Donor variation in in vitro HIV-1 susceptibility of monocyte-derived macrophages

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Primary human cells from different donors vary in their susceptibility to in vitro infection with HIV-1. In order to perform genetic analysis to identify host factors that affect HIV-1 susceptibility, it is important that a clear phenotype is defined. Here, we report a standardized method to study variation for in vitro HIV-1 infection in monocyte-derived macrophages (MDM) from large numbers of individuals. With this assay, HIV-1 susceptibility of MDM from 489 different donors shows more than 3 log variation and a good correlation with the 32 base pair deletion in the CCR5 co-receptor (ccr5 Δ32 genotype) of the donors. However, in 7 of 12 donors completely resistant to infection with CCR5-using HIV-1, this was not explained by the ccr5 Δ32 genotype, showing evidence that other host factors are likely to influence HIV-1 replication in MDM. Infections with VSV-G pseudotyped HIV-1 indeed confirmed the existence of post-entry level restrictions in MDM.

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Introduction

The limited number of proteins encoded by the genome of the human immunodeficiency virus type-1 (HIV-1) makes the virus highly dependent on host proteins for replication. Genetic variation between individuals may result in structural and functional differences in host proteins that are being used by the virus. Polymorphisms in non-coding regions can result in variation in expression levels of these proteins, which may affect entry and/or replication kinetics of HIV-1. Together with variation in viral factors, it is believed that these host genetic variations explain why people differ in susceptibility to infection with HIV-1 and in the clinical course of infection once infection has been established (Nelson and O’Brien, 2006; O’Brien and Nelson, 2004). Indeed, single nucleotide polymorphisms (SNPs), deletions and copy-number variations in a number of host factors are known to affect HIV-1 infection (reviewed by Lama and Planelles, 2007; O’Brien and Nelson, 2004; Telenti and Goldstein, 2006). For example, a 32 base pair deletion in the gene encoding CCR5, one of the major co-receptors for HIV-1, causes a reduced expression of CCR5 on the surface of T-cells and macrophages, thereby strongly influencing entry of the virus. Individuals homozygous for this deletion (1% of the Caucasian population) have no CCR5 expression on their cells, preventing entry of CCR5 using HIV-1 (Rana et al., 1997; Samson et al., 1996), and are relatively resistant to HIV-1 infection. Individuals heterozygous for this deletion have a delayed clinical course of infection relative to individuals with a CCR5 wild-type genotype (de Roda Husman et al., 1997; Dean et al., 1996).

Studies done on in vitro replication of HIV-1 showed variation in the replication capacity of HIV-1 between different individuals (Bleiber et al., 2005; Ciuffi et al., 2004; Loeuillet et al., 2008). While these studies used CD4+ T-cells, less is known about host factors influencing in vitro replication of HIV-1 in macrophages (Chang et al., 1996; Eiset et al., 2001; Foucher et al., 1994; Naif et al., 1999). Besides CD4+ T-lymphocytes and dendritic cells, macrophages are important target cells for HIV-1. They are early cellular targets and vectors for mucosal transmission of the virus. Together with resting T-lymphocytes, tissue macrophages are a reservoir for HIV-1 (Chun et al., 1997; Finzi et al., 1997; Igarashi et al., 2001). Tissue macrophages are long-lived cells (half-life of several months) and are less sensitive to anti-retroviral therapy because of poor tissue penetration of the drugs (reviewed by Carter and Ehrlich, 2008; Crowe and Sonza, 2000). Moreover, macrophages can produce large amounts of HIV-1 virions because they are relatively resistant to the cytopathic effects of HIV-1 (reviewed by Carter and Ehrlich, 2008; Crowe and Sonza, 2000).

Identifying host factors that are involved in regulating HIV-1 replication in monocyte-derived macrophages (MDM) will increase our understanding of HIV disease pathogenesis and may even result in identification of novel drug targets. The aim of our research is to determine the degree of host-determined variation in HIV-1 replication in MDM and to identify the host factors that give rise to this variation. In order to do this, an assay that can reliably screen HIV-1 susceptibility (measured by viral replication) of macrophages isolated...
from a large number of donors is essential. We here present a standardized method to quantify HIV-1 susceptibility in donor-derived MDM.

**Results**

*In vitro HIV-1 susceptibility of MDM shows large variation*

Data were normalized to correct for influence of isolation period, experimental operator and cell number (Fig. 1A, for details see Materials and methods). In concordance with previous studies (Eisert et al., 2001; Fouchier et al., 1994; Naif et al., 1999), we observed a large variation in donor MDM susceptibility to *in vitro* HIV-1 infection. Indeed, a more than 3 log difference in normalized p24 levels between donors was detected (Fig. 1B). To confirm that this was not HIV-1 strain-specific, MDM from 154 donors were inoculated in parallel with the molecular clone HIV-1 YU2 and the HIV-1 Bal isolate. There was a significant correlation between the levels of Gag p24 antigen produced by cells from the same donor infected with either YU2 or Bal. (Pearson \( r = 0.90, P < 0.0001 \)) (Fig. 2). Two different batches of HIV-1 YU2 produced more than 1 year apart also gave highly concordant results on MDM from the same donor (\( n = 9, \) Pearson \( r = 0.99, P < 0.0001 \)) (data not shown).

**HIV-1 susceptibility of MDM is a stable host characteristic**

Two separate blood donations (2.5 to 5 months apart) were collected from 14 of the 489 donors. Results from the first bleed were used for a within replication assay. We found a significant correlation between normalized p24 values observed in the 1st and 2nd susceptibility assays from the same bleed (Pearson \( r = 0.74, P = 0.0025 \)) (data not shown). Donor ranking based on p24 production by MDM within the group of donors from the day of monocyte isolation (typically \( n = 16 \)) was also compared between the 1st and 2nd time points. There was a significant correlation for rank between the two blood donations, both using normalized p24 levels and not normalized p24 levels (Spearman \( r = 0.69, P = 0.0064 \) and Spearman \( r = 0.63, P = 0.015 \) respectively) (data not shown). Three out of the 14 donors were HZ for the 32 base pair deletion in *ccr5* and were all ranked in the lower half of the group (14) (data not shown).

To further demonstrate that *in vitro* HIV-1 replication in MDM is a consistent donor-linked phenotype, monocytes were isolated at two different time points from an additional group of 16 healthy individuals. The second bleed was 7 days after the first bleed. Results from the infection with HIV-1 YU2 indeed showed a strong and highly significant correlation of p24 levels (pg/µl) produced by MDM obtained from the first and second bleeds (\( n = 16, \) Pearson \( r = 0.90, P < 0.0001 \)) (Fig. 3A). Interestingly, cell counts (obtained 18 days post infection) from the first time point were also highly similar to counts from the latter time point, both for non-infected MDM (\( n = 10, \) Pearson \( r = 0.83, P = 0.0027 \)) and for MDM infected with HIV-1 YU2 (\( n = 10, \) Pearson \( r = 0.86, P = 0.0004 \)) (data not shown). Moreover, the high correlation of p24 production between the 1st and 2nd bleeds was also observed after correction for cell number using counts from the uninfected MDM (\( n = 10, \) Pearson \( r = 0.90, P = 0.0004 \)) (data not shown).

**Correlation between *ccr5* genotype and HIV-1 susceptibility of MDM**

To confirm that our assay indeed captures HIV-1 susceptibility of MDM as determined by host genetic factors, we analyzed the correlation between MDM susceptibility and a host genetic factor that is well known to influence HIV-1 susceptibility *in vivo*, namely the *ccr5* Δ32 genotype. Virus replication in MDM was compared between groups without the deletion (MAJ), heterozygous for the deletion (HZ), or homozygous for the deletion (MIN) (Fig. 4). A highly significant negative effect of the 32 base pair (bp) deletion in
the CCR5 co-receptor gene on HIV-1 replication in MDM was observed 
(P < 0.0001, ANOVA). This genotype, however, could not explain all 
variation in susceptibility, as MDM from 9 ccr5 Δ32 heterozygous 
donors showed high virus replication, while MDM from 72 donors 
with a ccr5 wild-type genotype showed low virus replication. As 
expected, no HIV-1 replication was observed in MDM from 
ccr5 Δ32 homozygous donors (n = 5).

Variation in MDM HIV-1 susceptibility is only partially determined at 
viral entry

MDM from the 16 donors, that were isolated from 2 separate blood 
draws 1 week apart and inoculated with YU2, were inoculated in 
parallel with vesicular stomatitis virus glycoprotein (VSV-G) pseudo-
typed HIV-1 YU2. This VSV-G virus does not depend on cell surface 
expression of CD4 and CCR5 for cell entry, but enters the cells by 
endocytosis. A strong correlation of HIV-1 replication measured as p24 
production was observed between HIV-1 YU2 and its VSV-G pseu-
dotyped variant, both for MDM obtained from the 1st bleed (n = 16, 
Pearson r = 0.83, P < 0.0001) and those from the 2nd bleed (n = 16, 
Pearson r = 0.90, P < 0.0001) using an MOI of 0.006 (Fig. 3B). Similar 
results were obtained with higher MOIs (data not shown). Moreover, 
p24 production by MDM infected with VSV-G pseudotyped YU2 from 
the 1st and 2nd bleeds correlated signifi

cantly (n = 16, Pearson 
r = 0.83, P < 0.0001) (Fig. 3C) using an MOI of 0.006, as well as for 
higher MOIs (data not shown). These 
findings strongly suggest that 
host genetic factors other than those involved in entry are capable of 
restricting or enhancing HIV-1 replication in MDM.

Identification of high and low susceptible groups

In order to identify host genetic factors that determine HIV-1 
susceptibility of MDM in vitro, we compared the groups of donors at 
the extreme ends of the spectrum in the susceptibility assay, i.e. the 
donors showing high levels of p24 production and the donors 
showing low levels of p24 production (hereafter referred to as the 
high and low groups respectively) (Table 1, Fig. 1B, Fig. 5). There was a 
0.6 log difference in normalized p24 between the donor with lowest 
p24 production in the high group and the donor with highest p24

<table>
<thead>
<tr>
<th>In vitro HIV-1 replication</th>
<th>Low</th>
<th>High</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donors (number)</td>
<td>96</td>
<td>96</td>
<td>0.885&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gender (males:females)</td>
<td>52:44</td>
<td>53:43</td>
<td>0.672&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Age (years, mean ± SD)</td>
<td>46 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47 ± 12</td>
<td>0.342&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>European ancestry (number of donors)</td>
<td>92</td>
<td>88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.342&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt; lymphocytes (%, mean ± SD)</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>0.671&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Granulocytes (%, mean ± SD)</td>
<td>8 ± 5</td>
<td>7 ± 4</td>
<td>0.061&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD16&lt;sup&gt;+&lt;/sup&gt; monocytes (%, mean ± SD)</td>
<td>14 ± 5</td>
<td>13 ± 5</td>
<td>0.350&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDM per well (day 21, mean ± SD)</td>
<td>7906 ± 1784</td>
<td>9182 ± 2297</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normalized p24, log10 (mean ± SD)</td>
<td>−0.67 ± 0.38</td>
<td>0.50 ± 0.18</td>
<td>&lt;0.0001&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

SD, standard deviation.

<sup>a</sup> Pearson Chi-Square test.
<sup>b</sup> Information missing from 1 donor.
<sup>c</sup> Independent-Samples t-test.

Fig. 4. Normalized p24 values per ccr5 Δ32 genotype (wild-type or MAJ, heterozygous or HZ, and homozygous or MIN) show a strong in vitro effect of the 32 base pair deletion on HIV-1 entry and replication between the three different genotypes (P < 0.0001, ANOVA). Differences between wild-types and heterozygotes (P < 0.001), and heterozygotes and homozygotes (P < 0.001) are also significant (Bonferroni’s Multiple Comparison test). The horizontal lines display the mean normalized p24 level for each genotype.
production in the low group (Fig. 5). To exclude differences caused by gender, age, ancestry and purity of the analyzed cell fractions, we compared each of these parameters between both groups (Table 1). No significant differences in gender, age or ancestry were observed between donors of MDM with high or low HIV-1 susceptibility (Table 1). After magnetic bead sorting, the percentage of CD3+ lymphocytes present in the CD14+ fraction at day 0 did not differ between the 2 groups and there was also no difference in the percentage of granulocytes in the CD14+ fraction between the groups (Table 1). Moreover, after 21 days in culture (day 14 post infection), no non-MDM cells were observed by light microscopy and no difference was observed between the 2 groups. However, the average number of uninfected MDM counted per well was significantly lower for the low susceptible group as compared to the high susceptible group (Table 1) (P<0.0001, Independent-Samples t-test). No difference in cell morphology and cell number per well between infected and uninfected MDM was seen (n=7, P=0.620, Mann-Whitney test).

As expected, we also observed a significant difference in the frequency of the different ccr5 Δ32 genotypes between the low and high group (P=0.004, Pearson Chi-Square test), confirming the accuracy of our classification. Fig. 4 shows there is a highly significant difference in normalized p24 levels between the three ccr5 Δ32 genotypes (P<0.0001, ANOVA), and also between the wild-type and the heterozygous group (P<0.001), and the heterozygous and homozygous group (P<0.001, Bonferroni’s Multiple Comparison test). Furthermore, we identified 4 ccr5 Δ32 MAJ and 3 ccr5 Δ32 HZ donors in the low group with normalized p24 levels comparable to those from ccr5 Δ32 MIN donors (normalized p24<0.04).

A report by Quillent et al. (1998) showed that having the m303 mutation in one allele of the ccr5 gene while harboring the 32 bp deletion in the other allele, would confer an HIV-1 resistance phenotype similar to the ccr5 Δ32 homozygous phenotype. To investigate this, we also analyzed the ccr5 m303 genotype of our donors in the group with MDM that showed relatively low in vitro HIV-1 susceptibility (n=96). However, none of the three ccr5 Δ32 heterozygous donors of whom MDM showed HIV-1 replication below the limit of detection (i.e. normalized p24<0.04) carried the m303 mutation in the CCR5 gene (data not shown). The m303 mutation was also not detected in any of the remaining 93 individuals.

No association between monocyte CD16 expression and in vitro HIV-1 susceptibility of MDM

A previous study suggested that CD16+ monocytes are a preferential target for HIV-1 infection (Ellery et al., 2007). Here we analyzed whether the proportion of CD16 expressing cells within the CD14+ cell fraction at day 0 correlated with the HIV-1 susceptibility of the MDM. No difference in the proportion of CD16 positive monocytes at day 0 could be observed between the groups with high and low HIV-1 susceptibility (P=0.350, Independent-Samples t-test) (Table 1).

Discussion

Several studies have reported large variability in HIV-1 susceptibility of target cells isolated from different donors (Bleiber et al., 2005; Ciufti et al., 2004; Eisert et al., 2001; Fouchier et al., 1994; Loeuillet et al., 2008; Naif et al., 1999). As the host factors that influence this variability may be important in the viral life cycle and possibly are important anti-retroviral targets, reliable quantification of HIV-1 susceptibility for large numbers of donors is important. Given the paucity of data on host factors influencing HIV-1 susceptibility of macrophages, one of the important target cells of the virus, we developed an assay to determine HIV-1 susceptibility of monocyte-derived macrophages (MDM). Using this assay we observed more than 3 log difference in HIV-1 replication in MDM obtained from 429 different individuals. We showed that assay results were similar for two unrelated HIV-1 variants used to infect the MDM and found good correlation between assay results obtained with MDM from the same individual at different calendar dates. Moreover, the ccr5 Δ32 genotype was strongly correlated with the susceptibility as measured in our assay.

The large variation in in vitro HIV-1 replication in MDM that we observed, is in concordance with previous reports on HIV-1 replication in MDM (Eisert et al., 2001; Fouchier et al., 1994; Naif et al., 1999) but also with variation of HIV-1 replication in CD4+ T-cells and/or immortalized B-cell lines (Bleiber et al., 2005; Ciufti et al., 2004; Loeuillet et al., 2008). Al-Jabri et al. (2008) studied variation in HIV-1 infectibility of PBMC from 130 sero-negative donors. They also observed considerable variation in HIV-1 susceptibility of cells from different donors although, and in contrast to our findings, none of the individual’s PBMC in their study showed complete resistance to in vitro HIV-1 infection. This may point to differences in the ethnicity (and thus genetic factors, such as the frequency of the ccr5 Δ32 genotype) of the study population and/or the viruses used between the two studies, but may also reflect intrinsic differences in blocks to HIV-1 infection between PBMC and MDM. In addition, the high number of individuals that we have studied might also have contributed to identifying donor-derived MDM in which CCR5-using HIV-1 does not replicate. Furthermore, MDM-specific host factors may result in a complete block to HIV-1 irrespective of viral co-receptor use. Indeed, several host factors are known that restrict HIV-1 replication after entering the cell (Nathans et al., 2008; Sayah et al., 2004; Stremia et al., 2004). This explanation is supported by the observation that MDM from several donors (n=7) do not show any virus replication despite a MAJ or HZ ccr5 Δ32 genotype. Whether or not MDM from these specific individuals are also resistant to infection with different viruses (RSX4 or X4 HIV-1, HIV-2) is currently under investigation.

We observed a difference in MDM count between the low and high HIV-1 replication groups. It seems unlikely that the higher p24 values are due to the increased number of MDM per well, since p24 levels were expressed per 10,000 MDM for each donor, thus corrected for cell number. However, the increased number of cells may reflect an increased proliferative potential of the MDM. HIV-1 replicates preferentially in MDM with high proliferative capacity.
(Schuitemaker et al., 1994; Schuitemaker et al., 1992), although actual cell division is not required for HIV-1 infection in macrophages (Kooistra, Zwart, and Schuitemaker, 2000; Schmidtmaierova, Nuovo, and Bukrinsky, 1997). It is conceivable that the increased or decreased expression of specific host factors that affect HIV-1 susceptibility is regulated in concert with the cell cycle and/or differentiation of the MDM. For example, the expression of antiviral micro RNAs is specifically down-regulated during the transition from monocyte to macrophage (Wang et al., 2009).

We did not see a difference in the percentage of CD16\(^+\) monocytes between the low and high HIV-1 replication groups. This suggests that the level of CD16 on monocytes does not correlate with HIV-1 replication in MDM in vitro. Ellery et al. (2007) reported that CD16\(^+\) monocytes are more susceptible to HIV-1 infection in vivo because of higher CCR5 expression. In MDM, CCR5 expression levels are less likely to be a limiting factor as CCR5 levels are higher on MDM than on monocytes (Pesenti et al., 1999). Alternatively, the form of the molecular complex of APOBEC3G (low or high MW, corresponding with active and inactive enzyme, respectively (Ellery et al., 2007), may explain the differences in susceptibility of CD16\(^+\) and CD16\(^-\) monocytes. Further studies are required to see if differences in APOBEC3G MW can also explain differences in HIV-1 susceptibility of MDM.

In our present study we found a strong correlation between ccr5 Δ32 genotype and HIV-1 susceptibility in MDM. This is in concordance with results found by Ometto et al. (1999). Earlier studies showed that the ccr5 Δ32 allele is negatively associated with expression levels of CCR5 on CD4\(^+\) cells (Blaak et al., 2000; Paxton et al., 1999; Thomas et al., 2002; Venkatesan et al., 2002; Wu et al., 1997). However, Naif et al. (1999) found a poor correlation between CCR5 expression and HIV-1 susceptibility in MDM, except at very low expression levels, and others did not observe a correlation between the expression level of CCR5 and HIV-1 susceptibility in MDM (Eisert et al., 2001; Pesenti et al., 1999). The explanation for this difference with our findings may be that we specifically titrated the virus inoculum to obtain maximum discrepancy in virus production between different donors. When higher inocula are used, more donors reach the threshold for maximum virus production, while lower inocula result in more donors having no detectable virus replication. Thus our assay may allow better discrimination between MDM susceptibility of all donors.

Analysis of the ccr5 Δ32 genotype and m303 T to A polymorphisms in ccr5 revealed that only part of the variability in in vitro HIV-1 susceptibility of MDM could be explained by genetic variation in the ccr5 gene. MDM from several donors with the HZ genotype showed high susceptibility, while MDM from other donors with the MAJ genotype showed low susceptibility. In addition to other differences in ccr5 genotype/phenotype, it is likely that other host factors involved in viral replication and/or innate immunity against HIV-1 will explain the remaining variability. Infections with VSV-G pseudotyped HIV-1 indeed confirmed the existence of post-entry level restrictions in MDM.

In conclusion, we have shown high donor-linked variability between MDM from different donors for their in vitro HIV-1 susceptibility. Variability could only be partially explained by entry related factors. This variation is either due to differential expression of host factors that support HIV-1 replication or associated with innate cellular factors that may actively suppress viral infection. Further studies will be required to identify these host factors.

Materials and methods

Study population

Blood samples were obtained from 481 unique healthy blood bank donors. In addition, 8 HIV negative individuals from the Amsterdam Cohort Studies on HIV infection and AIDS (ACS) (van Griensven et al., 1987) were included in the study. All participants were HIV negative, at least 18 years of age and had given written informed consent. The average age of the study population was 47±12 years (n = 485; 4 donors with unknown date of birth). 454 donors (93%) were of European ancestry, whereas 31 donors (6%) were of ancestry from outside Europe, which was defined as having one or more (grand) parents born in a country outside Europe. Information on ancestry was missing from 4 donors. The 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 in Amsterdam and the Ethics Advisory Body of the Sanquin Blood Supply Foundation, The Netherlands.

Monocyte isolation

Monocyte isolations were performed between July and December 2007. In general, monocytes from 16 donors were isolated on one day. Peripheral blood mononuclear cells (PBMC) were isolated from EDTA blood by Ficoll-Paque density gradient centrifugation within 11 to 15 h after blood draw. Monocytes were subsequently isolated by magnetic-activated bead technology using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14\(^+\) magnetic microbeads (Miltenyi Biotec) were used to positively select monocytes. Prior to magnetic selection, PBMC were stained with CD3-PE, CD14-FITC and CD16-APC (Miltenyi Biotec). After cell separation, samples from each fraction were fixed using 2% paraformaldehyde. To confirm purity, FACS analysis was performed using a FACS Canto II flow cytometer (Becton Dickinson, La Jolla, CA). The remaining monocytes were seeded at a concentration of 5 \times 10^4 per well in a 96 wells plate in 100 μl Iscove’s modified Dulbecco medium supplemented with 10% (v/v) heat-inactivated human AB pool serum (HPS), penicillin (100 U/ml), streptomycin (100 μg/ml) and cyproxin (5 μg/ml) for 7 days at 37 °C and 5% CO₂.

Viruses

Viruses isolates were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, US National Institutes of Health.

HIV-1 BaL was grown on phytohemagglutinin (PHA) stimulated PBMC. PBMC were stimulated for 3 days in Iscove’s modified Dulbecco medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and PHA (5 μg/ml). Subsequently, the cells were grown in the absence of PHA in medium supplemented with recombinant interleukin-2 (20 U/ml; Chiron Benelux, Amsterdam, The Netherlands) and Polybrene (5 μg/ml; hexadimethrine bromide; Sigma, Zwijndrecht, The Netherlands). Cultures were expanded every 3–4 days and virus was harvested after 11 days.

HIV-1 YU2 virus stock was produced by transfecting 293T-cells with HIV-1 pYU2 plasmid DNA using the calcium phosphate method. In short, 293T-cells were cultured in Dulbecco’s Modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). For each 6 wells plate, 6 μg HIV-1 pYU2 DNA in 6 μl water was mixed with 750 μl 0.3 M CaCl₂, mixed with an equal volume of HEPES buffered saline pH 7.2, incubated at room temperature for 20 min and added to the culture medium. Culture supernatants were harvested after 2 and 3 days, and filtered through a 0.22 μm filter.

Using an in-house viral Gag p24 antigen capture enzyme-linked immunosorbent assay (ELISA) (Tersmette et al., 1989), the p24 concentration of virus isolate HIV-1 BaL and molecular clone HIV-1 YU2 stocks was determined to be 500 ng/ml and 780 ng/ml respectively. The 50% tissue culture infectious dose (TCID\(_{50}\)) was determined on U87 cells expressing CD4 and CCR5 cultured at 10%
CO₂ and 37 °C at day 14 after infection was 10^{5.87}/ml for BaL and 10^{1.93}/ml for YU2. Both HIV-1 BaL and YU2 stocks were sequence verified and tested for co-receptor usage and macrophage tropism (data not shown).

**HIV-1 infection assay**

Monocyte-derived macrophages (MDM) of all 489 donors were infected with 50 μl HIV-1 YU2 seven days after isolation (39 ng p24 per well, corresponding to an MOI of 0.0085). Parallel cultures of MDM of 154 donors were infected with 20 μl HIV-1 BaL (10 ng p24 per well, corresponding to an MOI of 0.27). The amount of virus added to the MDM was based on preliminary studies to assure maximum discrimination between donors (data not shown). Per donor, MDM in 12 replicate wells were incubated with HIV-1 BaL, HIV-1 YU2 or medium (negative control). All medium was removed prior to infection, and replaced with a mixture of pre-warmed (37 °C) monocyte/macrophage medium and virus stock (final volume 150 μl per well). Medium was also completely refreshed at days 4 and 7 post infection.

p24 levels were determined 14 days after infection by ELISA as described above. At day 14 post infection, MDM morphology was described above. At day 14 post infection, MDM morphology was assessed using phase contrast microscopy (Zeiss Axiovert 200) and viable cells were counted with Image Pro Plus (Media Cybernetics Inc.) after staining with Calcein AM (Invitrogen, Carlsbad, CA) (Fig. 6). HIV-1 infectivity results were only included for further analysis when at least 5000 cells per well could be counted, except for determination of correlation between HIV-1 BaL and HIV-1 YU2, where all available BaL and YU2 data from each donor was used. Dead and non-adherent cells were washed away when medium was replaced prior to infection and at days 4 and 7 post infection. 429 out of the 489 donors had at least 5000 viable cells after 21 days of culture.

Fourteen donors gave blood at two different time points (intermittence of 111 ± 33 days). Only results from the second blood donation were included in the overall analysis. Results from the two bleeds were compared for validation of the assay.

**Intra-donor variability and VSV-G pseudotype HIV-1 infections**

Blood samples were taken from 16 healthy sero-negative individuals on two occasions 7 days apart. All 16 participants, 4 males and 12 females, were older than 18 years of age (average 31 years) and had given written informed consent. Monocytes were isolated directly after the blood draw as described above. VSV-G pseudotyped HIV-1 YU2 stock was produced as described above, using 12 μg HIV-1 pYU2 plasmid and 3 μg VSV-G plasmid in 15 μl for each 6-well plate. The VSV-G pseudotyped HIV-1 YU2 virus stock had a p24 concentration of 1250 ng/ml and was titrated on TZM-bl cells expressing high levels of CD4 and CCR5. TCID₅₀ at day 2 post infection was 10^{4.45}/ml, as compared to 10^{4.75}/ml for HIV-1 YU2. Dependency of (co)receptor usage for entry was verified by inoculation of 293T cells with either the HIV-1 YU2 stock or the VSV-G pseudotyped YU2. MDM were infected with different concentrations of VSV-G pseudotyped HIV-1 YU2 (1, 6 and 13 ng p24 per well, corresponding to an MOI of 0.006, 0.028 and 0.056 respectively). All 16 donors were genotyped for the ccr5 Δ32 deletion.

**Peripheral blood lymphocytes (PBL) were used for DNA isolation using QiAamp DNA blood mini kit (Qiagen, Valencia, CA), ccr5 Δ32 genotyping was performed using PCR amplification of the ccr5 gene region encompassing the 32 bp deletion and subsequent amplified fragment analysis on agarose gels as described previously (van’t Wout, Schuitemaker, and Kootstra, 2008).**

For 303 genotyping we amplified DNA using GoTaq polymerase (Promega, Madison, WI) and primer pair 5′-GCTGAAGACGATGACTG-3′ (forward) and 5′-ACACGCCCCACATACTTCT-3′ (reverse). The following amplification cycles were used: 5 min 95 °C; 35 cycles of 30 sec 95 °C, 30 sec 58.5 °C, 1 min 72 °C; 10 min 72 °C. The m303 mutation results in loss of a unique HincII restriction site, amplicons were digested with HincII for 90 min at 37 °C.

**Data-analysis**

The number of cells per well differed between donors (mean number of cells per well 8887 ± 2243, n = 429). To correct for possible effects caused by cell number alone, total p24 in supernatant was calculated and expressed per 10,000 cells. Values from MDM in the wells to which no virus was added were used to correct for background signal. Since experiments were performed in 4 periods, p24 values were normalized by dividing through the median per period to avoid influences of time and effects of different operators (n = 2) that conducted experiments (Fig. 1A). The observed high correlation between the raw and normalized p24 levels (n = 429, Pearson r = 0.94, P < 0.0001) indicates that normalization led to a small improvement of the data, and not to a huge deviation from the raw p24 levels (data not shown). All 429 donors with valid results (MDM count at day 21 ≥ 5000) were ranked according to the normalized p24 values to select the two extreme groups (donor-derived MDM showing low (n = 96) and high (n = 96) viral replication). Homozygotes for ccr5 Δ32 (n = 5) were not included in the low viral replication group, but were analyzed separately. The number of 96 donors per group was chosen so the middle group (n = 232) would consist of at least double the number of individuals that make up an extreme group, ensuring adequate separation between the 2 groups of extremes. Data was log10 transformed to allow for parametric testing.

**Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) and SPSS 16 (SPSS Inc., Chicago, IL).**

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