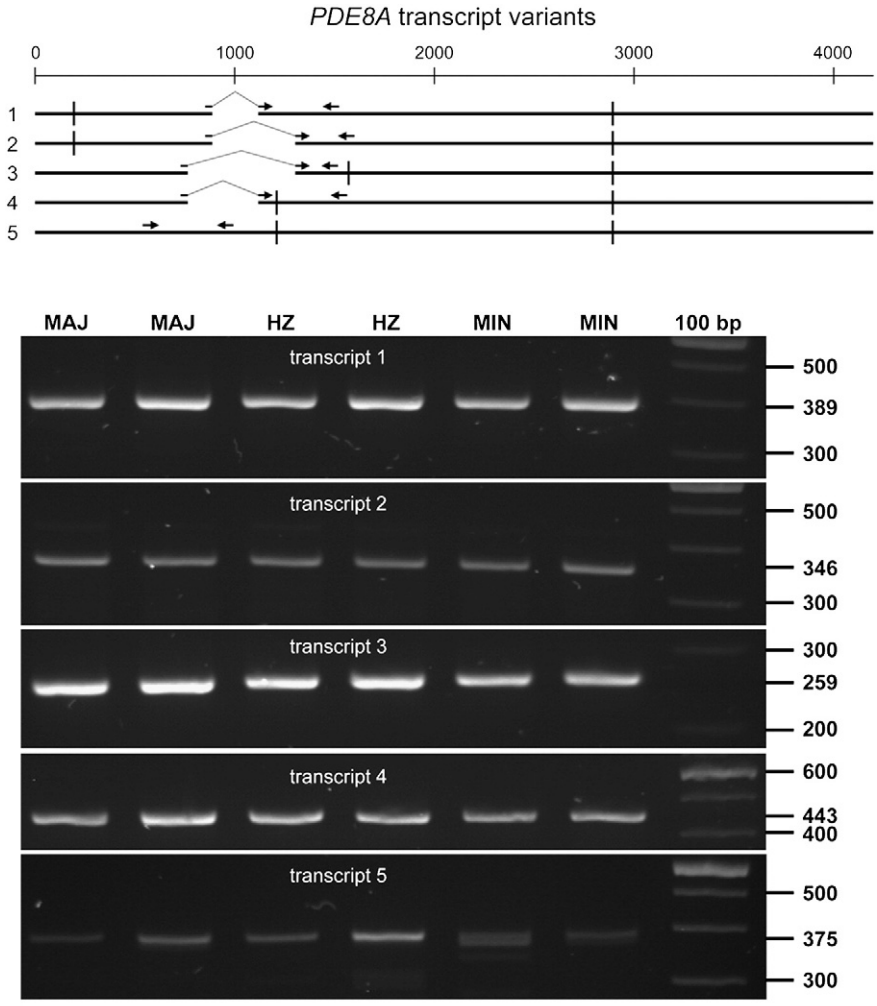

Fig. 3. Top: schematic alignment of the 5 different *PDE8A* transcript variants. Primers (arrows) were designed to allow for the unique detection of each splice variant. Vertical lines represent start or stop codon. Bottom: PCR products confirming the presence of multiple different *PDE8A* transcripts in monocyte-derived macrophages. The similarity in PCR results between MAJ, HZ and MIN donors suggests that SNP rs2304418 in *PDE8A* does not affect splicing of the *PDE8A* pre-mRNA. For transcript variant 5, additional nonspecific amplicons were seen for some donors. MAJ, homozygous for the major allele; HZ, heterozygous; MIN, homozygous for the minor allele; 100 bp, 100 base pair DNA marker.

Donors homozygous for the minor genotype show a two fold decrease of PDE8A mRNA levels in macrophages

Knock-down of *PDE8A* mRNA was described to have a negative effect on HIV-1 replication (Zhou et al., 2008). Therefore we next determined whether the SNP in *PDE8A* affects *PDE8A* mRNA levels in MDM. We performed a quantitative PCR (qPCR), using MDM cDNA from an additional group of 69 HIV-1 negative donors and used *GAPDH* and *ACTB* as reference genes. We found that the minor allele of SNP 12909130 (perfect proxy for rs2304418) was associated with lower levels of *PDE8A* transcript 1 in macrophages, and resulted in a PDE8A isoform 1 mRNA reduction of more than 20% for HZ donors and more than 55% for MIN donors ($p = 8.3 \times 10^{-5}$, linear regression) (Fig. 5A). Furthermore, the effect was additive, revealed by the highest average level of *PDE8A1* mRNA in the MAJ group, intermediate average level in the HZ group, and lowest average level in the MIN group, thereby perfectly corresponding with the effect of SNP rs2304418 on HIV-1 replication in macrophages (Fig. 1).

For 32 of the 69 donors mentioned above, data on *PDE8A* mRNA levels as well as HIV-1 replication (measured as Gag p24 levels) were available. The minor allele of SNP rs12909130 (perfect proxy for rs2304418) in *PDE8A* was associated with lower HIV-1 replication in MDM ($p = 0.0157$, Mann Whitney test) (Fig. 6A) as well as lower *PDE8A* mRNA levels in MDM ($p = 0.0085$, Mann Whitney test) (Fig. 6B). Indeed, there was a significant correlation between *PDE8A* mRNA level and HIV-1 replication in MDM (Pearson $r = 0.35$, significance of correlation, $p = 0.049$) (Fig. 6C).

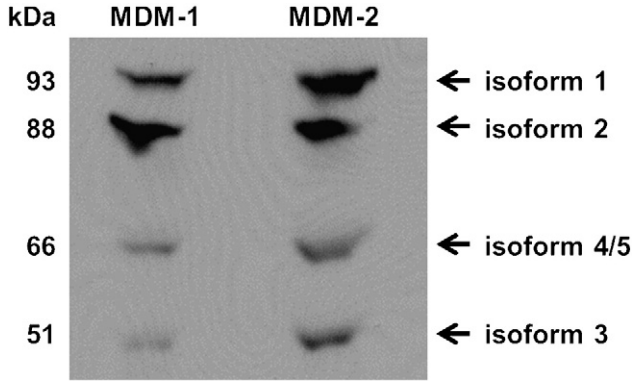

Fig. 4. Western blot demonstrating PDE8A expression in monocyte-derived macrophages derived from 2 healthy individuals. The bands correspond with PDE8A isoforms 1 (93 kDa), 2 (88 kDa), 3 (51 kDa), and isoforms 4 and 5 (both 66 kDa).

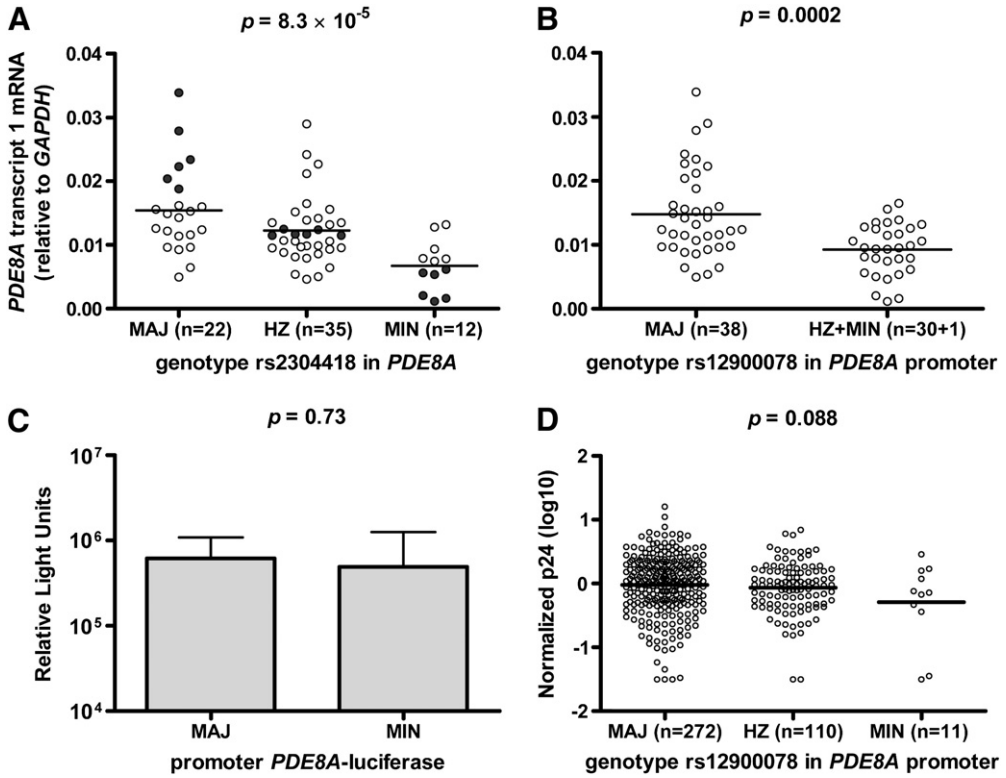

Fig. 5. SNP in *PDE8A* associated with messenger levels in monocyte-derived macrophages (MDM). (A) Quantitative PCR on MDM cDNA from 69 individuals showed a significant association between SNP rs12909130 (as a perfect proxy for rs2304418) genotype and *PDE8A* transcript 1 mRNA levels. *GAPDH* was used as reference gene; similar results were obtained when *ACTB* was used. Results are representative for transcript variants 1–4 (no data for transcript variant 5, since additional nonspecific amplicons were seen for some donors, Fig. 3) and multiple independent experiments. Filled circles represent samples used for resequencing. (B) When tested for association between transcript levels and SNP rs12900078 genotype (near perfect proxy for promoter SNP rs116893322), we again found a significant association. (C) *PDE8A*-promoter driven expression of luciferase did not differ between the variant containing the major (MAJ) or minor (MIN) allele of SNPs rs116893322. Bars represent the average measured value for one experiment and the standard deviation. Results are representative for multiple experiments, and different concentrations of *PDE8A*-promoter DNA used for the transfections (25, 50, 100 and 200 ng/well). (D) Trend towards association between SNP rs116893322 in the *PDE8A* promoter and HIV-1 replication as measured by normalized Gag p24 levels, tested for all donors (n = 393). MAJ, homozygous for the major allele; HZ, heterozygous; MIN, homozygous for the minor allele.

PDE8A overexpression increases HIV-1 replication, while PDE8A knock-down causes reduction

The direct effect of PDE8A on HIV-1 replication was analyzed in HEK293T cells. Overexpression of PDE8A resulted in a dose dependent increase in HIV-1 replication (Fig. 7A) ($p = 0.001$ and $p = 0.008$ with 1 and 0.5 μg of plasmid expressing *PDE8A* respectively; unpaired t-test). When we subsequently down-regulated PDE8A expression by co-transfection of short hairpin RNAs (shRNA's) targeting *PDE8A*, a significant decrease in HIV-1 replication was observed for 2 out of the 3 shRNA's used (Fig. 7B) ($p = 0.038$ and $p = 0.022$, unpaired t-test). A trend towards lower HIV-1 replication upon knock-down of *PDE8A* was also seen for a third shRNA ($p = 0.077$). Effectiveness of both overexpression and knock-down of *PDE8A* was verified by Western analysis (Figs. 7C–D). These data confirm that PDE8A is an HIV-1 dependency factor.

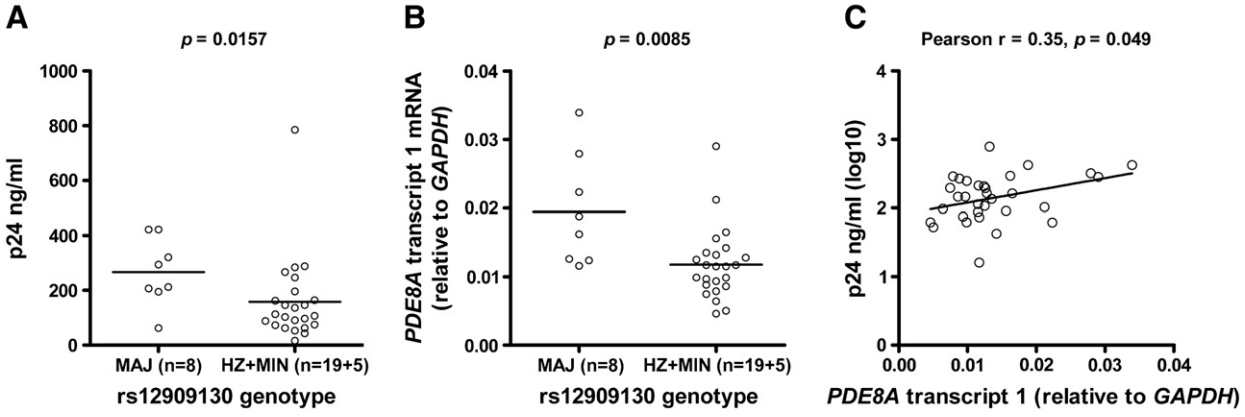

Fig. 6. Correlation between *PDE8A* mRNA levels and HIV-1 replication. SNP rs12909130 (perfect proxy for rs2304418) associated with (A) HIV-1 replication (measured as p24 ng/ml, corrected for the number of monocyte-derived macrophages per well) and (B) *PDE8A* mRNA levels. (C) *PDE8A1* mRNA levels are correlated with HIV-1 replication. MAJ, homozygous for the major allele; HZ, heterozygous; MIN, homozygous for the minor allele.

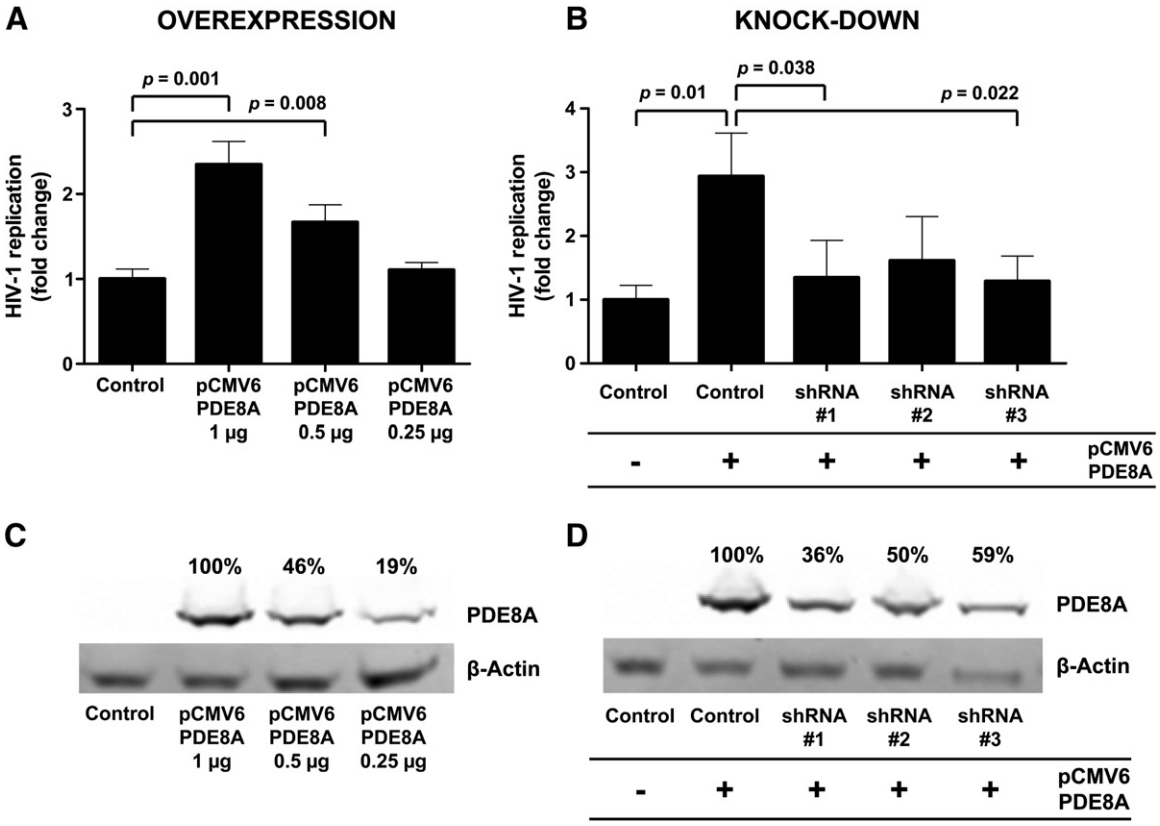

Fig. 7. PDE8A supports HIV-1 replication in HEK293T cells. (A) HEK293T cells were transfected with pCMV6-PDE8A and after 24 h infected with VSV-G-pseudotyped luciferase reporter virus. Luciferase activity was measured 2 days after infection. (B) HEK293T cells were co-transfected with pCMV6-PDE8A (1 µg) and constructs expressing shRNA's targeting *PDE8A* (1 µg). Twenty-four hours after transfection, the cells were infected with VSV-G-pseudotyped luciferase reporter virus and luciferase activity was measured at day 2 after infection. Combined results of 3 independent overexpression and knock-down experiments are shown. Western analysis demonstrating (C) dose-dependent overexpression of PDE8A and (D) knock-down of PDE8A by three different shRNA's in HEK293T cells. Results are representative for 2 independent experiments performed.

Resequencing of the PDE8A promoter and the untranslated regions does not reveal a more likely causal genetic variant

Screening the location of all SNPs in the *PDE8A* gene region (reported in the 1000 Genomes Pilot 1 project) that are in moderate or high LD with rs2304418 (r^2>0.6; 1000 Genomes Pilot 1) but not present on the Illumina SNP BeadChip (Table S3) did not result in the identification of a polymorphism more likely to cause the effect on HIV-1 replication in MDM. The online SNP function prediction (FuncPred) tool did not reveal potential causal variants in LD (r^2>0.6) with rs2304418 either (Xu and Taylor, 2009). Furthermore, we plotted location and *p* value for each SNP in the *PDE8A* gene region that was on the Illumina Human 610Q SNP BeadChip (Fig. S1) in an attempt to narrow down the gene region of interest. However, the large absolute genetic distance between SNP rs2304418 and rs12909130, the two most strongly associated SNPs in the *PDE8A* region, did not allow us to identify such a hotspot.

In a further attempt to find the causal genetic variant responsible for the differential mRNA levels and HIV-1 replication in MDM, we next selected 6 MAJ, 6 HZ and 6 MIN donors with high, intermediate and low *PDE8A* mRNA levels (Fig. 5A) for resequencing of the *PDE8A* promoter region (the ~800 bp region upstream of the ATG start codon) (Chen et al., 2009; Wang et al., 2001) as well as the *PDE8A* 5′ and 3′ untranslated region (UTR). No SNPs were found in the 5′and 3′ UTR compared to the reference sequence (NC_000015.9). However, we did find a polymorphism, identified as SNP rs116893322 (Fig. S2), located in the *PDE8A* promoter region 766 bp upstream of the ATG translation start codon (Fig. 2). For technical reasons, SNP rs12900078, a near perfect proxy for the promoter SNP rs116893322 (r^2 = 0.95), was used to genotype the remaining samples. The minor allele of SNP rs12900078 was significantly associated with lower levels of *PDE8A1* mRNA (p = 0.0002, dominant genetic model, unpaired t-test) (Fig. 5B). However, no differences were observed in luciferase expression in constructs that contained the putative *PDE8A* promoter with either the major or the minor allelic variant of SNP rs116893322 upstream of firefly luciferase (p = 0.73, unpaired t-test) (Fig. 5C), suggesting that the observed association between the SNP in the *PDE8A* promoter and *PDE8A* mRNA level is more likely due to the LD with the tag or causal SNP (r^2 = 0.36 in our study population (n = 393) and 0.52 in the 1000 Genomes Pilot 1 project). In agreement with this, there was only a trend for association with HIV-1 replication in MDM, either when analyzing only donors with MDM that had high or low HIV-1 replication (p = 0.052; n = 191) (data not shown) or when analyzing the total group of donors (p = 0.088; n = 393) (Fig. 5D). From these data we conclude that it is not likely that SNP rs116893322 in the promoter of *PDE8A*, or nearby SNP rs12900078 is the causal genetic variant that affects *PDE8A* transcript levels and HIV-1 replication in macrophages.

SNPs in PDE8A introns alter putative transcription factor binding sites

We found that all SNPs in high LD (r^2>0.6) with rs2304418 are located in intronic regions. Introns may be required for expression of a gene (Jeon et al., 2000; Jonsson et al., 1992) or may contain elements that regulate gene expression (Aronow et al., 1989; Bornstein et al., 1988; Schanke and Van Ness, 1994). This intron mediated enhancement or repression of gene expression may be affected by SNPs (Ju et al., 2010; Sturm et al., 2008; Wilkins et al., 2009), for instance by changing transcription factor bindings sites (TFBS) or short tandem repeats (Akagi et al., 2009; Albanese et al., 2001; Borrmann et al., 2003; Kersting et al., 2008; Sharma et al., 2007; Wagner et al., 2006). We first examined

for all SNPs in moderate to high LD with rs2304418 or rs12909130 ($r^2>0.6$) if the nucleotide change could affect possible human transcription factor binding, using the online Transcription Element Search System (TESS) (Schug, 2008). The log-likelihood score threshold was set at >10 in the search for SNP affected putative TFBS in *PDE8A* introns. Six putative binding sites affected by a SNP in LD with rs2304418 or rs12909130 in *PDE8A* were identified for the human transcription factors AP-1, Sp1, TCF7L2, MAZ, COUP-TF1 and ZEB1 (log-likelihood scores 11.22–18) (Table S4), four of which are located in the first intron of *PDE8A*. This finding indicates that several intronic variants may participate in *PDE8A* transcriptional activation. Furthermore, we searched for microsatellites throughout the complete *PDE8A* gene. Using the Microsatellite Repeats Finder (www.biophp.org) we found several large repeats: (GA)10(GT)17, (GT)19, (AC)29 and (AC)24, however only 2 small repeats were found affected by a SNP in LD with rs2304418 or rs12909130. The TCTCTCTC sequence located ~15 kb upstream of the *PDE8A* 3′ UTR is affected by the minor allele of SNP rs17541572 (r^2 with rs2304418 or rs12909130 = 0.66), changing it into CCTCTCTC, and the sequence ATATATANNNNNACACACA changes into ATATATANNNNNACACATA when harboring the minor allele for SNP rs62022528 (r^2 with rs12909130 = 0.86) located in intron 1. Therefore we conclude that several of the intronic SNPs in high LD with rs2304418 or rs12909130 could be involved in regulation of *PDE8A* transcription.

Discussion

The identification of common genetic variants with moderate or even large effects requires a large sample size. Using the upper and lower quartiles of a group of 393 donors whose MDM showed varying ability to support HIV-1 replication *in vitro*, we were able to detect a significant association between SNP genotype and HIV-1 replication in MDM for one out of 19,487 SNPs in 997 unique HDF genes. Additional studies with larger sample sizes are needed to validate associations of HIV-1 replication with the other SNPs listed in Table 1. We report here that SNP rs2304418 in *PDE8A* was associated with the variable *in vitro* HIV-1 replication in MDM from different donors and that this association was independent of the 32 base pair deletion in *CCR5*. Furthermore, the SNP was significantly associated with *PDE8A* mRNA levels in MDM, and lower *PDE8A* transcript levels in MDM was correlated with reduced HIV-1 replication in MDM. Not only did *PDE8A* knock-down result in reduced HIV-1 replication, as previously reported, we show that *PDE8A* overexpression also results in increased HIV-1 replication, further confirming the role of PDE8A as an HIV-1 dependency factor.

Despite the strong association, great variability in HIV-1 replication between MDM from donors with the same genotype for *PDE8A* SNP rs2304418 is observed. In our study we have only focused on common genetic variants and in general, most common variants have a small effect size. The total observed variation will most likely be explained by many common genetic variants with small effect sizes or few rare genetic variants with larger effect sizes, or a combination thereof.

Previous failure to replicate the association between SNP rs2304418 in *PDE8A* and HIV-1 replication in MDM (Bol et al., 2011b) is most likely a reflection of the sample size needed for replication. Resequencing of the *PDE8A* promoter region identified SNP rs11689332 that was in moderate LD with SNP rs2304418. In addition, two other SNPs in near perfect LD with SNP rs11689332 in the promoter were identified further upstream of the *PDE8A* promoter: SNPs rs62022525 and rs12900078, at −1716 and −1425 bp from the translation start site respectively. However, functional assays could not show an effect of these 2 SNPs on mRNA levels and only a trend towards association with HIV-1 replication was observed. This finding is in concordance with results of experiments on SNPs in the *PDE8A* promoter recently published by Chen et al. (2009). However, we did identify 6 intronic transcription factor binding sites and 2 microsatellites affected by SNPs in high LD ($r^2>0.6$) with our tag-SNP. Future functional assays will have to demonstrate whether any of these putative *cis*-regulatory elements is responsible for the differential *PDE8A* mRNA levels.

The observed positive correlation between *PDE8A* mRNA levels and HIV-1 replication is in concordance with previous findings (Zhou et al., 2008) that showed strong inhibition of HIV-1 replication upon expression of siRNAs targeting *PDE8A* mRNA. Furthermore, others have shown that the phosphodiesterase 8A inhibitor dipyridamole inhibited HIV-1 replication in MDM (Szebeni et al., 1989). However, it cannot be excluded that the known effect of dipyridamole on other members of the PDE family (Fawcett et al., 2000) and even other proteins may have contributed to the inhibition of HIV-1 replication, since selective inhibitors of PDE8A are not yet commercially available (Vasta et al., 2006). Methodological or biological differences between the RNAi screens might explain why PDE8A was not found as an HDF in the three other RNAi screens. However, a strong decrease in HIV-1 replication associated with *PDE8A* knock-down was also seen by König et al., but only for one out of the three siRNA pools used (Renate König, personal communication) (König et al., 2008). None of the other PDE family members were identified as HDF in the RNAi screens, suggesting that perhaps the Per-Arnt-Sim (PAS) domain unique for PDE8, might be involved in the regulation of HIV-1 replication in MDM. This regulatory domain involved in sensing and signaling was found to have a physical association with multiple IκB proteins which greatly enhanced PDE8A1 enzymatic activity (Wu and Wang, 2004).

PDE8A is a phosphodiesterase that specifically hydrolyzes cAMP to AMP. Different PDEs (PDE1–PDE11) regulate this second messenger level following exposure to different stimuli and in distinct subcellular compartments (Bornfeldt, 2006). Since only SNPs in already described HDF's were investigated, it cannot be excluded that other PDE family members may have an effect on HIV-1 replication (in macrophages) as well. The SNP BeadChip used for this study contained 1539 *PDE* gene SNP loci. Of these, only SNP rs4943927 located more than 15 kb downstream of *PDE2A* had an unadjusted $p<1\times10^{-3}$ ($p=9.3\times10^{-4}$). However, in general, the absence of an association between SNP genotype and a certain phenotype does not exclude that the gene product can have an effect, since the gene may be lacking any common genetic variants or SNPs in the gene region may not affect protein function. The inhibitory effect of knocking-down PDE8A on HIV-1 replication could be a result of altering the cyclic nucleotide signaling pathway, where PDE8A no longer, or to a lesser extent, inactivates the second messenger cAMP, which in turn could lead to higher levels of intracellular cAMP. Indeed, it has already been shown that high levels of cAMP are associated with lower HIV-1 replication in MDM (Hayes et al., 2002). Interestingly, a polymorphism in *ADCY1*, which catalyzes the conversion of ATP to cAMP, was also identified in our study (Table 1). Having one or two copies of the minor allele for rs4720510 in *ADCY1* was associated with lower HIV-1 replication in MDM, but the SNP had no effect on *ADCY1* mRNA levels (data not shown). Several statistical models of interaction as reviewed by H.J. Cordell (2009) did not reveal evidence for a significant interaction between rs2304418 in *PDE8A* and rs4720510 in *ADCY1* (data not shown).

While *PDE8A* transcripts are not expressed in unstimulated CD4+ T cells (data not shown and Glavas et al., 2001), they have been detected upon CD4+ T cell activation (Glavas et al., 2001). It is thus possible that the SNP rs2304418 in *PDE8A* also affects HIV-1 replication in CD4+ T cells. However, SNP rs2304418 in *PDE8A* is not associated with HIV-1 disease progression or viral load at set-point (Fellay et al., 2007, 2009; Le Clerc et al., 2009; Limou et al., 2009), arguing against an effect of this SNP in CD4+ T cells, and excluding HIV-1 infected macrophages as strong contributors to the VL set-point *in vivo*. While HIV-1 replication in macrophages may not affect overall disease progression, macrophages play important roles as viral reservoir and in specific pathologies and aspects of HIV-1 infection, such as HIV-1 associated dementia, cardiovascular disease and AIDS related

lymphomas (reviewed by Bol et al., 2011a). It will be worthwhile to evaluate the clinical significance of *PDE8A* in these HIV-1 related pathologies.

Our study is the first to confirm the importance of one of the RNAi screen hits in primary target cells for HIV-1. Macrophages are long lived cells that are less sensitive to certain antiretroviral drugs due to differences in their metabolism as compared to T cells. Moreover, the HIV-1 infection of macrophages is not lytic, and macrophages are sometimes sequestered which makes them less accessible to antiretroviral drugs (reviewed by Aquaro et al., 2002; Crowe et al., 2003). These characteristics make macrophages an almost ideal reservoir for the virus, which hinders complete eradication of HIV from the body. To identify novel therapeutic interventions specifically aimed at this viral reservoir, it is critical to better understand HIV-1's dependency of host macrophage proteins. Phosphodiesterases in general are promising targets for pharmacological intervention (Francis et al., 2011). PDE inhibitors are already used to treat multiple different diseases (Giembycz and Field, 2010; Reffelmann and Kloner, 2007; Schwartz and Kloner, 2010) and are probably best known for treatment of erectile dysfunction (sildenafil or VIAGRA®, Pfizer, New York City, NY, USA). Furthermore, they have been suggested for the treatment of many other pathologies (Furman and Pyne, 2006; Kumar et al., 2009; Menniti et al., 2006; Zhang, 2009). Therefore, PDE8A might be a promising candidate for pharmaceutical intervention in HIV-1 infection.

Materials and methods

Ethics statement

This study has been conducted in accordance with the ethical principles set out in the declaration of Helsinki, and was approved by the Medical Ethics Committee of the Academic Medical Center and the Ethics Advisory Body of the Sanquin Blood Supply Foundation in Amsterdam, The Netherlands. Written informed consent was obtained from all participants.

Study population

We previously determined the ability of HIV-1 to replicate in monocyte-derived macrophages (MDM) from 429 different healthy seronegative blood donors (Bol et al., 2009). In brief, Gag p24 levels were measured in MDM culture supernatant 14 days post infection with HIV-1 YU2 by an in-house enzyme-linked immunosorbent assay. To correct for differences in the number of viable MDM present at day 14 post infection, p24 levels were expressed per 10,000 cells. Since monocyte isolations were performed in four time frames and by two operators, p24 levels were normalized by dividing through the median per period and operator. These normalized p24 levels were subsequently used as a measure for *in vitro* HIV-1 replication in MDM. The population used in this study is as described in (Bol et al., 2011b). In short, 192 individuals whose MDM gave the highest (n = 96) or lowest (n = 96) p24 production *in vitro*, were selected for SNP genotyping; thus representing two groups of donors with MDM that had a more extreme phenotype. Inclusion of donors with extreme phenotypes is known to increase power in genetic association studies (Van Gestel et al., 2000). One donor from the group with low *in vitro* HIV replication in MDM was excluded for further analysis because the corresponding DNA samples did not pass the quality control.

Selected studies and genes

We selected all genes that encode for the HIV-1 dependency factors (HDFs) identified in four recent genome-wide RNAi studies (Brass et al., 2008; König et al., 2008; Yeung et al., 2009; Zhou et al., 2008). Lists of genes with gene symbol or gene ID were available for three of the studies (Brass et al., 2008; König et al., 2008; Zhou et al., 2008). For the HDFs found by Yeung et al. (2009) GenBank (accession) numbers were used to find the corresponding genes. After removing duplicates from the resulting total of 1039 genes for all four studies combined, 997 unique genes coding for HDFs were used for further study (Table S5). For these genes, 23,340 SNPs were present on the Illumina HumanHap 610-Quad BeadChip (Table S6, first column).

Genotyping

For the two groups of donors whose MDM had highest or lowest *in vitro* HIV Gag p24 production, we used SNP data that was generated in a genome-wide association study on *in vitro* HIV-1 replication in MDM (Bol et al., 2011b) using the Illumina Infinium Human Hap 610-Quad BeadChip (Illumina, San Diego, CA, USA) (Steemers and Gunderson, 2007). Genotypes of rs2304418 in *PDE8A* from the donors with MDM that had intermediate HIV-1 replication *in vitro* (n = 202) were determined by sequencing the *PDE8A* gene region encompassing the C/T SNP. Genomic DNA was amplified using GoTaq polymerase (Promega, Madison, WI, USA) and primers PDE8A-G F and PDE8A-G R (Table S2). The following amplification cycles were used: 5 min 95 °C, 40 cycles of 30 s 95 °C, 30 s 58 °C, 1 min 72 °C; 10 min 72 °C. The ABI TaqMan® (Applied Biosystems, Carlsbad, CA, USA) SNP genotyping assay was used to genotype SNP rs12900078 (C_1342278_10) in the promoter of *PDE8A*. The assay was run on a LightCycler® 480 system (Roche, Basel, Switzerland) using Probes Master (Roche), and the following amplification cycles: 10 min 95 °C; 50 cycles of 15 s 95 °C, 1 min 60 °C.

Quality control of SNP data and statistical analysis

Markers to detect copy number variation of genes coding for an HDF (n = 1112) were not included in this study. DNA samples with a SNP call frequency < 98% (n = 1 out of 192), SNPs with a call frequency < 98% (n = 375), SNPs with a minor allele frequency (MAF) < 5% (n = 2612) and SNPs for which there were no donors homozygous for the minor allele (n = 3425) were excluded from analysis. A total of 3853 SNPs (which is not the sum of the above mentioned number of excluded SNPs per criteria, since an excluded SNP often met more than one of the exclusion criteria) were excluded from the selected 23,340 SNPs. The remaining 19,487 SNPs in 997 unique genes known to be associated with HIV-1 replication were used to test for association with *in vitro* replication of HIV-1 in MDM (Table S6, second column). Additional quality control steps, identification of population stratification and statistical analysis were performed as described in Bol et al. (2011b). SNAP (version 2.2) and Haploview (version 4.2) were used for testing linkage disequilibrium (LD) (Barrett et al., 2005; Johnson et al., 2008).

Transcript detection and quantitative PCR

Buffy coat or full blood was obtained from 69 additional healthy blood donors, and data on HIV-1 replication in MDM was available from 32 of these. Monocyte isolation, MDM culture and HIV-1 infection was performed as previously described (Bol et al., 2009). Total RNA was extracted from day 7 uninfected MDM using the High Pure RNA Isolation kit (Roche). A maximum of 1 μg total RNA and oligo (dT) primers were used for reverse transcription of mRNA, using Roche's Transcriptor First Strand cDNA Synthesis kit (60 min at 50 °C). Resulting cDNA was used for PCR to detect the presence of different transcript variants, as well as quantitative PCR (qPCR) analysis, using transcript specific primers (Table S2). qPCRs were performed using SYBR Green I Master (Roche) and were run on a LightCycler® 480 system (Roche). All procedures were carried out according to

manufacturer's protocol. Messenger RNA levels are reported relative to *GAPDH*. Gene expression values were obtained using Roche's LightCycler® relative quantification software (release 1.5.0). To facilitate accurate and reliable between-donor comparison, cDNA synthesis and qPCR experiments for all 69 samples were performed simultaneously. PBL were used for DNA isolation using the High Pure PCR Template Preparation kit (Roche). The ABI TaqMan® SNP genotyping assay was used to genotype rs12909130 (C_1342209_10) as a proxy for rs2304418 ($r^2 = 1.00$, 1000 Genomes Pilot 1) (Applied Biosystems, Carlsbad, CA, USA). The TaqMan® assay was run on the LightCycler® 480 system (Roche), using Probes Master (Roche), and the following amplification cycles: 10 min 95 °C; 50 cycles of 15 s 95 °C, 1 min 60 °C. Restriction fragment length polymorphism analysis was used to genotype SNP rs4720510 (T/C) in *ADCY1*. A 372 bp amplicon encompassing rs4720510 was generated using the primer pair ADCY1-G F and ADCY1-G R (Table S2). Recognition of TTATAA and subsequent DNA digestion by the restriction enzyme PsiI allowed for discrimination between the wild-type, heterozygous and homozygous minor genotype.

Western blot analysis

Seven days after monocyte isolation, MDM were lysed in RIPA-buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing Complete® EDTA free protease inhibitor (Roche). After adding NuPAGE LDS 4× sample buffer (Invitrogen) and 0.1 M DTT, samples were heated at 95 °C for 10 min. The Odyssey Protein Weight Marker was loaded as a reference for protein size (LI-COR, Lincoln, NE, USA). Proteins were separated by SDS-PAGE (NuPAGE 10% Bis–Tris precast gel and MES SDS running buffer (Invitrogen) and transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell, Dassel, Germany) using NuPAGE transfer buffer. After blocking for 2 h with PBS containing 1% Protifar (Nutricia, Schiphol, The Netherlands) and 0.5% bovine serum albumin, the blot was probed with a 1:500 dilution of PDE8A 121AP (C-terminal Ab IgG, 98–102 kDa) antibody (FabGennix, Frisco, TX, USA). After washing, membranes were incubated with a secondary anti-rabbit horseradish peroxidase-linked whole antibody Ab at 1:2000 (GE Healthcare, Waukesha, WI, USA). PDE8A protein was detected using the SuperSignal West Femto Sensitivity reagent (Pierce, Rockford, IL, USA).

Expression of MYC-tagged PDE8A and ß-actin was detected using OP10L anti-c-Myc antibody (1:5000; Calbiochem, San Diego, CA, USA) and SC-1616 anti-ß-actin antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) respectively. IRDye 800CW conjugated Goat anti-Mouse IgG (1:15,000; 926–32210, LI-COR, Lincoln, NE, USA) and IRDye 680LT conjugated Donkey anti-Goat IgG (1:15,000; 926–32224, LI-COR) were used as secondary antibodies to visualize expression using the Odyssey infrared image system (LI-COR).

Sequencing

A 2096 base-pair product (spanning −1614 to +482, relative to the translation start site) was amplified using the Roche GC-rich PCR system, and the primers PDE8A-P F and PDE8A-P R (Table S2). Amplification was done according to manufacturer's protocol, using 1 M of resolution solution. Amplification conditions were 3 min at 95 °C, 10 cycles of 30 s at 95 °C, 30 s at 58 °C, 95 s at 72 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 58 °C and 95 s at 72 °C with 5 s additional elongation after each cycle, and a final elongation of 7 min at 72 °C. PCR products were purified using the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare) according to the manufacturer's protocol. Sequencing conditions were 5 min at 94 °C, 45 cycles of 15 s at 94 °C, 10 s at 50 °C, 2 min at 60 °C, and a 10 min extension at 60 °C. Sequencing was performed using primers PDE8A-P FI-1, FI-2, RI-1, RI-2, RI-3 (Table S2) and BigDye Terminator v1.1 Cycle Sequencing kit (ABI Prism, Applied Biosystems), according to the manufacturer's protocol on a 3730xl DNA analyzer (Applied Biosystems). Nucleotide sequences were assembled using the SeqMan application in the software package Lasergene (DNASTAR), were subsequently aligned using ClustalW in the software package of BioEdit (Hall, 1999) and were edited manually.

An additional 504 bp long fragment further upstream of the *PDE8A* promoter (−2065 to −1561) and the *PDE8A* 3′ untranslated region (UTR) were amplified using GoTaq polymerase (Promega, Madison, WI, USA) and primer pair PDE8A-P F2 + R2 and PDE8A 3UTR F + R respectively (Table S2). PCR products were purified by using ExoSAP-IT (USB, Cleveland, OH, USA) according to the manufacturer's protocol. Sequencing conditions were 5 min at 94 °C, 30 cycles of 15 s at 94 °C, 10 s at 50 °C, 2 min at 60 °C, and a 10 min extension at 60 °C. Sequencing was performed using primers mentioned above and an additional internal primer PDE8A 3UTR FI for the 3′ UTR fragment (Table S2).

Plasmid constructs

A 1578 bp *PDE8A* promoter fragment (−1559 to −1, relative to ATG translation start site) encompassing SNPs rs12900078 and rs116893322 was cloned from genomic DNA isolated from both a donor homozygous for major allele and from a donor homozygous for the minor allele using primer pairs PDE8A-PC F1 and PDE8A-PC R1, introducing XhoI and NcoI restriction sites respectively (Table S2). Amplification was done using the GC-rich PCR system (Roche) and according to manufacturer's protocol, using 1 M of resolution solution. Amplification conditions were 3 min at 95 °C, 10 cycles of 30 s at 95 °C, 30 s at 58 °C, 70 s at 72 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 58 °C and 70 s at 72 °C with 5 s additional elongation after each cycle, and a final elongation of 7 min at 72 °C. XhoI/NcoI digested amplicons were purified using the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare) and subsequently ligated into a XhoI/NcoI digested pBlue3′ LTR-luc (Jeeninga et al., 2000) (a kind gift from Dr. R. Jeeninga and Dr. B. Berkhout, Academic Medical Center, Amsterdam, The Netherlands) using Roche's Rapid Ligation kit, and transformed into DH5α *E. coli* (Invitrogen, Carlsbad, CA, USA). Primers PDE8A-PC F1 to F4 and PDE8A-PC R2 to R6 were used to verify construct sequence and the presence of the SNPs (Table S2).

For *PDE8A* overexpression and knock-down the pCMV6-entry expressing a MYC-tagged PDE8A (Origene, Rockville, MD, USA) and pLKO.1 constructs expressing shRNA candidates from the MISSION™ TRC-Hs 1.0 library (PDE8A TRCN48874, PDE8A TRCN48875, PDE8A TRCN48876 and empty vector SHC001; Sigma-Aldrich St. Louis, MO, USA) (Root et al., 2006) were used.

Transfection and infection of HEK293T cells

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium without Hepes (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% (v/v) inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml), and maintained in a humidified 10% CO_2 incubator at 37 °C. For transfection, HEK293T cells were plated at a cell density of 1×10^4 per well in a 96-well culture plate. After 24 h, cells were transfected with plasmid DNA (concentration as indicated in the results) using calcium phosphate: plasmid DNA was mixed with 0.30 M $CaCl_2$, subsequently mixed with an equal volume of 2× HEPES buffered saline pH 7.2, incubated at room temperature for 15 min and added to the culture medium. After 24 h incubation in a humidified 3% CO_2 incubator at 37 °C culture medium was replaced and cultures were continued at 10% CO_2 at 37 °C.

VSV-G-pseudotyped single-round luciferase virus was produced by co-transfection of pNL4-3.Luc.R-E-construct and the pCMV-VSV-G in HEK293T using the calcium phosphate method as described above. Virus was harvested at 48 and 72 h after transfection and filtered through a 0.22 µm filter. Virus titers were quantified by

determining the $TCID_{50}$ on HEK293T cells. Forty-eight hours after transfection, the cells were inoculated with 800 $TCID_{50}$ of VSV-G-pseudotyped single-round luciferase reporter virus. Luciferase activity was measured by adding 25 µl substrate (0.83 mM ATP, 0.83 mM D-luciferin (Duchefa, Haarlem, The Netherlands), 18.7 mM $MgCl_2$, 0.78 µM $Na_2H_2P_2O_7$, 38.9 mM Tris pH 7.8, 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 µM dithiothreitol) directly to the culture medium. Luminescence was measured for 1 s per well using a luminometer (Berthold, Bad Wildbad, Germany).

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.08.013.

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