Susceptibility of in Vitro Stimulated PBMC to Infection with NSI HIV-1 Is Associated with Levels of CCR5 Expression and β-Chemokine Production

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INTRODUCTION

Non-syncytium-inducing (NSI) variants of HIV-1 use the β-chemokine receptor CCR5 as a cofactor for entry of target cells (Deng et al., 1996; Dragic et al., 1996; Alkhatib et al., 1996; Choe et al., 1996; De Roda Husman et al., 1999b; Zhang, L., et al., 1998; Zhang, Y.-J., et al., 1998; Björndal et al., 1997). The relevance of this receptor in HIV-1 infection and AIDS pathogenesis has become clear by virtue of genetic polymorphisms influencing its expression levels. In the human Caucasian population the most prevalent polymorphism in the coding region of CCR5 is a 32-bp deletion (CCR5Δ32). Individuals homozygous for CCR5Δ32 (CCR5Δ32/Δ32), who consequently lack CCR5 cell surface expression (Wu et al., 1997; Liu et al., 1996), are protected from HIV-1 infection (Liu et al., 1996; Huang et al., 1996; Samson et al., 1996; Dean et al., 1996; Michael et al., 1997; Zimmerman et al., 1997). However, the existence of HIV-1-infected CCR5 Δ32/Δ32 individuals showed that this protection is not absolute (Balotta et al., 1997; Theodorou et al., 1997; O’Brien et al., 1997; Biti et al., 1997), which might be explained by transmission of viruses that use CXCR4 as a coreceptor (Michael et al., 1998). CCR5Δ32 heterozygous individuals (CCR5 Δ32/+) were shown not to be protected from infection, but CCR5Δ32 heterozygosity has been associated with a mean elongated AIDS-free survival period compared to individuals with the wild-type CCR5 genotype (CCR5 +/+ ) (Dean et al., 1996; De Roda Husman et al., 1997; Huang et al., 1996; Meyer et al., 1997; Eugen-Olsen et al., 1997; Michael et al., 1997; Zimmerman et al., 1997). More recently, also other polymorphisms in the coding region (Quillent et al., 1998) and the promoter region (McDermott et al., 1998; Martin et al., 1998; Mummidii et al., 1998) of CCR5 have been shown to be associated with disease progression. In line with the in vivo observations, PBMC from CCR5 Δ32/ + individuals were shown to be less susceptible to NSI HIV-1 in vitro (Paxton et al., 1998; Wu et al., 1997; Blaak et al., 1998; Kim et al., 1998) and in vivo in PBL-SCID mice (Picchio et al., 1997) compared to PBMC from CCR5 +/+ individuals. The on average lower proportion of CCR5-expressing CD4+ T cells and lower CCR5 surface expression levels on PBMC (Wu et al., 1997; De Roda Husman et al., 1999a) may result in slower spread of the virus and hence explain reduced NSI HIV-1 susceptibility of PBMC and slower disease progression in CCR5 Δ32/+ individuals.

An additional factor influencing HIV-1 replication is the presence of the natural ligands of CCR5. These ligands, RANTES, MIP-1α, and MIP-1β, were shown to inhibit NSI replication in vitro (Paxton et al., 1996; Deng et al., 1996; Dragic et al., 1996; Alkhatib et al., 1996; Cocchi et al., 1995; Margolis et al., 1998; Kinter et al., 1998) and high endogenous production by PBMC and/or CD4+ T lymphocytes was associated with protection from HIV-1 in-
fection in vivo (Paxton et al., 1998; Furci et al., 1997; Zagury et al., 1998) and HIV-1 disease progression (Saha et al., 1998). The inhibitory effect of β-chemokines is proposed to act through blocking of the coreceptor as well as through down-regulation of CCR5 on the cell surface (Alkhatib et al., 1997). The relevance of β-chemokine production in HIV-1 disease progression was recently supported by the identification of a polymorphism in the RANTES promoter that associates with reduced RANTES secretion and concomitantly with reduced CD4+ T cell decline (Liu et al., 1999).

We analyzed here CCR5 expression levels and β-chemokine production of PBMC and CD8-depleted PBMC after in vitro stimulation with phytohemagglutinin (PHA) and rIL-2. In parallel, the susceptibility for a panel of primary NSI variants was determined and the relation with CCR5 and β-chemokine levels was studied.

RESULTS

Susceptibility of total PBMC and CD8-depleted PBMC

Three-day PHA-stimulated PBMC and CD8−PBMC derived from 14 healthy donors were inoculated with a panel of seven primary NSI variants and further cultured in the presence of rIL-2. The donors were selected based on their CCR5 genotype: 7 had the CCR5 Δ32/+ and 7 the CCR5 +/+ genotype. After 14 days of culture the TCID50 was determined for each virus/donor combination. Since cells from each donor were inoculated with the same stock of each virus, the differences in the virus titers are a reflection of differences in NSI HIV-1 susceptibility of the cells. Paired analysis of PBMC and CD8−PBMC showed that the susceptibility was similar in 11/14 cases (Fig. 1). For 1 donor the susceptibility of PBMC was higher than that of CD8−PBMC (donor 1, average 50% tissue culture infections dose (TCID50) 10^4.60 vs 10^4.22, respectively, P = 0.028, paired t test). For 2 donors the inverse was observed, with reduced susceptible PBMC compared to CD8−PBMC (donor 7, average TCID50 10^4.05 vs 10^4.30, respectively, P = 0.058; donor 10, average TCID50 10^3.40 vs 10^4.25, respectively, P = 0.002). Although interindividual differences in susceptibility were observed, these differences were not related to differences in CCR5 genotype (Fig. 1).

Correlates of NSI HIV-1 susceptibility

For the PBMC of each donor the average TCID50 of seven NSI variants was used as a measure for NSI HIV-1 susceptibility. In order to classify the relative NSI HIV-1 susceptibility of the PBMC from each of the 14 donors, the median of the 14 average TCID50 values was determined (10^4.15). PBMC with an average TCID50 below that of the group median were defined as having relatively low NSI HIV-1 susceptibility, PBMC with an average TCID50 above that of the group median were defined as having relatively high NSI HIV-1 susceptibility (Table 1). Thus 7 donors with less and 7 donors with more susceptible PBMC could be identified (group average TCID50 10^4.59 and 10^3.59, respectively). For these two groups of donors CCR5 expression prior to stimulation (t = −3) and CCR5 expression and β-chemokine production after 3 days of PHA stimulation (i.e., just prior to inoculation, t = 0) were compared. In parallel cultures that were mock infected, the CCR5 expression and β-chemokine production after further propagation in the presence of rIL-2 (t = 6) were analyzed.
The percentage of CCR5$^+$ cells and the MFI of CCR5 in the CD4$^+$ cell population prior to stimulation and at the moment of inoculation did not differ between donors with relatively low and donors with relatively high in vitro NSI HIV-1 susceptibility (Fig. 2). However, in vitro susceptibility of PBMC was highly associated with the levels of CCR5 expression observed after 6 days of mock infection (42 and 20% CCR5$^+$ CD4$^+$ T cells in PBMC with high and low susceptibility, respectively, $P < 0.002$; MFI of CCR5 of 25 and 14 in PBMC with high and low susceptibility, respectively, $P = 0.013$, Student’s $t$ test; Fig. 2).

Similarly, at the end of 3-day PHA stimulation no difference was observed in the mean production of MIP-1$\alpha$, MIP-1$\beta$, and RANTES between PBMC with relatively high and relatively low NSI HIV-1 susceptibility (Fig. 3a). After 6 days of mock infection, the mean level of each $\beta$-chemokine was higher in the cell cultures with the lowest susceptibility (mean values: MIP-1$\alpha$, 10.6 vs 2.7 ng/ml, $P = 0.025$; MIP-1$\beta$, 5.5 vs 2.3 ng/ml, $P = 0.042$; RANTES, 6.4 vs 0.6 ng/ml, $P = 0.010$, Student’s $t$ test; Fig. 3b). Note that the approximately 4-fold lower level of chemokine production observed at $t = 6$ compared to $t = 0$ (Fig. 3) is likely explained by a 10-fold lower cell concentration ($0.5 \times 10^6$ vs $5 \times 10^5$ cells/ml) in combination with a 2-fold longer cultivation period (6 vs 3 days).

Similar to what we observed for PBMC, the NSI HIV-1 susceptibility of CD8$^+$ PBMC was associated with the levels of CCR5 surface expression and $\beta$-chemokine production at $t = 6$ (data not shown).

### Association between CCR5 surface expression and $\beta$-chemokine production

Both high CCR5 surface expression and low $\beta$-chemokine production after PHA/rIL-2 stimulation (i.e., at $t = 6$)

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**TABLE 1**

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<th>PBMC with relatively low in vitro NSI HIV-1 susceptibility</th>
<th>PBMC with relatively high in vitro NSI HIV-1 susceptibility</th>
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<td><strong>Donor</strong></td>
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$^a$ Calculated average $^{10}$log TCID$_{50}$ values of seven NSI variants.

$^b$ +/-, CCR5 wild-type individuals; $\Delta$32/+, individuals heterozygous for the 32-bp deletion in CCR5.

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**FIG. 2.** CCR5 expression on cells with high and low susceptibility for NSI HIV-1 infection. The percentage of CCR5-expressing CD4$^+$ T cells (a) and the mean fluorescence intensity of the CD4$^+$ T cell population (b) was compared for PBMC with relative high (□) and relative low (□) susceptibility. CCR5 expression was analyzed prior to stimulation ($t = -3$), after 3 days of PHA stimulation, just prior to virus inoculation ($t = 0$) and after 6 days of mock infection in the presence of rIL-2 ($t = 6$). Error bars represent the standard error of the mean. Differences were analyzed with the Student $t$ test; statistical significance is indicated by $^*P < 0.05$. 

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**IN VITRO SUSCEPTIBILITY TO NSI HIV-1 INFECTION**

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were associated with high in vitro NSI HIV-1 susceptibility. Since it was shown previously that low CCR5 cell surface expression is associated with high \( \beta \)-chemokine levels (Paxton et al., 1998), likely due to internalization of the receptor after chemokine binding (Alkhatib et al., 1997), we analyzed the correlation between \( \beta \)-chemokine production and CCR5 expression, in relation to susceptibility. No (or a weak) correlation was observed between the percentage of CCR5-expressing \( CD_4^+ \) T cells and the log-transformed values of levels MIP-1\( \alpha \) and MIP-1\( \beta \) after PHA/rIL-2 stimulation (\( R_p = 0.455, P = 0.10 \); and \( R_p = -0.301, P = 0.30 \), respectively; not shown). A strong inverse correlation was observed, however, for the percentage of CCR5-expressing \( CD_4^+ \) T cells and the level of RANTES production (\( R_p = -0.630, P = 0.016 \); Fig. 4). It is clear from Fig. 4 that the PBMC with low susceptibility have both a relatively low proportion of CCR5-expressing \( CD_4^+ \) T cells and a relatively high level of RANTES production.

**CCR5 genotype and in vitro NSI HIV-1 susceptibility**

In contrast to what was found previously by some (Paxton et al., 1998; Blaak et al., 1998; Wu et al., 1997; Kim et al., 1998), but in agreement with others (Picchio et al., 1997), we did not observe that susceptibility to NSI HIV-1 infection in vitro was associated with the CCR5 genotype (Table 1). In order to understand the basis of this dissociation, we analyzed the association of CCR5 genotype with in vitro induced CCR5 surface expression and \( \beta \)-chemokine production after PHA/rIL-2 stimulation. As was shown previously (Wu et al., 1997; De Roda Husman et al., 1999a), the percentage of CCR5-expressing \( CD_4^+ \) T cells was associated with the CCR5 genotype prior to stimulation (24\% in CCR5 \( +/+ \) and 14\% in CCR5 \( \Delta 32/+ \), \( P = 0.006 \), Students t test; Fig. 5a, left). However, after PHA/rIL-2 stimulation this association was lost (31\% CCR5-expressing \( CD_4^+ \) T cells both in CCR5 \( +/+ \) and in CCR5 \( \Delta 32/+ \) PBMC, \( P = 0.96 \), Student's t test; Fig. 5a, right). The similar CCR5 expression levels in CCR5 \( +/+ \) and CCR5 \( \Delta 32/+ \) cells after stimulation could be ex-
plained by an on average higher up-regulation in CCR5Δ32/+ (on average, +17%; range, +2 to +40%) compared to CCR5+/+ (on average, +7%; range, −16 to +31%) cells. Similar observations were made for CCR5 expression as measured by the MFI (not shown).

In addition, no CCR5 genotype-related differences in β-chemokine production were observed after PHA/rIL-2 stimulation (mean values of 22.1 and 6.0 ng/ml for CCR5+/+ and CCR5Δ32/+ PBMC, respectively; P = 0.13; Fig. 5b). Of interest, the three CCR5+/+ PBMC with the highest β-chemokine production levels were the three CCR5+/+ PBMC with reduced HIV-1 susceptibility (Table 1). From the inverse relation between β-chemokine production and CCR5 expression (Fig. 4) it follows that the relatively high β-chemokine production by some of the CCR5+/+ PBMC coincides with low CCR5 expression levels, which might account for the on average equal CCR5 expression levels, which might account for the on average equal CCR5 expression levels between CCR5+/+ and CCR5Δ32/+ PBMC. Indeed, stratification of the PBMC according to genotype suggests that the percentage of CCR5-expressing cells may largely be determined by the combination of the genotype and the level of β-chemokine production (Fig. 6).

**CCR5 genotype and CCR5 surface expression on in vivo activated cells**

The absence of an association between CCR5 genotype and CCR5 expression after in vitro stimulation prompted us to study the association between CCR5 genotype and CCR5 surface expression on in vivo activated cells. To this purpose CCR5 expression was analyzed on resting and activated CD4+ T cells in unstimulated PBMC from the 14 healthy blood donors. In vivo activation was identified by cellular expression of CD45RO in the absence of CD27 expression (Hamann et al., 1997; Baars et al., 1995) or in combination with either HLA-DR or CD69 expression. The proportion of CCR5-expressing cells was very low within the naïve CD4+ T cell population (average values: CD45RO−/HLA-DR−, 7%; CD45RO−/CD69−, 3%; CD45RO−CD27−, 0.7%; Fig. 7a) and not statistically different between CCR5+/+ and CCR5Δ32/+ PBMC (not shown). The proportion of CCR5-expressing cells was highest in the activated memory CD4+ T cells (average values: CD45RO+/HLA-DR+, 50%; CD45RO+/CD69+, 62%; CD45RO+CD27+, 60%; Fig. 7a) and intermediate in the resting memory CD4+ T cells (average values: CD45RO+/HLA-DR+, 29%; CD45RO+/CD69+, 25%; CD45RO+CD27+, 22%; Fig. 7a). The association between the level of CCR5 expression and activation status of the cells is in agreement with previous observations (Bleul et al., 1997; Ostrowski et al., 1998; De Roda Husman et al., 1999a). Both in the resting and in the activated memory CD4+ T cell populations the proportion of CCR5 surface-expressing cells (Fig. 7bc) and CCR5 expression as measured by MFI (not shown) were on average lower in CCR5Δ32/+ compared to CCR5+/+ PBMC.

**DISCUSSION**

Susceptibility of PBMC and CD8-depleted PBMC for in vitro NSI HIV-1 infection was associated with the expression of the coreceptor for NSI variants, CCR5, and the levels of its natural ligands, the β-chemokines, produced...
during culture. This association was only observed for 3-day PHA-stimulated PBMC that had subsequently been cultured for 6 days in the presence of rIL-2, paralleling 6 days of infection. The absence of an association of HIV-1 susceptibility with CCR5 surface expression and β-chemokine levels immediately after 3-day PHA stimulation, so at the moment of inoculation, suggests that under the presently used conditions virus production is determined by the capacity of the virus to spread through culture, rather than by the efficiency of the initial inoculation.

In the present study we confirmed the existence of an association between ex vivo CCR5 expression levels and CCR5 genotype (Wu et al., 1997; De Roda Husman et al., 1999a) and extended these observations by showing that this association exists for both resting and in vivo activated CD4\(^+\) T cells. However, after in vitro PHA and rIL-2 stimulation this association was lost, apparently due to a relatively higher up-regulation of CCR5 in the CCR5\(_{32}\) heterozygous PBMC. CCR5 can be down-regulated upon binding of its natural ligands (Alkhatib et al., 1997) and high β-chemokine levels may consequently result in reduced CCR5 expression. In agreement with this is the inverse correlation we here observed between RANTES production and CCR5 surface expression. In this view, the dissociation between CCR5 genotype and CCR5 expression in vitro might be due to levels of β-chemokines and CCR5 genotype together determining the ultimate levels of CCR5 cell surface expression. In vivo, however, the CCR5 genotype may be decisive and a role for down-modulation by chemokines limited to inflammatory sites. Down-modulation of CCR5 by RANTES (and to a lesser extent by MIP-1α and MIP-β) may explain the association between β-chemokine production and reduced in vitro susceptibility for NSI HIV-1. In addition the β-chemokines may directly interfere with HIV-1 entry (Alkhatib et al., 1997).

The absent correlation between CCR5 genotype and CCR5 expression levels under the currently used experimental conditions likely translates into the lack of an association between CCR5 genotype and NSI HIV-1 susceptibility. This observation contrasts with previous studies (Paxton et al., 1998; Wu et al., 1997; Blaak et al., 1998; Kim et al., 1998), which might reflect different cultivation conditions that differentially influence β-chemokine expression and hence CCR5 expression levels. While the present data indicate that the CCR5 expression level, and not CCR5 genotype per se, is an important determinant for NSI HIV-1 susceptibility in vitro, they do not exclude the possibility that CCR5 genotype, by determining in vivo CCR5 expression, influences HIV-1 replication in vivo. Indeed, in the PBL-SCID mouse model, replication of NSI HIV-1 was significantly lower in mice repopulated with PBMC from a CCR5\(_{32}\) heterozygous donor compared to mice repopulated with CCR5\(_{1}\)/PBMC (Picchio et al., 1997). In analogy, reduced percentages of CCR5-expressing CD4\(^+\) T cells and reduced CCR5 density at the cell surface may interfere with the efficiency of in vivo HIV-1 infection and viral spread through the human body. In agreement, we previously demonstrated that among individuals with a CCR5 \(+/+\) genotype, a reduced CCR5 surface expression correlated with a better prognosis of HIV-1 infection (De Roda Husman et al., 1999a). The in vivo association between CCR5 genotype and expression levels (this study; Wu et al., 1997; De Roda Husman et al., 1999a) might offer an explanation for the observed protective effect of the CCR5\(_{32}\) heterozygous genotype on disease progression of HIV-1-infected individuals (Dean et al., 1996; De Roda Husman et al., 1997; Huang et al., 1996; Meyer et al., 1997; Eugen-

![FIG. 6. Correlation between the percentages of CCR5-expressing cells and β-chemokine production stratified according to CCR5 genotype. Depicted are the percentages of CCR5-expressing cells in the CD4\(^+\) T cell populations in relation to the total levels of β-chemokines (a) and the levels of RANTES (b) in culture supernatants, all measured 6 days after mock infection. Each symbol represents the PBMC from one donor; the different colors indicate the different CCR5 genotypes: ○, CCR5\(_{32}/1\) PBMC; ●, CCR5 \(+/+\) PBMC.](image-url)
Olsen et al., 1997; Michael et al., 1997; Zimmerman et al., 1997; Morawetz et al., 1997). Indeed, the observed lower mean viral load early in infection in CCR5 Δ32 heterozygous compared to CCR5 1/1 individuals may be a reflection of impaired virus replication (De Roda Husman et al., 1997; Huang et al., 1996; Meyer et al., 1997).

MATERIALS AND METHODS

Cells and viruses

PBMC were isolated from buffy coats from 14 healthy plasmapheresis donors by Ficoll–Paque isolation. PBMC were stored in liquid nitrogen until usage. Three days prior to infection (t = −3) thawed PBMC (5 × 10⁶/ml) were stimulated with PHA (1 μg/ml). At the day of infection (t = 0), PHA-supplemented medium was removed and from part of the PHA-stimulated PBL (PHA-PBL), CD8⁺ T cells were depleted by the use of CD8-coated immunomagnetic beads (miniMACS, CLB, Amsterdam, The Netherlands). Both total PHA-PBL and CD8⁺ PHA-PBL were suspended in rIL-2 (Proleukin, Chiron Benelux BV, Amsterdam, The Netherlands; 20 U/ml) supplemented medium (0.5 × 10⁶ cells/ml). Cells used in the susceptibility assay were plated in 96-well plates (50,000 cells per well) and cultured for 14 days. Parallel (mock-infected) cultures for FACS analysis and determination of β-chemokine production in supernatant were grown in 6-well plates, at the same cell concentration.
Viruses were seven biological HIV-1 clones obtained during the early, asymptomatic stage of infection from three participants [ACH15 (n = 4), ACH372 (n = 2), and ACH456 (n = 1)] of the Amsterdam Cohort Studies on HIV-1 infection and AIDS. Participants and the moment of virus isolation were described in more detail elsewhere (nontransmitters from couples D4, D8, and D7, respectively (BlaaK et al., 1998). All HIV-1 clones had the NSI phenotype as determined on the MT2 cell line (Koot et al., 1992).

Susceptibility assay

The seven NSI HIV-1 clones were titrated on PHA-PBL (on average 67% of CD3+ T cells were CD4+ and 25% were CD8−) and CD8− PHA-PBL (on average 93% of CD3+ T cells were CD4+ and 0.2% were CD8−) from each of the 14 blood donors. Briefly, the cells were inoculated with serial fivefold dilutions of each virus stock and cultures were maintained for 14 days. At day 7, one-third of the medium was removed and replaced by fresh rIL-2-supplemented medium. At day 14 virus production was determined in an in-house p24 ELISA (Tersmette et al., 1989) and the TCID50 per milliliter of virus stock was determined. For each donor the average TCID50 of seven NSI viruses was used as a measure of NSI HIV-1 susceptibility of their PBMC and CD8− PBMC.

CCR5 genotyping

Genomic DNA was isolated from fresh PBMC (Qiagen, Westburg, Hilden, Germany). CCR5 genotyping was performed by PCR analysis using primers flanking the 32-bp deletion in CCR5 (De Roda Husman et al., 1997).

FACS analysis

Prior to stimulation (t = 3), after PHA stimulation (i.e., just before inoculation, t = 0), and after 6 days of mock infection in the presence of rIL-2 (t = 6), cells were stained with a combination of monoclonal antibodies (mAbs) directed against CD4 (–TC, Caltag, Burlingame, CA) and CCR5 (5G7–FITC, PharMingen, San Diego, CA). Since after 1 week of culture the proportion of viable CD4+ T cells decreases rapidly (unpublished observation), and hence most of the viral spread through the culture will occur during the first week, no analysis was performed on cells during the second week of the culture period. To monitor the efficiency of CD8 depletion, cells were stained with a combination of mAbs directed against CD3 (–PE) CD8 (–FITC), and CD4 (–TC) (all mAbs from Caltag).

In order to determine CCR5 expression levels on in vivo activated CD4+ T cells, cryopreserved, unstimulated PBMC were stained with a combination of (1) CD4 (–PERCP, Becton Dickinson, San Jose, CA), CD45RO (–APC, Becton Dickinson), HLA-DR (–PE, Caltag), and CCR5 (5G7–FITC, PharMingen); (2) CD4 (–PERCP, Becton Dickinson), CD45RO (–PE, Dako, Glostrup, Denmark), CD69 (–APC, Becton Dickinson), CCR5 (5G7–FITC, PharMingen); (3) CD4 (–PERCP, Becton Dickinson), CD45RO (–APC, Becton Dickinson), CD27 (–FITC, CLB, Amsterdam, The Netherlands), CCR5 (5G7–PE, PharMingen). All incubation steps were performed for 20 min at 4°C. Expression of the markers was analyzed with a FACScan or a FACScaliber (both from Becton Dickinson).

β-Chemokine production

At t = 0 and t = 6, 200 μl of cell-free culture supernatant was sampled and stored at −20°C until analysis. The presence of MIP-1α, MIP-1β, and RANTES in the supernatant was determined by ELISA (R&D Systems, Minneapolis, MN).

Statistical analyses

Comparisons between cells with the CCR5 +/+ and CCR5 Δ32/+ genotype, between cells with high and low susceptibility, and between total PBMC and CD8− PBMC were made with the Student’s t test. In the cases of comparisons of β-chemokine levels, statistics were performed on the log-transformed values. The correlation between CCR5 expression and the log-transformed values of RANTES was determined with the Pearson correlation coefficient. Normality of the samples was determined by the Shapiro–Wilks test for normality. All statistical analyses were performed by the use of SPSS 7.5 for windows.

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