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Low-Level CD4$^+$ T Cell Activation Is Associated with Low Susceptibility to HIV-1 Infection[1]

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Different features have been associated with low susceptibility to HIV type 1 (HIV-1) infection in exposed seronegative individuals. These include genetic make-up such as homozygosity for the *CCR5-Δ32* allele and the presence of HIV-specific CTLs. We studied immune activation and immune responsiveness in relation to HIV-1 susceptibility in 42 high-risk seronegative (HRSN) participants of the Amsterdam Cohort Studies and 54 men from the same cohort who were seronegative at the moment of analysis but later became HIV seropositive. HRSN had higher naive (CD45RO CD27) CD4 and CD8 T cell numbers and lower percentages of activated (HLADR CD38, CD70) CD4 and proliferating (Ki67) CD4 and CD8 T cells, irrespective of previous episodes of sexually transmittable infections. Furthermore, whole blood cultures from HRSN showed lower lymphoproliferative responses than healthy laboratory controls. These data suggest that low levels of immune activation and low T cell responsiveness may contribute to low HIV susceptibility. ***The Journal of Immunology,*** **2005, 175: 6117–6122.**

Rare individuals have been described who remain persistently seronegative for Abs against HIV type 1 (HIV-1)[4] despite multiple high-risk sexual exposures to the virus. These high-risk seronegative (HRSN) individuals include children born from HIV-infected mothers (1–4), female sex workers in areas where HIV infection is epidemic (5–9), hemophiliacs who received HIV-1-contaminated blood preparations (10, 11), and sexual partners of HIV-1-infected individuals (12–17).

Different mechanisms may influence host susceptibility to HIV infection. Infection is generally initiated by CCR5-using HIV-1 variants and individuals whose target cells lack CCR5 expression due to a homozygous 32-bp deletion in the *CCR5* gene are highly resistant for HIV-1 infection (18–21). Heterozygosity for the *CCR5-Δ32* allele and high expression levels of the CCR5 ligand RANTES have also been associated with resistance to HIV infection (22–24). HIV-specific CTLs have been identified in many exposed seronegative individuals, suggesting a role for HIV-specific CTL responses in resistance to HIV-1 infection (5, 15, 25–30). However, these responses do not necessarily reflect protective cellular immunity from infection but may merely reflect exposure to the virus since we found similar amounts and specificity of HIV-specific CTLs in seronegative men who later became HIV-1 seropositive (31, 32). For many HRSN individuals, the underlying mechanism of resistance to HIV-1 infection remains unclear. Insight into this mechanism may help to understand the mechanism of HIV-1 transmission and to improve the development of preventive measures.

It has been shown that after vaginal exposure of rhesus macaques to SIV, the majority of infected cells in the mucosa are resting CD4 T cells (33, 34). Zhang et al. (34) proposed a model for HIV transmission where initial low-level virus replication in resting CD4 T cells sustains local infection, while spread of infection from the initial site and subsequent seroconversion depends on infection of activated CD4 T cells. Higher levels of virus production by activated CD4 T cells may increase HIV-1 transmission efficiency as is observed in the setting of pre-existing mucosal inflammation due to other sexually transmittable infections (STIs) or induced by some microbicides (35–37).

We studied the association between immune activation and susceptibility to HIV-1 infection in HRSN homosexual men from the Amsterdam Cohort Studies (ACS). We hypothesized that having low levels of activated CD4$^+$ T cells could protect against HIV-1 seroconversion, despite high-risk sexual behavior. Therefore, we compared the expression of cellular activation markers between HRSN individuals and ACS participants who were seronegative at the moment of analysis but later did become HIV-1 positive. In addition, we compared in vitro stimulatory T cell responses in fresh blood samples from HRSN individuals to those in healthy blood donor samples. Our data suggest that low levels of immune activation are associated with resistance to HIV-1 seroconversion.

Materials and Methods

Study participants

From the ACS, we selected 42 homosexual men who had an HIV-negative follow-up of at least 5 years despite unprotected receptive anal sex with at least 6 different partners and from whom cryopreserved PBMC samples were available. As a control group, we selected 54 ACS participants who seroconverted during follow-up in the same time period, from 1983 until January 1996, and from whom preseroconversion (pre-SC) cryopreserved samples were available. Pre-SC control samples were taken at least 6 mo before HIV-1 seroconversion. Plasma viral RNA loads in these samples

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[4] Abbreviations used in this paper: HIV-1, HIV type 1; HRSN, high-risk seronegative; STI, sexually transmittable infection; ACS, Amsterdam cohort studies; pre-SC, preseroconversion; IQR, interquartile range; UAR, unprotected anal receptive intercourse.

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Table I. *Risk behavior of study participants*

Subjects	*n*	Lifetime Partners at Study Entry[a]	UAR Partners (per year)[a]	HIV-Negative Follow-Up (years)[a]
HRSN	42	500 (128–1325)	3.4 (1.8–4.8)	10.5 (9.2–10.8)
Pre-SC	54	120 (30–800)	1.6 (0.4–4.1)	4.3 (1.8–5.6)

[a] Median numbers (and IQR) are depicted.

Table II. *Other infections in study participants*

Subjects	*n*	Syphilis[a]	Gonorrhea[a]	HSV-1[b]	HSV-2[b]	HHV8[b]
HRSN	42	24/42 (57%)	25/42 (59%)	33/39 (84%)	22/42 (52%)	19/42 (45%)
Pre-SC	54	16/53 (30%)	34/53 (64%)	36/46 (78%)	29/54 (54%)	20/54 (37%)

[a] Proportion of individuals who reported a syphilis/gonorrhea episode before the moment of sampling for activation marker measurement; of one pre-SC control no syphilis/gonorrhea record was available.
[b] Proportion of individuals who tested positive for HSV-1 or HSV-2 or HHV8. For three HRSN participants and 8 pre-SC controls, no data on HSV-1 status were available.

were below the limit of detection of 1000 copies/ml plasma at 6 mo before seroconversion for all participants. There was no difference in age between the groups (HRSN individuals, $n = 42$, median: 36.1 years, interquartile range (IQR): 32.3–41.7; pre-SC controls, $n = 54$, median: 35.2 years, IQR: 29.5–40.2). Table I shows risk behavior of the study participants. History of other STIs (Table II) were self reported (syphilis and gonorrhea episodes) or measured in HSV-1, HSV-2, or HHV-8 Ab-specific ELISAs (38, 39). In addition to cryopreserved samples from HRSN participants and pre-SC controls for FACS analyses, we used fresh blood samples from 10 HRSN participants from whom updated contact addresses were available and who were willing to donate fresh blood. The HIV-seronegative status of HRSN individuals was confirmed using a standard HIV Ab test (Vidas HIV DUO; bioMerieux). Fresh blood samples of 193 healthy blood donors with no reported risk behavior served as controls.

This study was approved by the medical ethical committee of the Academic Medical Centre Amsterdam (AMC) and informed consent was obtained from all study participants.

FACS analysis

Cryopreserved PBMC samples were thawed and stained with the following combinations of mAbs: 1) CD4 (-APC; BD Biosciences), CD8 (-PerCP; BD Biosciences), HLADR (-FITC; BD Biosciences), and CD38 (-PE; Sanquin); 2) CD4 (-PerCP; BD Biosciences), CD45RO (-APC; BD Biosciences), CCR5 (-FITC; BD Pharmingen), and CXCR4 (-PE; BD Pharmingen); 3) CD4 (-PerCP; BD Biosciences), CD8 (-FITC; BD Biosciences), biotinylated CD70 (a kind gift of Dr. R. A. W. van Lier, AMC, Amsterdam, The Netherlands), and streptavidin-APC (BD Biosciences); 4) CD4 or CD8 (-PerCP; BD Biosciences), CD45RO (-PE; BD Biosciences), biotinylated CD27 (Sanquin), and streptavidin-APC (BD Biosciences). Cells were then fixated (FACS lysing solution; BD Biosciences), permeabilized (FACS permeabilization buffer; BD Biosciences), and stained intracellular with Ki67 (-FITC; DakoCytomation). Incubations were performed for 20 min at 4°C. Analysis was performed on FACSCalibur (BD Biosciences) with CellQuest software. Absolute numbers of cell subsets were calculated using the absolute numbers of $CD3^+$ lymphocytes that were determined at the time of sample collection, before PBMC isolation and cryopreservation.

Proliferation assay

Lymphocyte proliferation was measured by [^{3}H]thymidine incorporation. Briefly, fresh whole blood samples were cultured in IMDM alone or with Abs directed against human CD3 (anti-CD3) either with or without anti-CD28, a combination of anti-CD2 and anti-CD28, or with 5 μg/ml PHA. After 3 days, [^{3}H]thymidine was added to the culture, and cellular DNA was harvested after 24 h. [^{3}H]Thymidine incorporation was counted in a 1205 Betaplate scintillation counter and corrected for T cell input using CD3 counts obtained by FACS analysis.

Statistical analyses

Mann-Whitney *U* test was used to compare groups because most data were not normally distributed. Statistical analyses were performed using SPSS software (version 11.5; SPSS).

Results

Expression of activation markers in HRSN and pre-SC samples

To examine if immune activation plays a role in the host susceptibility for HIV-1, we compared the expression of CD4 and CD8 lymphocyte activation markers between HRSN and pre-SC samples from ACS participants who became HIV-1 seropositive later during follow-up. The pre-SC control samples were chosen at least 6 mo before HIV-1 seroconversion (median: 36.7 mo, IQR: 15.5–52.4). There was no difference between the groups in the calendar period of sampling or in age.

The absolute number of CD4 T cells was higher in HRSN participants than in pre-SC controls, while there was no significant difference in the number of CD8 T cells (Fig. 1*A*). The numbers of

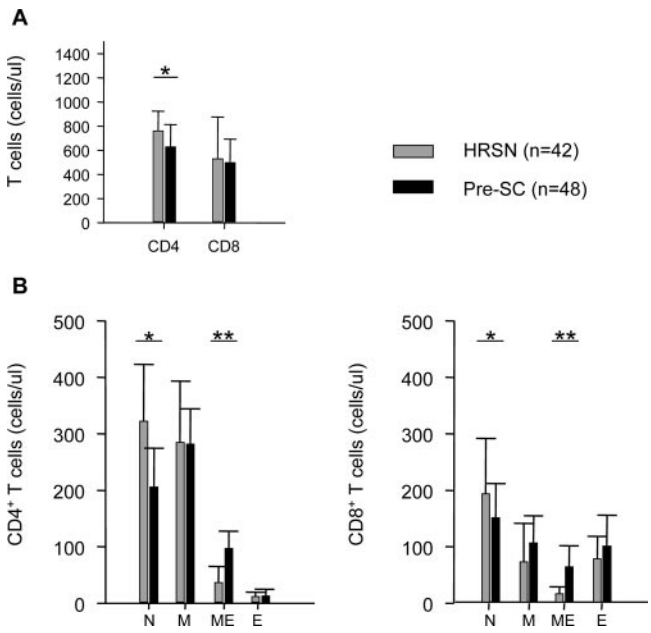

FIGURE 1. CD4 and CD8 T cell numbers. *A*, Median numbers of $CD4^+$ and $CD8^+$ T cells of HRSN participants (▨) and pre-SC controls (■). *B*, Median numbers of $CD4^+$ (*left panel*) and $CD8^+$ (*right panel*) T cell subsets of HRSN participants and pre-SC controls. N, naive ($CD45RO^-CD27^+$); M, memory ($CD45RO^+CD27^+$); ME, memory-effector ($CD45RO^+CD27^-$); and E, effector ($CD45RO^-CD27^-$). Error bars indicate interquartile range. *, $p < 0.05$; **, $p < 0.001$.

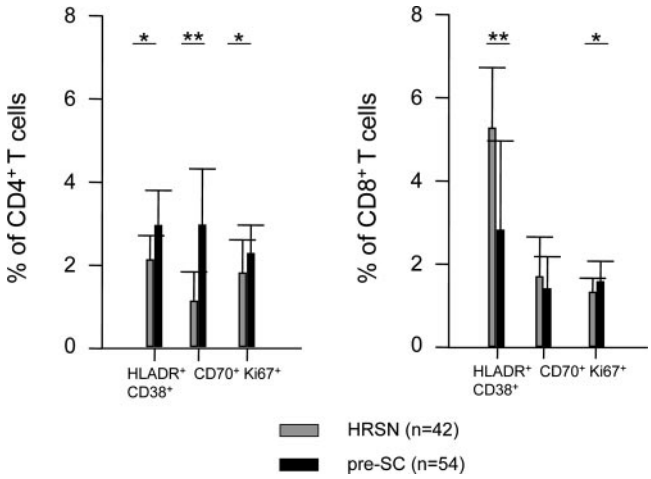

FIGURE 2. Expression of activation markers on $CD4^+$ and $CD8^+$ T cells. Median percentages of $HLADR^+/CD38^+$, $CD70^+$, and $Ki67^+$ cells within the $CD4^+$ (*left panel*) and $CD8^+$ (*right panel*) T cells of HRSN participants (▨) and pre-SC controls (■). Error bars indicate interquartile range. *, $p < 0.05$; **, $p < 0.001$.

naive ($CD45RO^-CD27^+$) CD4 and CD8 T cells were higher in HRSN participants compared with pre-SC controls, and the numbers of memory effector ($CD45RO^+CD27^-$) CD4 and CD8 T cells were lower (Fig. 1*B*). The percentages of proliferating ($Ki67^+$) CD4 and CD8 T cells and of activated ($HLADR^+CD38^+$ and $CD70^+$) CD4 T cells were lower, but the percentage of activated ($HLADR^+CD38^+$) CD8 T cells was significantly higher in the HRSN participants than in pre-SC controls (Fig. 2). Also, when CD38 expression was analyzed independent of HLADR, we found higher percentages of $CD8^+$ CD38-expressing cells in HRSN participants than pre-SC controls ($p < 0.001$; data not shown). No differences were found in CD38 expression on $CD4^+$ T cells (data not shown).

Analysis of the expression of HIV-1 coreceptors CCR5 and CXCR4 on CD4 T cells showed that HRSN participants had lower percentages of CXCR4-expressing CD4 T cells compared with pre-SC controls, also within the $CD45RO^+$ subset (Table III). The percentage of CCR5-expressing CD4 T cells was not significantly different between HRSN individuals and pre-SC controls, but within the CD4 $CD45RO^+$ T cells, a significantly lower percentage of $CCR5^+$ cells was found in HRSN individuals. Because of higher CD4 T cell numbers in HRSN participants, no statistically significant differences between absolute numbers of $CXCR4^+$ or $CCR5^+$ CD4 T cells were found between HRSN and pre-SC samples.

Expression of activation markers in relation to STI occurrence

To examine whether the differences found in the expression levels of activation markers might be explained by differences in risk behavior or associated with the occurrence of STIs, we determined the number of lifetime partners, the number of different unprotected anal receptive intercourse partners, and the occurrence of syphilis, gonorrhea, HSV-1, HSV-2, and HHV8 in HRSN individuals and pre-SC controls. As expected, HRSN participants reported higher risk behavior (Table I), and a higher proportion of HRSN participants had experienced a syphilis episode compared with the pre-SC controls (Table II). HRSN individuals and pre-SC controls were indifferent for infections with gonorrhea, HSV-1 and HSV-2, and HHV8 (Table II). Comparing individuals who had and who had not reported a syphilis and/or gonorrhea episode, we found that pre-SC controls who reported at least one such episode in their lifetime had a statistically significant higher percentage of both $CD70^+$ and $Ki67^+$ CD4 T cells compared with pre-SC controls who never reported a syphilis or gonorrhea episode (Fig. 3). Interestingly, no significant differences in T cell activation levels were found between HRSN individuals who did and who did not report a syphilis or gonorrhea episode. Inclusion of other STIs in this analysis resulted in too small no-STI control groups. Except for the differences mentioned above, no other recurrent trends were observed when this analysis was performed for each STI separately (data not shown).

Finally, excluding participants who did not report a syphilis episode, we found similar differences in T cell markers between HRSN individuals and pre-SC controls as reported above (data not shown). The same holds true for gonorrhea, HSV-1, HSV-2, and HHV8, confirming that the lower T cell activation levels found in HRSN participants compared with pre-SC controls are not explained by differences in STI history.

In vitro proliferation

The observation that HRSN individuals have lower CD4 T cell activation levels than pre-SC controls despite high STI incidence suggests that CD4 T cells from HRSN participants are less responsive to Ag exposure than T cells from pre-SC controls. To test whether HRSN individuals also have low lymphoproliferative responses compared with healthy blood donor controls, we collected fresh blood samples from the HRSN participants. Collected samples were tested for HIV Abs and proved seronegative.

When compared with laboratory controls, we found statistically significant lower levels of [^{3}H]thymidine incorporation in fresh whole-blood samples from HRSN participants after stimulation with PHA (HRSN ($n = 10$), median: 799 cpm/10^3 T cells, IQR: 547–997; controls ($n = 193$), median: 1294 cpm/10^3 T cells, IQR:

Table III. *HIV coreceptor expression on $CD4^+$ T cells*

$CD4^+$ T Cells	HRSN			Pre-SC			p Value[a]
	n	Median	IQR	n	Median	IQR	
%$CCR5^+$	42	10.6	8.2–14.5	51	10.1	8.4–17.0	0.717
%$CCR5^+$ of $CD45RO^+$	42	18.0	15.9–22.7	50	24.5	16.5–29.7	**0.008**
%$CXCR4^+$	42	80.4	71.9–88.6	51	91.7	88.0–95.2	**<0.001**
%$CXCR4^+$ of $CD45RO^+$	42	68.4	58.7–74.6	50	81.7	73.7–87.6	**<0.001**
$CCR5^+$ (cells/μl)	42	89	61–108	46	75	52–117	0.452
$CCR5^+$ $CD45RO^+$ (cells/μl)	42	69	45–98	45	77	51–113	0.405
$CXCR4^+$ (cells/μl)	42	556	460–784	46	540	462–725	0.559
$CXCR4^+$ $CD45RO^+$ (cells/μl)	42	247	168–342	45	241	51–113	0.872

[a] Mann-Whitney U test; values of $p < 0.05$ are depicted in bold type.

1037–1600; $p < 0.001$) and after CD3/CD28 stimulation (HRSN ($n = 10$), median: 1072 cpm/10^3 T cells, IQR: 727-1393; controls ($n = 193$), median: 1324 cpm/10^3 T cells, IQR: 1039–1556; $p < 0.05$) (Fig. 4). Also, after stimulation of CD3 only or CD2/CD28, responses in HRSN whole-blood samples were lower than controls, but these differences were not statistically significant. Remarkably, also the spontaneous ex vivo lymphoproliferation (without any stimulatory agent added) was lower in HRSN than in control samples (HRSN ($n = 10$), median: 1.28 cpm/10^3 T cells, IQR: 0.50–2.02; controls ($n = 182$), median: 1.91 cpm/10^3 T cells, IQR: 1.21–2.86; $p < 0.05$) (Fig. 4).

Discussion

Immune activation may play a role in HIV-1 transmission efficiency. Zhang et al. (34) have shown that at the site of SIV infection, mostly resting CD4 T cells are infected. They hypothesized that initial low-level replication in these resting CD4 T cells sustains local infection but that only higher levels of virus production by activated CD4 T cells may enable spread of infection from the initial site throughout the lymphatic tissue. High levels of activated CD4 T cells at the site of HIV-1 exposure may increase HIV-1 transmission efficiency while low levels of activated CD4 T cells may be associated with a persistent HIV-seronegative status despite high-risk sexual behavior.

To test this hypothesis, we examined whether low expression levels of cellular activation markers correlated with persistent HIV-seronegative status despite high-risk sexual behavior. Indeed, HRSN participants showed lower levels of CD4 T cell activation ($HLADR^+CD38^+$ and $CD70^+$) and CD4 and CD8 T cell proliferation ($Ki67^+$) compared with pre-SC controls, suggesting lower levels of activated CD4 target cells. Differences in activation levels were significant but small, which does not exclude a relevant biological effect. Whether the differences in CD4 cell activation level are indeed mechanistically relevant for HIV susceptibility remains to be established.

HRSN samples did show higher percentages of $HLADR^+$ $CD38^+$ CD8 T cells compared with pre-SC controls. Differences in CD38 and/or HLA-DR expression on CD4 and CD8 T cells have been reported before (17) and may be indicative of an active, CD4-independent, CD8 immune response, possibly, but not necessarily, directed against HIV-1. Nevertheless, we and others (31, 32, 40) have shown that the detection of HIV-1-specific CTLs is not necessarily associated with resistance to HIV-1 infection. Excluding either participants from our analyses, who did not report a syphilis episode, a gonorrhea episode, or who tested negative for HSV-1, HSV-2, or HHV-8, gave the same results (data not shown). This suggests that the reported differences between HRSN participants and pre-SC controls are not related to differences in pathogen exposure. In fact, pre-SC controls who reported a syphilis or gonorrhea episode had elevated levels of CD4 T cell activation compared with pre-SC controls who did not report a syphilis or gonorrhea episode while this difference was not found in HRSN participants.

It has been shown in healthy individuals that increased levels of immune activation are associated with low naive T cell numbers and increased numbers of memory and effector T cells (41). HRSN individuals had higher numbers of naive T cells and lower numbers of memory-effector T cells compared with pre-SC controls despite high pathogen exposure, again suggesting that in these individuals pathogen exposure did not induce the same level of immune activation. Indeed, lymphoproliferative responses in HRSN individuals were significantly lower than in healthy controls, confirming previous results by Salkowitz et al. (11), who compared lymphoproliferative capacity of HRSN hemophiliacs with healthy blood donors. These data suggest that a less reactive immune system results in lower immune activation levels, which may lower the chance of HIV-1 seroconversion. This protective effect may be due to lower numbers of activated CD4 T cells at the site of infection that otherwise would have contributed to the spread of the infection to draining lymph nodes.

In contrast with our data, one study reported no differences in CD4 T cell numbers, elevated CD8 effector ($CD62L^-RA^+$) numbers and lower naïve, and $CD28^+$ CD8 T cells subsets in HRSN men compared with blood bank donors (17). In another study, elevated percentages of $CD38^+$ CD8 T cells but no difference in $HLADR^+CD38^+$ CD8 percentages nor in naive or memory CD8 T cell numbers were found in HIV-exposed seronegative female sex workers compared with low-risk blood donors (7). Conceivably, differences in levels of immune activation reported in these

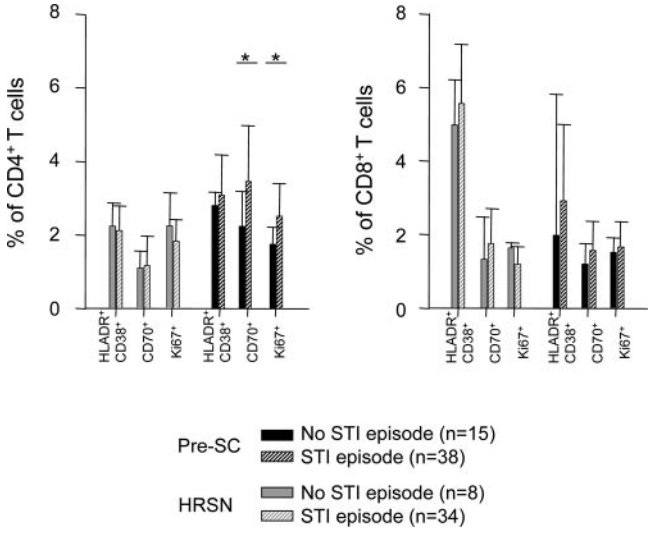

FIGURE 3. Expression of activation markers in relation to reported syphilis and gonorrhea episodes. Median percentages of $HLADR^+$/$CD38^+$, $CD70^+$, and $Ki67^+$ cells within the $CD4^+$ (*left panel*) and $CD8^+$ (*right panel*) T cells of HRSN participants (▨) and pre-SC controls (■) who did (▨) or did not (▩) report a syphilis or gonorrhea episode. Error bars indicate interquartile range. *, $p < 0.05$.

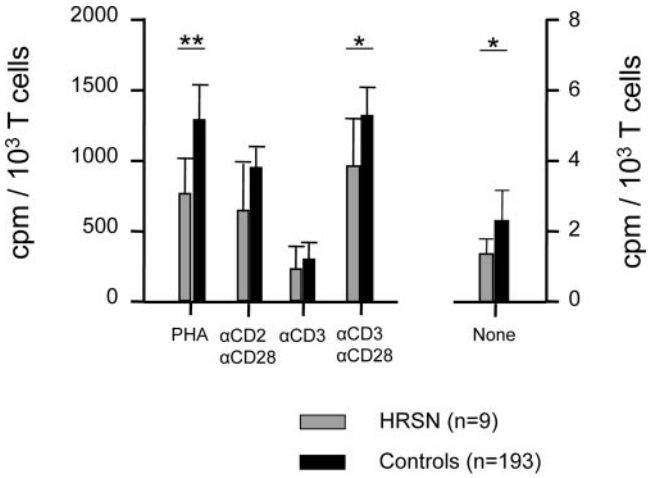

FIGURE 4. T cell proliferation capacities of HRSN participants and healthy controls. Median [^{3}H]thymidine incorporation by T cells stimulated with PHA, CD2/CD28, CD3, or CD3/CD28 mAbs and by unstimulated T cells of HRSN (▨) and blood donor controls (■). Error bars indicate interquartile range. *, $p < 0.05$; **, $p < 0.001$.

studies may reflect higher pathogen exposure in HRSN individuals compared with the low-risk blood donors that were used as controls. To be able to distinguish between effects of pathogen exposure and exposed seronegative status, in the present study we used samples taken before HIV seroconversion from a control group that consisted of participants of the same cohort study who eventually became HIV-1 seropositive.

As mentioned, other known factors may be involved in protection from HIV-1 seroconversion. In agreement with our previous observations (31) and other reports (12, 14, 16, 42), we found significantly higher RANTES production of PHA-PBMC in vitro before and after CD8 T cell depletion in HRSN individuals compared with low-risk blood donors (data not shown). In addition, because the *CCR5-Δ32* genotype has been associated with resistance to HIV infection (22, 23), we analyzed our data excluding those individuals who carried the *CCR5-Δ32* allele. One of 42 HRSN participants was homozygous for the 32-bp deletion and 3 were heterozygous, whereas 4 of 54 pre-SC controls were heterozygous for the deletion. Exclusion of these individuals from our analyses did not change our findings (data not shown).

As compared with the pre-SC samples, HRSN participants showed a significantly lower proportion of CXCR4-expressing $CD4^+$ T cells both in the CD45RO-negative and CD45RO-positive subsets and a lower CCR5 expression only in the CD45RO-positive subset. In agreement, reduced CXCR4 expression has been associated with duration of commercial sex work in seronegative female sex workers (7). However, others have reported higher CXCR4 expression levels in exposed seronegative female sex workers as compared with low-risk blood donors (8, 43). The relevance of CXCR4 expression for HIV transmission efficiency is unclear as CCR5-using and not CXCR4-using variants are generally considered to establish new infections. Moreover, we found no difference in absolute numbers of CXCR4-expressing $CD4^+$ T cells between HRSN and preseroconverters, which makes it unlikely that CXCR4 expression is directly involved in a low HIV susceptibility.

The lower activation level of CD4 T cells in HRSN participants compared with pre-SC controls suggests a role for CD4 T cell activation in HIV-1 susceptibility. Local infection of resting CD4 T cells may occur in HRSN individuals, resulting in low HIV-1 DNA levels detected in some exposed seronegative individuals (44, 45). This would provide an explanation for the presence of HIV-1-specific CTL responses to regulatory HIV-1 proteins in many exposed seronegative individuals, including participants of our present study (28, 30, 31).

Taken together, our data suggest that HRSN individuals have less reactive CD4 T cells, which may result in low numbers of activated CD4 T cells at the site of initial infection, preventing high level HIV-1 replication and seroconversion after transmission. The underlying mechanism of this low CD4 T cell activation remains to be elucidated. To our knowledge, these individuals do not have a clinical record of frequent infections. Apparently, low CD4 T cell activation and low T cell responsiveness do not hamper efficacious responses to common pathogens as may be reflected by the selectively activated $HLADR^+CD38^+$ CD8 T cells.

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Disclosures

The authors have no financial conflict of interest.

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