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Marked differences in CCR5 expression and activation levels in two South African populations

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Summary

The chemokine receptor CCR5 is pivotal in determining an individual's susceptibility to HIV-1 infection and rate of disease progression. To establish whether population-based differences exist in cell surface expression of CCR5 we evaluated the extent of CCR5 expression across all peripheral blood cell types in individuals from two populations, South African Africans (SAA) and South African Caucasians (SAC). Significant differences in CCR5 expression, both in number of CCR5 molecules per cell (density) and the percentage of CCR5-expressing cells, were observed between the two study groups, within all cell subsets. Most notably, the percentage of all $CCR5^+$ cell subsets was significantly lower in SAC compared with SAA individuals ($P < 0{\cdot}01$) among natural killer (NK) -cell subsets ($CD56^+$, $CD16^+$ $CD56^+$ and $CD56^{dim}$) whereas CCR5 density was significantly higher in SAC compared with SAA individuals in $CCR5^+$ $CD8^+$ T-cell subsets and $CCR5^+$ NK-cell subsets ($CD56^+$, $CD16^+$ $CD56^+$ and $CD56^{dim}$) (all $P < 0{\cdot}05$). These relationships were maintained after exclusion of *CCR5Δ32* heterozygous individuals ($n = 7$) from the SAC dataset. The SAA individuals exhibited significantly higher cell activation levels, as measured by HLA-DR expression, than SAC individuals in $CD4^+$ T-cell subsets ($P = 0{\cdot}002$) and $CD56^+$ NK-cell subsets ($P < 0{\cdot}001$). This study serves to demonstrate that ethnically divergent populations show marked differences in both cell activation and CCR5 expression, which are likely to impact on both susceptibility to HIV-1 infection and the rate of HIV-1 disease progression.

Keywords: CCR5; HLA-DR; South African populations

Introduction

Shortly after the discovery of CCR5 as an HIV-1 co-receptor, it was identified as one of the host cell proteins that play an important role in the transmission and pathogenesis of HIV infection. Numerous studies have demonstrated the importance of CCR5 receptor density in the context of HIV-1 infection in that the amount of CCR5 expressed on the cell surface can directly influence an individual's susceptibility to HIV-1.[1–3] *In vitro* studies by Platt *et al.*[3] have demonstrated that a co-receptor density threshold of between 7×10^2 and 2×10^3 CCR5 molecules/cell is required for efficient replication of R5 HIV-1 in cell lines expressing CD4 levels similar to those on primary T cells. Furthermore, the density of CCR5 molecules on CD4 T cells correlates positively with the replication of R5 HIV-1.[4,5] Increased CCR5 density, determined as the mean number of molecules per cell, in HIV-1 infected individuals correlates with high viral loads,[6] faster disease progression,[7,8] as well as poorer response to antiretroviral treatment.[7,9,10] In addition, CCR5 density is also a determinant of the efficiency of CCR5 in chemotactic response to its ligands.[11]

The best possible example of the importance of CCR5 in HIV-1 infection has been demonstrated by a 32-bp deletion in the *CCR5* open reading frame, *CCR5Δ32*, which was first discovered in high-risk individuals resisting HIV infection.[2,12,13] Individuals heterozygous for the *CCR5Δ32* allele have a marked reduction in CCR5 surface expression in comparison to individuals lacking this allele[14] and individuals homozygous for this mutant fail to express detectable CCR5 protein on cell surfaces.[2]

Population studies of *CCR5Δ32* show that it is present at an average allele frequency of 10% in Europe; however, it is very rare or absent in Africans[15,16] suggesting that this allele is fairly recent in terms of human evolution.[15] There is also considerable individual–to-individual variability in surface expression on blood lymphocytes in CCR5 'wild-type' individuals, i.e. individuals lacking polymorphisms in the *CCR5* open reading frame.[1,14,17]

In addition to *CCR5* genetic polymorphisms, CCR5 surface expression can also be influenced by its chemokine ligands. For example, inverse associations between gene copy number of the CCR5 ligand, CCL3L, and CCR5 expression levels have been reported.[10,18] Similarly, CCL5, the CCR5 ligand most abundant in human plasma, regulates CCR5 density by inducing internalization of the receptor.[19] Other molecules, such as interleukin-2, interleukin-12 and interferon-α have also been shown to up-regulate CCR5 expression.[20–23] An increase in cell activation levels has also been associated with increased CCR5 expression.[14,24–26] Furthermore, HIV-1-infected individuals have significantly greater percentages of CCR5-expressing $CD4^+$ T cells when compared with healthy controls.[26,27] No study to date has considered CCR5 expression across different peripheral blood immune cell subsets between ethnically divergent populations, and earlier studies have largely focused on $CD4^+$ T cells and have assessed CCR5 expression mainly in the context of HIV-1 infection. To gain further insight into the many roles that can be attributed to CCR5 in the immune response and its role as an HIV co-receptor, requires an in-depth look at how this molecule is distributed across immune cell types in the absence of any chronic infections or immune disorders, and how this might vary between individuals and ethnic groups. Given that CCR5 expression plays an important role in HIV-1 infection and rate of disease progression, the overall CCR5 expression profile may predispose to these infection/disease outcomes. We therefore performed a cross-sectional study in which we evaluated the expression of the receptor CCR5, as both percentage of CCR5-expressing cells and CCR5 density, on various cell types in whole blood samples taken from healthy, HIV-1-uninfected individuals to evaluate baseline expression in two South African population groups, South African Africans (SAA) and South African Caucasians (SAC).

Materials and methods

Study participants

This study cohort comprised 22 SAA and 31 SAC healthy, HIV-1-uninfected individuals. The SAA cohort had a median age of 33·5 years (range 23–62 years) and comprised 14 (63·6%) women and eight (36·4%) men. The SAC cohort had a median age of 40·0 years (range 25–67 years) and comprised 20 (64·5%) women and 11 (35·5%) men. There were no statistical differences among the median ages or the male : female ratio of the two groups (Mann–Whitney $P = 0{\cdot}129$ and Fisher's exact $P = 1$, respectively). Since the *CCR5Δ32* allele has been shown to impact upon CCR5 expression, participants in this study were genotyped as described previously.[28] Seven SAC (22·6%) individuals were heterozygous for the *CCR5Δ32* allele. This allele was absent in the SAA population. If *CCR5Δ32* allele-bearing individuals were removed from the analysis, the population group remained age matched ($P = 0{\cdot}209$: SAA median 33·5 years; SAC median 39·5 years) and gender matched ($P = 1$; SAA: 14 females and 8 males; SAC: 16 females and 8 males). This study was approved by the University of the Witwatersrand Committee for Research on Human Subjects, and informed written consent was obtained from all participants.

Whole blood surface staining and flow cytometry

Ethylenediaminetetraacetic acid (EDTA) –anti-coagulated whole blood obtained from each of the study participants was stained within 1 hr of blood collection. The following antibody panels were used for each donor: (i) T cells: CD3-allophycocyanin (APC), CD4-FITC, CD8-peridinin chlorophyll protein (PerCP), CCR5-phycoerythrin (PE); (ii) B cells: CD19-APC, CCR5-PE; (iii) natural killer (NK) cells: CD3-PerCP, CD16-FITC, CD56-APC, CCR5-PE; and (iv) granulocytes and monocytes: CD45-APC, CD14-FITC, CCR5-PE. An HLA-DR marker to assess the extent of cell activation (i.e. HLA-DR) was also included in a subset of the cohort in the following panel: (v) CD3-PerCP, CD8-FITC, CD56-APC, HLA-DR-PE. All antibodies were obtained from BD Pharmingen (BD Biosciences, San Jose, CA). The CCR5 antibody used was conjugated to PE at a ratio of 1 : 1, thereby allowing for CCR5 quantification, as the mean number of CCR5 molecules per cell (CCR5 density), in addition to the percentage of CCR5-expressing cells within a cell subset. Quantification was carried out using the QuantiBRITE system (BD Biosciences), which is a set of four pre-calibrated beads to calibrate the fluorescence 2 (FL2) axis in terms of PE molecules.

Fifty microlitres of whole blood was used for each antibody panel described above. After incubating stained samples for 15 min, the red blood cells were lysed with 2 ml FACS® lysing solution (BD Biosciences) for 7 min. The cells were then pelleted by centrifugation at 100 ***g*** for 5 min at room temperature and washed with FACS flow (BD Biosciences). The stained cells were suspended in 150 μl paraformaldehyde (Electron Microscopy Services, Pretoria, South Africa) and stored at 4° until flow cytometric acquisition within 6 hr of sample collection. All incubations were performed at room temperature in the dark.

Flow cytometric acquisition and analysis was performed on the FACSCalibur (BD Biosciences). The flow cytometer was set up by running FACSComp 5.2 (four-colour lyse-wash) software (BD Biosciences) with CaliBRITE beads (BD Biosciences). Daily compensation using whole blood stained with a single antibody was conducted to optimize instrument settings for the assay. The lymphocyte population was identified on the basis of forward and side scatter parameters. T cells were defined as CD3-expressing lymphocytes and were further classified as $CD4^+$ or $CD8^+$ T cells. Lymphocytes expressing CD19 were defined as B cells. The NK cells were defined as lymphocytes negative for CD3 and positive for CD56 expression. NK cell subsets analysed were, $CD56^+$, i.e. total CD56-expressing NK cells, $CD56^{dim}$, $CD56^{bright}$ and $CD16^+$ $CD56^+$. Monocyte and granulocyte populations were identified on the basis of side scatter and CD14 parameters. Monocytes were identified by the presence of CD14 markers, whereas granulocytes are negative for CD14. The mean number of CCR5 receptors per cell was determined for $CCR5^+$ cell subsets. Data were analysed using FloJo 7·6·1 (Tree Star, San Carlos, CA).

Statistical analysis

Mann–Whitney *U*-tests were conducted to compare CCR5 density between individuals, grouped by population or by the presence or absence of the *CCR5Δ32* allele. Correlations between CCR5 density and age of individuals were calculated by bivariate Spearman's rank coefficients. All statistical analyses were performed using PASW Statistics 18 software (SPSS Inc., Chicago, IL).

Fisher exact tests were performed using the Simple Interactive Statistical Analysis software (Uitenbroek, D. G, Binomial. SISA. 1997. http://www.quantitativeskills.com/sisa/distributions/binomial.htm [1 January 2004]) to test whether there was any significant difference in composition of population groups.

Results

CCR5 expression on lymphocyte populations

Isolation of peripheral blood mononuclear cells by Ficoll purification and a delay in whole blood sample processing is documented to result in acute down-regulation of CCR5 expression on various cell types.[29–31] Therefore, flow cytometry was performed on whole blood samples. Data presented as mean/medium fluorescence intensity or as the proportion of positive cells are always relative to controls that are specific for any given experiment. Hence, we used a method for enumerating CCR5 molecules, which allowed better comparison between individuals using a CCR5 antibody conjugated to PE at a ratio of 1 : 1 in combination with antibodies that define different cell types. This allowed for CCR5 quantification as the mean number of CCR5 molecules per cell, i.e. CCR5 density, in addition to the percentage of CCR5-expressing cells within a cell subset.

In agreement with previous studies, a large inter-individual variability on CCR5 expression was observed.[1,6] The mean number of CCR5 molecules per cell differed by as much as sixfold between individuals, most notably in the $CD8^+$ T-cell and $CD56^{bright}$ NK-cell subsets.

The potential influence of gender upon expression was examined. Female participants demonstrated a significantly higher percentage of CCR5-expressing cells in the $CD56^{bright}$ NK subset than the men ($P = 0{\cdot}042$) when the whole cohort was examined. However, this significance was lost when the population groups were examined separately. No differences in expression between male and female participants were observed in all other cell subsets (data not shown).

It is interesting to note that a high percentage of CCR5-expressing cells within a subset does not necessarily correlate with high CCR5 density, i.e. some individuals have a small percentage of CCR5-expressing cells within a cell subpopulation but express CCR5 at high density on this small proportion of cells and vice versa. This is in agreement with other reports.[6]

The cell subsets with the highest CCR5 density were T cells and monocytes: T cells (ranging from 1369 to 4820 molecules/cell); $CD4^+$ T cells (ranging from 945 to 3678 molecules/cell); $CD8^+$ T cells (ranging from 1055 to 5953 molecules/cell) and $CCR5^+$ monocytes (ranging from 1628 to 8773 molecules/cell). The subsets with the greatest percentage of cells expressing CCR5 were $CD8^+$ T cells ($CD3^+$ $CD8^+$, 57·8% median) and $CD56^{bright}$ NK cells (64·8% median). Although the $CD56^{bright}$ NK-cell and B-cell populations were observed as high CCR5-expressing subsets, these cell types were present in low numbers, which may affect the accuracy of the CCR5 density values.

CCR5 expression differs between SAA and SAC individuals within all cell subsets

Significant differences in CCR5 expression were noted between SAA and SAC individuals, both in terms of CCR5 density and in the percentage of individual cell types that express CCR5 in the different lymphocyte subpopulations (Table 1). When *CCR5Δ32*-bearing SAC individuals ($n = 7$) were removed from the analysis, these differences were maintained or even strengthened (Table 1).

As NK cells showed significant differences in CCR5 expression, we examined expression on various NK cell subsets in detail (Fig. 1). Overall, SAC individuals tend to express a greater number of CCR5 receptor molecules per cell, compared with SAA individuals, but on a significantly smaller proportion of CCR5-positive cells.

Table 1. Differences in CCR5 expression, both as percentage of CCR5-expressing cells and CCR5 density, between two South African population groups, South African Africans (SAA) ($n = 22$) and South African Caucasians (SAC) ($n = 31$) across all peripheral blood cell populations*

	Median percentage CCR5-expressing cells (%)						Median number of CCR5 molecules per cell					
	Population			Population (excluding *CCR5Δ32*)			Population			Population (excluding *CCR5Δ32*)		
Cell subset	SAA	SAC	*P*	SAA	SAC	*P* (excluding *CCR5Δ32*)	SAA	SAC	*P*	SAA	SAC	*P* (excluding *CCR5Δ32*)
B cells												
$CD19^+$ $CCR5^+$	26·0	19·7	< 0·001	26·0	20·1	0·001	1761	1492	0·028	1761	1474	0·012
T cells												
$CD3^+$ $CCR5^+$	40·8	38·4	0·610	40·8	42·8	0·775	2435	2901	0·066	2435	3045	0·004
$CD4^+$ $CCR5^+$	29·0	26·5	0·698	29·0	31·4	0·652	2150	2252	0·626	2150	2357	0·113
$CD8^+$ $CCR5^+$	55·8	59·1	0·773	55·8	60·5	0·524	2150	3217	0·008	2150	3502	0·001
Monocytes												
$CD14^+$ $CCR5^+$	10·1	12·4	0·093	10·1	12·5	0·050	4802	4318	0·220	4802	4646	0·403
Natural killer cells												
$CD56^+$ $CCR5^+$	34·6	22·8	0·001	34·6	24·6	0·007	1265	1450	0·017	1265	1440	0·031

*Values are indicated for analysis including and excluding individuals heterozygous for the *CCR5Δ32* allele present in the SAC population. *P*-values are indicated. Where significant differences are observed ($P < 0·05$), the highest value in the comparison is highlighted.

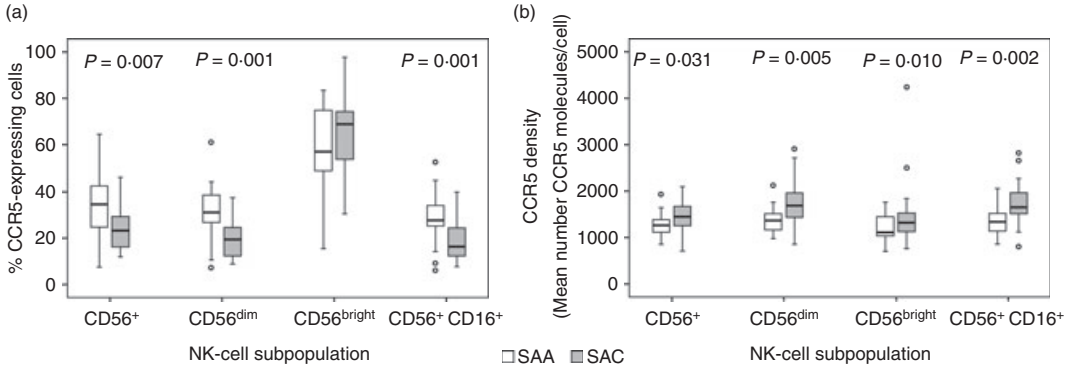

Figure 1. CCR5 expression in South African African (SAA; $n = 22$) and South African Caucasian (SAC; $n = 24$) individuals within natural killer (NK) cell subsets. (a) Percentage of CCR5-expressing cells within NK-cell subsets. (b) CCR5 density on CCR5-expressing NK-cell subsets. Heterozygous *CCR5Δ32* individuals have been excluded. Box-whisker plots depicting the median (horizontal black line), 25th and 75th centiles (margins of the box) and the 10th and 90th centiles (whiskers). Outliers are indicated with (○). Where significant, *P*-values have been indicated.

Relationship between CCR5 expression and age of individuals

The age range of participants in our study was broad, ranging between 23 and 67 years of age. Hence, we investigated the relationship between CCR5 expression and age on all peripheral blood mononuclear cell subsets studied. A significant negative correlation with age and the percentage of CCR5-expressing cells was seen in the B-cell subset in SAA individuals ($R = -0·478$, $P = 0·024$) but not in CCR5 'wild-type' SAC individuals ($R = -0·352$, $P = 0·092$). When grouped together (SAA+SAC), a significant positive correlation was noted on $CD8^+$ T-cell subsets ($R = 0·320$, $P = 0·020$). This relationship was lost when the populations were analysed separately, although a trend was still maintained in the SAC population excluding *CCR5Δ32* allele-bearing individuals ($R = 0·374$, $P = 0·072$). Significant negative correlations were observed in the SAC population in $CD56^+$ NK-cell subsets whether the population was examined as a whole or with the exclusion of *CCR5Δ32* heterozygous individuals ($R = -0·461$, $P = 0·009$ and $R = -0·448$, $P = 0·028$, respectively). The relationship appears to be the result of the $CD16^+$ $CD56^+$ NK-cell subset ($R = -0·417$, $P = 0·043$ in SAC CCR5 'wild-type' individuals), because no significant correlations were observed in the other NK-cell subsets. No similar relationships were

observed in SAA individuals so the proportion of CCR5-expressing NK cells appear to decrease with age in SAC individuals.

Although correlations between the proportion of CCR5-expressing cell subsets and age were observed in B-cell, T-cell and NK-cell subsets, these relationships were not observed when correlations with CCR5 density and age were analysed in the same cell subsets (data not shown). In monocyte cells, however, a negative correlation between CCR5 density and age only in SAA individuals was observed ($R = 0{\cdot}468$, $P = 0{\cdot}028$) (Fig. 2).

SAA and SAC individuals differ in cell activation levels

The levels of activation for a subset of individuals from both population groups, SAA ($n = 16$) and SAC ($n = 24$), were measured for T cells and NK cells using HLA-DR as a marker of activation. The SAA individuals exhibited significantly higher expression of HLA-DR, measured as the percentage of HLA-DR-expressing cells, in $CD4^+$ T-cell ($P = 0{\cdot}002$) and NK-cell ($CD56^+$, $P < 0{\cdot}001$) subsets (Fig. 3). When we investigated the correlations between HLA-DR and CCR5 expression levels, a positive correlation was observed in SAA individuals, between HLA-DR expression (%) and the percentage of CCR5-expressing cells on T-cell ($CD3^+$) and $CD4^+$ T-cell subsets ($P = 0{\cdot}021$ and $0{\cdot}037$, respectively, Table 2). However, the proportion of HLA-DR-expressing cells did not correlate with CCR5 density on these same subsets (Table 2). The SAC individuals, on the other hand, demonstrated a positive relationship between proportions of HLA-DR-expressing cells and CCR5 density, i.e. individuals with higher proportions of T-cell subsets expressing HLA-DR had higher numbers of mean CCR5 molecules per cell on $CD3^+$, $CD4^+$ and $CD8^+$ T-cell subsets (Table 2), but no significant correlation was observed when analysing the relationship between the percentage of CCR5-expressing subsets and of HLA-DR-expressing subsets. No significant correlations were observed in any NK-cell subsets (data not shown).

To investigate possible relationships between CCR5 density and HLA-DR density on the same cell subsets, we used the geometric mean of $HLA\text{-}DR^+$ subsets as a substitute measure for the mean number of molecules per cell as we did not use an HLA-DR antibody conjugated to PE at a 1 : 1 ratio. No significant differences in HLA-DR mean fluorescence intensity between the two populations were observed (data not shown). Furthermore, no correlations between HLA-DR mean fluorescence intensity and CCR5 density were observed.

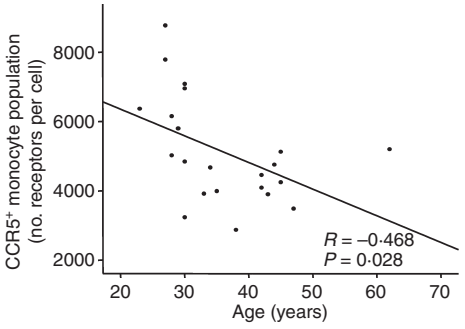

Figure 2. Correlation between CCR5 density (mean number of molecules per cell) on monocyte cells and age of South African African (SAA) study participants ($n = 22$). *P*-value is indicated.

Influence of CCR5Δ32 allele on CCR5 expression within the SAC population

Individuals bearing the *CCR5Δ32* allele tended to have a lower percentage of CCR5-expressing cell subsets in all cell populations examined, yet statistical significance was only observed in $CD4^+$ T cells ($P = 0{\cdot}026$) (Fig. 4a). Individuals heterozygous for the *CCR5Δ32* allele demonstrated lower CCR5 density on T-cell populations relative to individuals that lack the allele ($P < 0{\cdot}005$) (Fig. 4b).

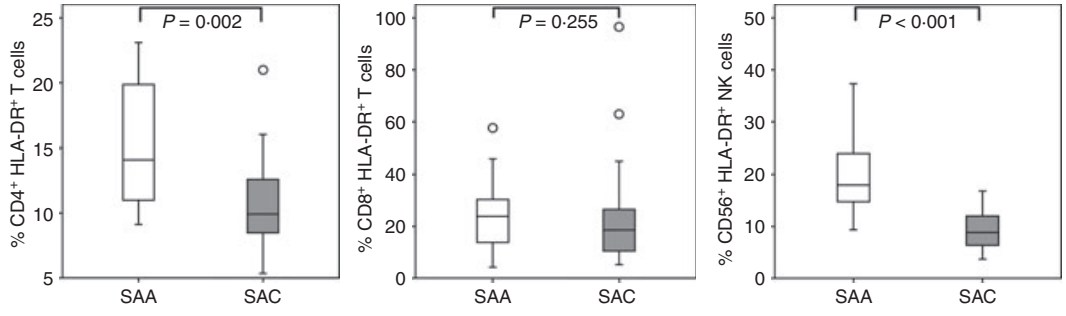

Figure 3. Activation levels, as demonstrated by percentage HLA-DR expression in the two study population groups, South African Africans (SAA; $n = 16$) and South African Caucasians (SAC; $n = 23$) within natural killer (NK) -cell and T-cell subsets. Box-whisker plots depicting the median (horizontal black line), 25th and 75th centiles (margins of the box) and the 10th and 90th centiles (whiskers). Outliers are indicated with (○). *P*-values have been indicated.

Table 2. Correlation between cell activation, as measured by HLA-DR percentage expression, and CCR5 expression (percentage CCR5-expressing cells and density)*

Correlation between HLA-DR expression and % CCR5-expressing cells in T-cell subsets						
	% HLA-DR$^+$ CD4$^+$ T cells*			% HLA-DR$^+$ CD8$^+$ T cells		
Cell subset	SAA+SAC (n = 39)	SAA (n = 16)	SAC (n = 23)	SAA+SAC (n = 39)	SAA (n = 16)	SAC (n = 23)
% CCR5-expressing cells						
CD3$^+$	R = 0·367	R = 0·569	R = 0·254	R = 0·159	R = 0·141	R = 0·172
	P = 0·022	P = 0·021	P = 0·242	P = 0·334	P = 0·602	P = 0·434
CD3$^+$ CD4$^+$	R = 0·308	R = 0·525	R = 0·071	R = 0·273	R = 0·200	R = 0·315
	P = 0·056	P = 0·037	P = 0·746	P = 0·093	P = 0·457	P = 0·144
CD3$^+$ CD8$^+$	R = 0·407	R = 0·366	R = 0·569	R = 0·172	R = 0·349	R = 0·106
	P = 0·010	P = 0·164	P = 0·005	P = 0·294	P = 0·185	P = 0·630
Number of CCR5 molecules/cell						
CD3$^+$	R = −0·058	R = 0·242	R = −0·020	R = 0·334	R = −0·058	R = 0·526
	P = 0·725	P = 0·367	P = 0·929	P = 0·038	P = 0·832	P = 0·010
CD3$^+$ CD4$^+$	R = −0·033	R = 0·164	R = −0·051	R = 0·286	R = −0·077	R = 0·444
	P = 0·840	P = 0·544	P = 0·817	P = 0·077	P = 0·777	P = 0·034
CD3$^+$ CD8$^+$	R = −0·145	R = 0·320	R = −0·044	R = 0·317	R = −0·005	R = 0·484
	P = 0·377	P = 0·227	P = 0·843	P = 0·050	P = 0·984	P = 0·019

SAA, South African Africans; SAC South African Caucasians.
*Where significant, *P*-values have been highlighted.

This is consistent with other reports.[14] However, it is interesting to note that differences in expression in other cell populations are not significant with the exception of CD56^{bright} NK cells (Fig. 4b). In addition, some SAC individuals heterozygous for the *CCR5Δ32* allele had comparable CCR5 expression, both in the percentage of CCR5-expressing cells and CCR5 density, compared with homozygous 'wild-type' individuals (Fig. 4a,b).

Discussion

Extensive variation exists between individuals, in their susceptibility to HIV-1 and the rate of disease progression to AIDS. Cell surface expression of the HIV-1 co-receptor, CCR5, is highly variable, even in individuals homozygous for the wild-type open reading frame region.[1,14,17] Although the influence of the *CCR5Δ32* mutation on CCR5 expression on T cells has been studied extensively in the context of HIV-1 infection, we lack information on how healthy HIV-1 uninfected individuals may differ in terms of individual CCR5 expression levels. Given that the extent of expression of CCR5 plays a pivotal role in determining HIV-1 susceptibility and that striking ethnic or population differences in SNP frequencies of CCR5 exist, it is surprising that very few studies have questioned how populations may differ in terms of their CCR5 expression levels.

Transsexual men receiving oestrogens and anti-androgens have been reported to have increased CCR5 expression on T cells[32] and female mice have higher CCR5 expression on CD4$^+$ T cells compared to their male counterparts.[33] Oestrogen therefore appears to influence CCR5 expression, so we looked at potential differences in expression between genders in the current study. On the whole, no differences in expression were observed between male and female study participants, whether examined collectively or separately in the two population groups.

Previous studies have demonstrated CCR5 receptor expression to be stable within individuals over multiple time-points despite the wide range of variability that exists between individuals.[6,26,34] However, aging has been associated with increased CCR5 expression on T cells both in mice through gene expression studies[35] and in humans using ribonuclease protection assays and Western blots.[36] Hence, we investigated the relationship between CCR5 expression and age on all studied cell subsets in the two population groups (age and sex matched). The proportion of CCR5-expressing CD8$^+$ T cells was observed to significantly increase with age in the pooled cohort but no correlations were observed in T cells when the two populations were analysed separately. In a human study in which CCR5 expression on CD4$^+$ T cells was shown to be higher in older individuals, CCR5 expression was compared between two groups, young adults (aged 18–40 years) and older subjects ($\geq$ 60 years, mean age 73 years).[36] Only three of our participants were aged $\geq$ 60 years (one SAA individual, aged 62, and two SAC individuals, aged 64 and 67), so it is likely that our study did not have sufficient 'older' individuals to show this

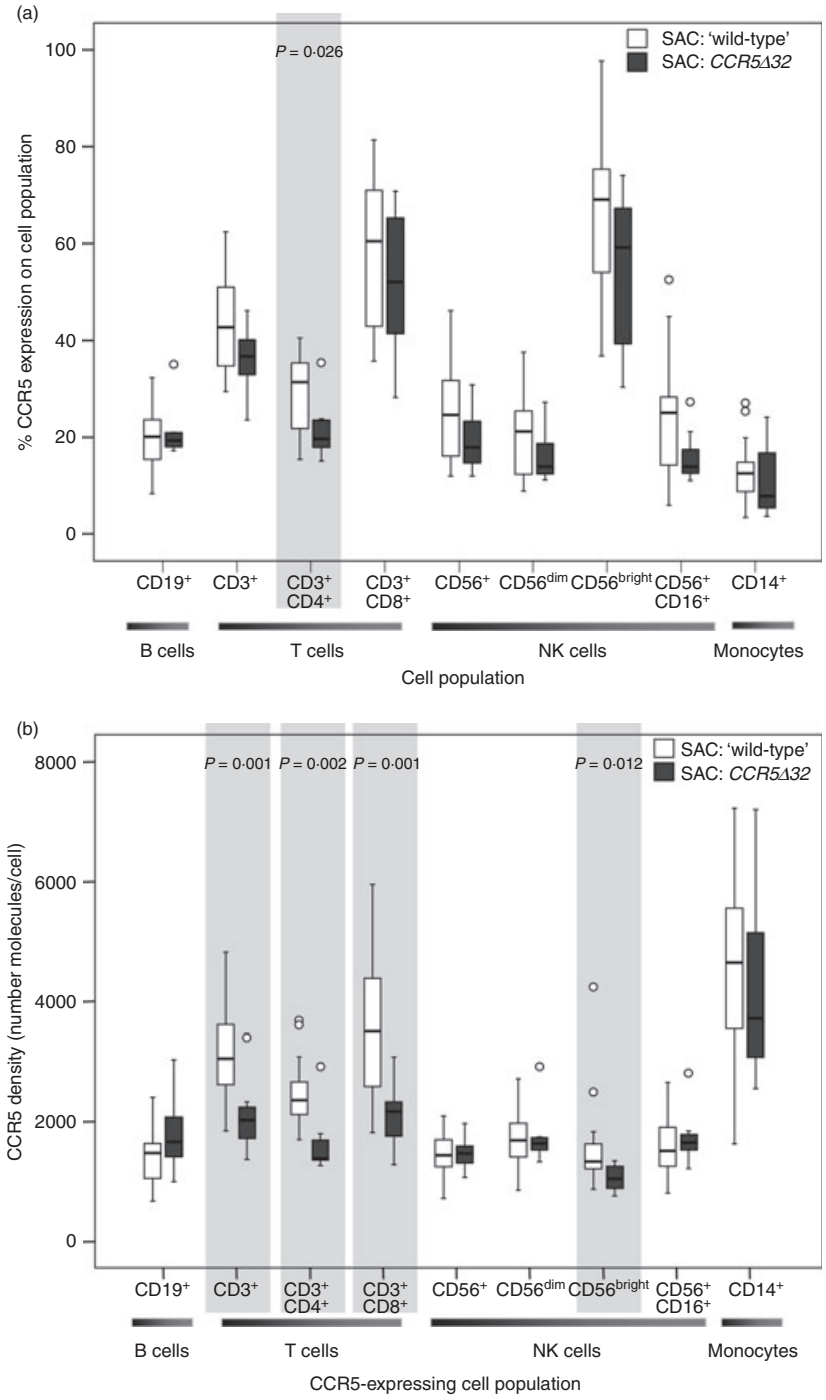

Figure 4. Influence of *CCR5Δ32* bearing alleles on CCR5 expression in South African Caucasian (SAC) population within all cell populations examined. (a) Percentage of CCR5-expressing cells in the different cell subsets examined in SAC individuals heterozygous for the *CCR5Δ32* allele ($n = 7$) and CCR5 'wild-type' SAC individuals ($n = 24$). (b) CCR5 density (mean number of molecules/cell) on the different cell subsets examined in SAC individuals heterozygous for the *CCR5Δ32* allele ($n = 7$) and CCR5 'wild-type' SAC individuals ($n = 24$). Where significant, *P*-values have been indicated. Box-whisker plots depicting the median (horizontal black line), 25th and 75th centiles (margins of the box) and the 10th and 90th centiles (whiskers). Outliers are indicated with (○).

relationship. Also, levels of expression were determined differently, where we have looked at cell surface expression by means of flow cytometry, Yung and Mo[36] used ribonuclease protection assays and Western blots. However, in SAA individuals, but not SAC individuals, we observed a decrease in CCR5 density on $CCR5^+$

monocytes in older individuals. Further studies with a larger cohort would be necessary to unequivocally establish the effects of age on CCR5 expression in healthy adults.

Although only a few studies have compared CCR5 expression in different population groups, differences have been noted. For example, Ethiopians living in Israel have been shown to express CCR5 at higher levels, both in terms of proportions of CCR5-expressing $CD4^+$ cells and the number of molecules per $CD4^+$ cell, than Israeli Caucasians, despite comparable activation levels as measured by HLA-DR expression.[37] However, the prevalence, and hence potential influence upon expression, of the *CCR5Δ32* allele in this study was not indicated. In the present study, we show significant expression differences, both in terms of percentage of CCR5-expressing cells and the CCR5 density, between our two study populations, in all cell subsets. The SAA individuals tended to express fewer CCR5 molecules per cell but on a larger proportion of cells than SAC individuals. The most significant differences in CCR5 expression between the two study groups were seen in the NK-cell subsets. The role of NK cells in HIV-1 infection is complex and is not fully understood. Although NK cells are able to directly mediate the killing of HIV-1-infected cells, they are also thought to be involved in the killing of uninfected $CD4^+$ T cells and so contribute towards $CD4^+$ T-cell decline (reviewed by Funke *et al.*[38]). Cytotoxic NK-cell subsets, expressing CD4, CCR5 and CXCR4 have been identified.[39,40] Furthermore, *in vitro* studies have shown HIV-1 to infect stimulated $CD4^+$ NK cells expressing HIV-1 co-receptors,[39–41] an indication that these cells could serve as HIV-1 reservoirs. It would follow that, similar to T-cell subsets, both the proportion of CCR5-expressing and CCR5 density of NK cells could impact on the progression of HIV-1 infection.

Immune activation levels have previously been reported as environmentally driven.[42] Individuals living in Africa, of Italian (Caucasian) and Ugandan origin, were demonstrated to have higher activation levels when compared with individuals from the same population groups living in Italy.[42] Interestingly, comparable activation levels, as measured by HLA-DR expression, were seen in both the Italian and Ugandan group living in Uganda,[42] contrasting the results in our study where SAA and SAC individuals, largely controlled for environment, differ significantly in HLA-DR expression, i.e. activation levels, in $CD4^+$ T-cell and NK-cell subsets. These differences could be indicative of either the two South African population groups being exposed to differing environmental/activation triggers or that corresponding geographically distinct population groups (SAA versus Ugandans and SAC versus Italians) differ with respect to their genetic background. Similarly, in a study comprising participants of Ethiopian and Israeli origin, Ethiopian individuals that had recently moved to Israel had higher $CD4^+$ $HLA\text{-}DR^+$ and $CD8^+$ $CD38^+$ levels compared with Ethiopian individuals who had been living in Israel for > 7 years and Caucasian Israelis.[37] Ethiopians who had been living in Israel for > 7 years still had higher activation levels than their Caucasian Israeli counterparts, implying that there must be factors other than environmental, i.e. genetic, to which the higher activation levels can be attributed.[37] Furthermore, Kenyan women negative for HIV-1 and other sexually transmitted infections, have increased activated mucosal T cells ($CD4^+$ $CD69^+$ T-cell, $CD4^+$ $CD69^+$ $CCR5^+$ T-cell and $CD8^+$ $CD69^+$ T-cell subsets) compared with women of mixed ethnicity in the USA.[43] It was postulated that these differences could be attributed to differences in host genetics or from immune activation caused by chronic systemic infections, which may be more prevalent in Africa.[43] Given that the participants in our study were all living in the same environment and had no overt infections or known immune conditions, we postulate that differences in activation levels are more likely to be driven by genetic factors. These studies collectively highlight the complexity of understanding what influences the level of immune activation measurable in a healthy individual and point towards an interplay between the genetics and environment of the individual.

Cell activation results in an increase in CCR5 expression.[14,24–26] Activated ($HLA\text{-}DR^+$) $CD4^+$ cells have been shown to express CCR5 at higher levels than non-activated ($HLA\text{-}DR^-$) $CD4^+$ cells.[6,14,37,44] Our results in healthy SAC individuals are in keeping with this; higher proportions of HLA-DR-expressing $CD4^+$ and $CD8^+$ T cells correlated with correspondingly higher density of CCR5 expression. Furthermore, the low expression of activation markers HLA-DR and CD38 on $CD4^+$ T cells is linked to low HIV-1 susceptibility in HIV-1-exposed uninfected individuals.[44,45] It is interesting to note that in a report by Reynes *et al.*,[6] the observation is made that although activation, measured by HLA-DR expression on $CD4^+$ T cells, leads to a significant increase in both the number of CCR5 molecules and the proportion of CCR5-expressing cells, the difference in percentage of CCR5-expressing cells between $HLA\text{-}DR^-$ and $HLA\text{-}DR^+$ cell subsets is much greater than differences seen in CCR5 density. A recent report has demonstrated *in vivo* that activated ($HLA\text{-}DR^+$ $CD38^+$) T cells in lymphoid tissues are highly susceptible to infection by R5 tropic HIV-1 viruses and postulated that this is probably because of the observed higher expression of CCR5 on these cells in comparison to other $CD4^+$ T-cell subsets.[24]

Consistent with the significantly higher activation levels observed in T and NK cells, SAA individuals had greater proportions of CCR5-expressing subsets compared with SAC individuals. However, an inverse relationship was observed in terms of CCR5 density. Several studies have shown that CCR5 density is capable of influencing HIV-1 susceptibility, with higher density associated with increased

susceptibility to infection.[1–3] Furthermore, it has been reported that CCR5 density on $CD4^+$ T cells of slow progressors versus normal progressors, but not the percentage of CCR5-expressing cells, correlate positively with viral loads.[8] Interestingly, CCR5 density was also lower in the slow progressors compared with the uninfected controls.[8] However, Meditz *et al.*[24] have recently demonstrated that both CCR5 density and the percentage of CCR5-expressing cells are important determinants of HIV-1 susceptibility *in vivo*. Hence, both density and the percentage of CCR5-expressing cells appear to be important determinants, coupled with the extent of immune activation, and it is the combinatory effects of these that may predispose to different infection and disease outcome phenotypes. It can be envisaged that larger proportions of CCR5-expressing cells in the periphery would result in larger numbers of cells entering an immune response at the site of infectious encounter; if these are $CD4^+$ cells then more target cells would be available for HIV-1 infection, if among these there are CCR5-expressing NK cells, for example, then those co-expressing CD4 may be vulnerable to infection but others may be more effective killer cells – providing a counter response to control the extent of infection. The overall outcome would be dependent on many factors, including also the concentration of the natural chemokines that bind CCR5, which aside from their role as chemoattractants would also prevent HIV-1 entry by blocking or down-regulating CCR5 expression.

Although increased cellular activation in $CD4^+$ T-cell subsets is associated with increased susceptibility to HIV-1 infection, it is not clear what the functional significance of HLA-DR expression on NK cells may be. The expression of class II MHC proteins such as HLA-DR is usually considered to be restricted to professional antigen-presenting cells (reviewed by Chaplin[46]). However, HLA-DR expression on both NK-cell and T-cell subsets has been documented and in both these subsets, HLA-DR expression is often used as a biomarker of activation.[47–49] HLA-expressing NK cells have been shown to present antigen to trigger T-cell proliferation and interleukin-2 production.[50,51] In addition, HLA-DR-expressing NK cells have significantly stronger cytolytic activity in comparison to other NK cells.[49] Evans *et al.*[49] demonstrate that the magnitude of an individual's NK-cell interferon-γ response triggered by the model pathogen, *Mycobacterium bovis* bacillus Calmette–Guérin, was associated with the initial proportion of HLA-DR-expressing NK cells in peripheral blood mononuclear cells. Hence, HLA-DR-expressing NK cells appear to play an important role in differences in individual responses to bacillus Calmette–Guérin and potentially other pathogens. Although SAA individuals appear to have a larger proportion of activated $CD4^+$ T cells in comparison to SAC individuals, and therefore potentially increased risk of acquiring HIV-1 if exposed, we hypothesize that the concomitant higher NK-cell activation may counteract this potential risk either entirely or to some extent through enhanced killing of activated/infected $CD4^+$ T cells.

Across all subsets examined, the influence of *CCR5Δ32* on CCR5 expression was most notable in the T-cell subsets. We observed individuals heterozygous for the *CCR5Δ32* allele to have significantly lower percentages of CCR5-expressing $CD4^+$ T cells in comparison to CCR5 'wild-type' individuals, an observation which is in agreement with previous studies.[14,52,53] SAC individuals bearing this allele had significantly reduced receptor density on T-cell ($CD4^+$ and $CD8^+$ subsets) and $CD56^{bright}$ NK-subsets. Interestingly, the statistical relationships between CCR5 expression of *CCR5Δ32* and 'wild-type' individuals were stronger when analysing CCR5 density than when analysing the percentage of CCR5-expressing cells. Similar results have been reported by Shalekoff and Tiemessen.[53] Although *CCR5Δ32* heterozygosity does have an impact on CCR5 expression, this was not observed across all subsets and furthermore, in agreement with previous studies, some 'wild-type' individuals lacking *CCR5Δ32* had CCR5 densities comparable to individuals bearing this allele.[14,52,53] Consequently, although a significant factor, heterozygosity for the *CCR5Δ32* allele is not the only determinant of CCR5 cell surface density.

Although several studies have described differences in CCR5 expression levels between individuals, these have been mainly restricted to a single lymphocyte population (eg. $CD4^+$ T cells), and have mainly addressed only the role of CCR5 as co-receptor for entry of HIV-1 into CD4 T cells. Importantly, we should not overlook that CCR5 also plays a role in T-cell immunity.[54,55] In this study we analysed CCR5 expression on T cells, NK cells, B cells and monocytes. Although HIV-1 mainly infects $CD4^+$ T lymphocytes, it is known to have a broad cellular host range infecting a number of different cell populations of the brain, bowel, heart, kidney and liver in addition to haematopoietic cells.[56] Aside from the ability of HIV-1 to infect cell types other than $CD4^+$ T cells, the role of differential CCR5 expression on other cell types and their role in the immune response on initial encounter with HIV-1, or in existing chronic infection, need to be better understood to establish the overall impact of differing degrees of CCR5 expression *in vivo*.

We found CCR5 expression to differ substantially between SAA and SAC individuals and that these differences can be attributed to factors other than the *CCR5Δ32* allele. In addition, these two groups differ substantially in their activation levels as measured by HLA-DR. Further understanding of what contributes towards the large inter-individual and inter-population variability in CCR5 expression, and understanding the implications thereof, will assist in the design of prophylactic and

therapeutic strategies that work in conjunction with the host's immune response to HIV-1.

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Disclosures

All authors declare that there is no conflict of interest.

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