Interleukin 21 (IL-21)/microRNA-29 (miR-29) axis is associated with natural resistance to HIV-1 infection

Paula A.S. Ortega[a,*], Irma Saulle[a,*], Vincenzo Mercurio[a], Salomè V. Ibba[a], Elisa M. Lori[a], Claudio Fenizia[a], Michela Masetti[a], Daria Trabattoni[a], Sergio L. Caputo[b], Francesca Vichi[b], Francesco Mazzotta[b], Mario Clerici[c,d] and Mara Biasin[a]

Background: Interleukin-21 (IL-21) modulates HIV-1 infection through the elicitation of different antiviral mechanisms, including Th17 lineage commitment and induction of microRNA (miR)-29, a miRNA endowed with anti-HIV activity. As miR-29 expression is significantly increased in HIV-1-exposed seronegative individuals (HESN), we investigated the role of miR-29/IL21 axis in the natural control of HIV-1 infection.

Methods: Peripheral blood mononuclear cells (PBMCs) isolated from 15 Italian sexually exposed HESN and 15 HIV-unexposed healthy controls were in-vitro infected with an R5-tropic HIV-1_{Ba-L} strain. Seven days post HIV-1 infection we evaluated: 1) p24 production (ELISA); 2) $CD4^+$/$IL\text{-}21^+$ and $CD4^+$/$IL\text{-}17^+$ T lymphocytes (FACS); 3) IL-17 concentration in supernatants (ELISA); and 4) IL-6, IL-17, IL-21, and miR-29a,b,c expression by $CD4^+$ T lymphocytes as well as perforin and granzyme by peripheral blood mononuclear cells (qPCR). The same analyses were performed on the 15 HIV+ partners.

Results: At baseline IL-6 expression alone was increased in HESN compared to healthy controls. Seven days after in-vitro HIV-1 infection, nevertheless, differences emerged. Thus, $CD4^+$/$IL21^+$ and $CD4^+$/$IL17^+$ T lymphocytes, as well as IL-21 and IL-17 expression and production were significantly augmented in HESN compared to healthy controls. Interestingly, IL-21 upregulation correlated with a significantly increased expression of miR-29a,b,c and a reduced susceptibility to in-vitro HIV-1 infection in HESN alone. No differences were observed in perforin and granzyme expression.

Conclusion: The IL-21/miR-29 axis is upregulated by HIV-1 infection in HESN suggesting its involvement in the natural resistance to HIV-1 infection in HESN. Approaches that exogenously increase IL-21 production or prompt preexisting cellular IL-21 reservoir could confine the magnitude of the initial HIV-1 infection.

AIDS 2018, **32**:000–000

Keywords: HIV-1-exposed seronegative individual, HIV, interleukin-21, miRNA-29

Introduction

Interleukin 21 (IL-21) is a pleiotropic cytokine composed of four α helical bundles and produced by natural killer T (NKT) cells, $CD4^+$, T lymphocytes, T follicular helper (TFH) cells and TH17 lymphocytes, with lower levels of production by several other populations of lympho-haematopoietic cells [1,2].

[a]Department of Biomedical and Clinical Sciences 'L. Sacco', University of Milan, Milan, [b]S. Maria Annunziata Hospital, Florence, [c]Don C. Gnocchi Foundation ONLUS, IRCCS, and [d]Department of Physiopathology and Transplantation, University of Milan, Milan, Italy.

Correspondence to Prof. Mara Biasin, Chair of Immunology, Department of Biomedical and Clinical Sciences 'L. Sacco', University of Milan. Via G.B.Grassi 74, 20157 Milan, Italy.

Tel: +39 0250319679; fax: +39 02 50319677; e-mail: mara.biasin@unimi.it

* Paula A.S. Ortega and Irma Saulle contributed equally to the writing of the article.

Received: 22 December 2017; revised: 1 June 2018; accepted: 2 June 2018.

DOI:10.1097/QAD.0000000000001938

The functional IL-21 receptor (IL-21R) is broadly expressed on lympho-haematopoietic populations [1]. Accordingly, IL-21 exerts its effects on a wide range of cell types. Indeed, in B cells, IL-21 stimulates immunoglobulin responses and increases the number of plasma cells [1], but it can also potently induce B cell apoptosis, a mechanism that could determine the elimination of improperly activated autoreactive B lymphocytes [2]. IL-21 also promotes the functional differentiation of several $CD4^+$ T cell subsets and stimulates $CD8^+$ T cell proliferation and functional responses, including the autocrine production of granzyme B [3]. In particular, IL-21 production by Th17 cells stabilizes and enlarges this cellular population [4] through the induction of retinoic acid receptor-related orphan receptor-γt (RORγt), a transcription factor that works as a master regulator of Th17 cells [5]. This effect results in the potentiation of IL-17 production, a proinflammatory cytokine, which effectively mediates immune response against microorganisms [6].

IL-21 has been convincingly correlated with the control of HIV-1 infection. Thus, analyses performed in different groups of HIV+ patients have shown that, whereas IL-21 production negatively correlates with viral load in the early stages of HIV-1 infection [7], IL-21 plasma levels are considerably diminished when the viral load reaches 20 000 copies/ml, at which point peripheral blood IL-21-secreting $CD4^+$ T cells are not detectable [8]. Conversely, HIV-1 infected elite controllers who do not progress to AIDS despite the absence of antiretroviral therapy, maintain normal levels of IL-21-producing $CD4^+$ T cells [9]. Notably, stimulation of HIV-1 specific $CD8^+$ T cells in the presence of IL-21 leads to enhanced degranulation and cytotoxic effector function [10]. Moreover, $CD8^+$ T cells in HIV-infected patients produce IL-21, and the frequencies of these cells are closely associated with viral control [11,12].

Recently, Adoro *et al*. reported a novel antiviral activity for IL-21 that is mediated by microRNA (miRNA or miR)-29 and results in suppressed HIV-1 infection in primary lymphoid $CD4^+$ T cells [13]. MiRNAs are small noncoding RNA sequences (about 22 nucleotides), which negatively regulate gene expression at the posttranscriptional level and repress the target messenger RNA through binding to their 3′ untranslated region (UTR) [14]. In particular, by blocking STAT3, the ability of IL-21 to induce miR-29 is suppressed. It is therefore plausible that STAT3 binds a putative regulatory site within MIR29 genes, thus acting as a positive regulator of miR-29 expression in $CD4^+$ T cells during HIV-1 infection [13].

The human miRNA-29 family of microRNAs is composed by miR-29a, miR-29b, and miR-29c [15]. MiR-29a binds its seed region to the 3′ UTR of HIV-1 mRNA and redirects it to cellular P bodies, resulting in viral mRNA degradation and suppression of translation. MiR-29 also downregulates HIV-1 Nef transcripts and Nef protein expression, which decreases HIV-1 replication [16]. Moreover, miR-29b inhibits cyclin T1 expression in resting $CD4^+$ T cells, which regulates the positive transcription elongation factor b (p-TEFb), necessary for Tat-dependent transactivation of viral gene expression [17]. Accordingly, reporter assay by transfection of miR-29 as well as ectopically expressed premiRNA results in a strong reduction of p24 levels, while knockdown of endogenous miR-29a/b led to enhanced HIV-1 infection [18]. These data suggest that the IL-21/miR-29 axis contributes to the control of HIV-1 replication and could be exploited in the design of new therapeutic strategies. Consistently with this observation, we recently showed that miR-29 is highly expressed in plasma and peripheral blood mononuclear cells (PBMCs) of HIV-1 exposed seronegative individuals (HESN), suggesting a role of this miRNA in the natural resistance to HIV-1 infection [19]. Based on these premises we performed an in-depth analysis of the possible role played by the IL-21/miR-29 axis in the natural resistance to HIV-1 infection observed in HESN.

Materials and methods

Study population

Blood samples were collected from 15 HESN and 15 HIV-1 positive individuals who are part of a serodiscordant cohort of heterosexual couples recruited at the S. Maria Annunziata Hospital in Florence, Italy, that has been followed since 1997 [20]. Fifteen healthy controls, without known risk factor for HIV-1 infection, were also included in the study. All these individuals are Italian of Caucasian origin. Inclusion criteria for HESN were a history of multiple unprotected sexual episodes for more than 4 years at the time of the enrolment, with at least 3 episodes of at-risk intercourse within 4 months prior to study entry, and an average of 30 (range, 18 to >100) reported unprotected sexual contacts per year. The HESN, HIV+, and healthy controls have similar demographic background: age (mean years ± SD: 44.5 ± 10.4 for HESN; 43.3 ± 12 for HIV+, and 46.7 ± 10.9 for healthy controls), gender (53.3, 46.6, and 45.4%, males for HESN, HIV+, and healthy control, respectively), and citizenship as well as share the same genetic background (European–Tuscan ascendancy) and the same exposure to environmental factors. The presence of any chronic disease was an exclusion criterion when the HESN and healthy control were recruited and no other pathologies were detected at sampling in any of the individuals.

In the HIV+ patients, the median (range) of $CD4^+$ cell counts were 617 cells/mL (224–1007 cells/mL), and viral loads were under the detection limit (>20 copies/mL) for

all the patients except for one (44 copies/ml). All the patients were undergoing antiretroviral (ARV) treatment at the time of the study (supplementary Table 1, http://links.lww.com/QAD/B321).

The study was designed and performed according to the Helsinki declaration and was approved by the Ethics Committee of the participating units. All subjects provided written informed consent to participate in this study.

Isolation of peripheral blood mononuclear cells and cell count

Whole blood was collected by venepuncture in Vacutainer tubes containing EDTA (Ethylene diamine tetra acetic acid) (BD Vacutainer, San Diego, California, USA). Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood by density gradient centrifugation on Ficoll (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) and washed twice in phosphate-buffered saline (PBS) (PBI, Milan, Italy). Cell count was performed with the automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Korea).

Isolation of $CD4^+$ T cells

PBMCs were cultured for two hours in treated plates at 37°C to allow the adhesion of CD4-expressing monocytes. $CD4^+$ T cells were isolated in basal conditions as well as at the end of the in-vitro HIV-1 infection assay by direct magnetic labelling using CD4 microbeads (Miltenyi Biotech, Germany) according to manufacturer's protocol. $CD4^+$ T cell purity, assessed by flow cytometry, ranged between 92% and 97%.

HIV-1 strain

The R5 tropic HIV-1_{BaL} (contributed by Drs. S. Gartner, M. Popovic and R. Gallo, courtesy of the National Institutes of Health AIDS Research and Reference Reagent Program) was used to perform in-vitro HIV-1 infection. The virus was provided through the EU programme EVA Centre for AIDS Reagents (The National Institute for Biological Standards and Control NIBSC, Potter Bars, UK).

In-vitro peripheral blood mononuclear cells HIV-1 infection

Forty $\times 10^6$ PBMCs isolated from HESN and healthy control were cultured in RPMI 1640 containing 20% fetal bovine serum, with or without 0.5 ng/1×10^6 cells HIV-$1_{Ba\text{-}L}$ virus with a cellular density of 2×10^6/ml, and incubated for 24 h at 37°C and 5% CO_2. Cells were then washed and resuspended in medium containing IL-2 (15 ng/ml) (R&D systems, Minneapolis, Minnesota, USA) and PHA (1 μg/ml) (Sigma-Aldrich, Saint Louis, Missouri, USA). Two days later, cells were washed, resuspended in complete medium with IL-2, plated in 6-well tissue culture plates and incubated at 37°C and 5% CO_2. Seven days post-HIV-1 infection 1×10^6 PBMCs were used to determine perforin and granzyme expression and to perform flow cytometry analyses; ELISA was performed on 7 days post-infection supernatants. $CD4^+$ T cells were separated from the remaining PBMCs, as previously described, to allow gene expression and miRNA analyses.

p24 ELISA

An HIV-1 p24 Elisa assay kit (XpressBio, Frederick, Maryland, USA) was used to measure viral p24 in supernatant after 7 days HIV-1 infection, according to the manufacturer's protocol. Plates were read at 450 nm, using the IMark microplate reader equipped with Microplate Manager 6 software (both from Biorad, Hercules, California, USA). The absorbance of each microplate well was calibrated against the absorbance of an HIV-1 p24 antigen standard curve. Samples with absorbance values equal to or greater than the cutoff factor were considered initially reactive and were retested in duplicate to determine whether the reactivity was reproducible. The assay limit of detection was 1.7 pg/ml.

Interleukin-17 protein concentration in supernatant

The human IL-17A High Sensitivity ELISA Kit (eBioscience, San Diego, California, USA) was used to measure interleukin-17A in 7 days post HIV-1 infection supernatant, according to the manufacturer's protocol. The limit of detection of the assay was 0.01 pg/ml.

Flow cytometry

Flow cytometric analyses were performed on 7 days post in-vitro HIV-1 infection PBMCs. 0.5×10^6 PBMCs were stained with antihuman CD4 labeled with PE-Cyanine7 (eBioscience), followed by fixation, permeabilization and incubation with antihuman IL-21 labeled with APC (Biolegend, San Diego, California, USA) and antihuman IL-17 labeled with FITC (eBioscience). At least 200 000 events were acquired in the gate of $CD4^+$ cells, using a FC500 flow cytometer (Beckman-Coulter, California, USA). AQ6

Gene expression analysis

RNA was extracted from basal and in-vitro HIV-1 infection $CD4^+$ T cells and PBMCs using the acid guanidium thiocyanate–phenol–chloroform method. RNA was dissolved in RNase-free water, and purified from genomic DNA with RNase-free DNase (RQ1 DNase, Promega, Madison, Wisconsin, USA). One microgram of RNA was reverse transcribed into first-strand cDNA in a 20-μl final volume containing 1 μmol/l random hexanucleotide primers, 1 μmol/l oligo dT and 200 U reverse transcriptase (Promega). cDNA quantification for IL-6, IL-21 and IL-17, perforin and granzyme was performed by real-time PCR using a CFX ConnectTM real-time PCR system (Bio Rad, Hercules, California, USA) and a SYBR Green PCR mix (Bio Rad). The results are presented as the media of the relative

expression units to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and beta-actin (β-actin) reference genes calculated by the $2^{-\Delta\Delta Ct}$ equation using the CFX manager 3.1 (Bio-Rad). Reactions were performed according to the following thermal profile: an initial 95°C for 15 min (denaturation) followed by 40 cycles of 15 s at 95°C (denaturation), 1 min at 60°C (annealing) and 20 s at 72°C (extension). Melting curve analysis was also performed for amplicon identification. Ct values of 35 or higher were excluded from the analyses.

MicroRNA-29a,b,c reverse transcription and real-time PCR analysis

One microgram of RNA isolated from in-vitro HIV-1 infection $CD4^+$ T cells was reverse transcribed into first-strand cDNA in a 20 μl final volume at 37°C for 60 min using miScript II RT Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Samples were amplified using the miScript SYBR Green PCR Kit with the same running protocol used for array analyses. The primers (Qiagen) were: hsa-miR-29a-3p, hsa-miR-29b-3p, hsa-miR-29c-3p. RNAU6 was used as endogenous control to normalize the relative miRNA expression as previously described by others [21–23].

Statistical analysis

The relative expression levels of miRNAs and target genes were calculated using the comparative $\Delta\Delta C_t$. The fold changes were calculated by the equation $2^{-\Delta\Delta Ct}$. Data were analyzed using Student's T or ANOVA test by GRAPHPAD PRISM version 5 (Graphpad software, La Jolla, Ca, USA), and P values of 0.05 or less were considered to be significant.

Results

Susceptibility to in-vitro HIV-1 infection is reduced in HIV-1-exposed seronegative individual

We evaluated the susceptibility of PBMCs obtained from 15 HESN and from 15 healthy controls to HIV-1_{Ba-L} infection by measuring p24 levels in supernatants seven days after in-vitro HIV-1 infection. The results confirmed a reduced susceptibility to HIV-1_{Ba-L} infection in HESN compared to healthy controls (mean ± SD of 52682 pg/ml and 92969 pg/ml, $P < 0.05$) (Fig. 1).

Increased interleukin-21 expression in in-vitro HIV-1-infected $CD4^+$ T cells from HIV-1-exposed seronegative individual

To verify if the reduced susceptibility to HIV-1 infection observed in HESN is associated with an increased production of the antiviral cytokine IL-21, the expression levels of this cytokine were evaluated in $CD4^+$ T cells, both in basal condition and 7 days post in-vitro HIV-1 infection. In basal condition, IL-21 mRNA expression

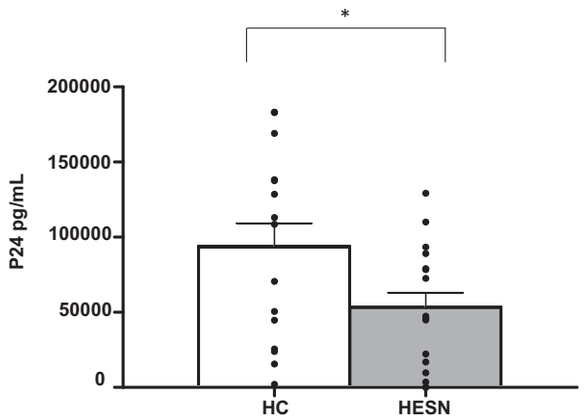

Fig. 1. HIV-1-exposed seronegative individual (HESN) peripheral blood mononuclear cells (PBMCs) were less susceptible to in-vitro HIV-1_{Ba-L} infection compared to healthy controls. PBMCs from 15 HESNs (light grey bar) and healthy controlss (white bar), were in-vitro infected with a R5 HIV-1_{Ba-L}. P24 concentration was measured by ELISA on 7 day post in-vitro HIV-1 infection supernatant. Mean values and SE are shown. $^*P < 0.05$.

levels were comparable in HESN, healthy controls and HIV+ (Fig. 2a). Notably, 7 days post in-vitro HIV-1 infection, IL-21 expression was not modulated in healthy controls $CD4^+$ T cells but in the HESN cohort it was twice as high than the uninfected conditions; these differences were statistically significant ($P < 0.05$) (Fig. 2b). Similarly, 7-days post HIV-1 infection the percentage of IL-21 producing $CD4^+$ T cells was higher in HESN in comparison with healthy controls, although these differences approached but did not reach statistical significance (Fig. 2c).

Interleukin-6 expression is increased in basal and in-vitro HIV-1-infected $CD4^+$ T cells from HIV-1-exposed seronegative individual

As the antiviral effects of IL-21 are at least partially mediated by the activation of the transcription factor STAT3 [12], we investigated the expression level of other STAT3-inducing factors. We first took into consideration IL-6, a pro-inflammatory cytokine whose expression level was shown to increase in HESN individuals following Toll-like receptor stimulation [18]. In basal condition, IL-6 expression was similar in $CD4^+$ T cells of HESN and HIV-1 individuals and was significantly higher compared to healthy controls ($P < 0.05$ in both comparisons) (Fig. 3a). These results suggest that IL-6 expression is elevated in HESN even in the absence of an overt HIV-1 infection and could be a key element in the natural resistance to HIV-1. In support of this hypothesis, these differences were maintained even after in-vitro HIV-1 infection as IL-6 expression was significantly higher in HESN compared to healthy controls ($P < 0.05$) (Fig. 3b).

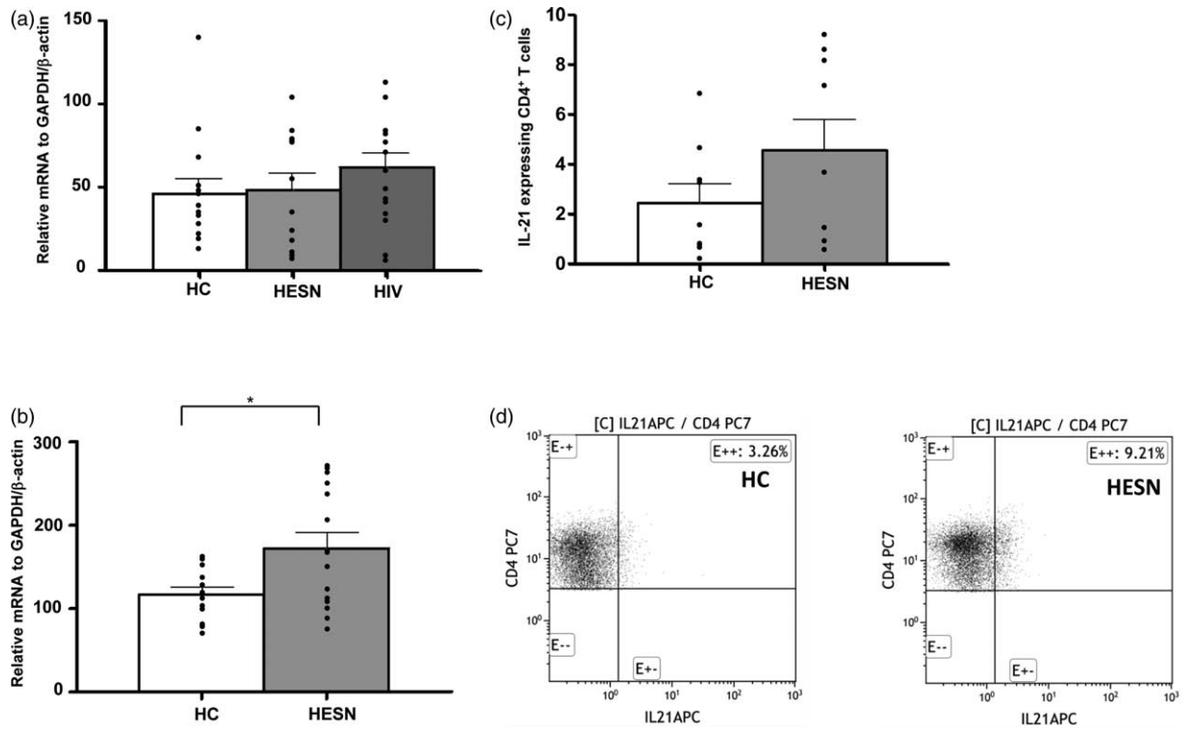

Fig. 2. Interleukin-21 (IL-21) expression was higher in HIV-1 infected CD4$^+$ T cells from HIV-1-exposed seronegative individual (HESN). (a) IL-21 mRNA expression in unstimulated (baseline) CD4$^+$ T cells isolated from healthy controls (white bars), HESN (light grey bars) and HIV+ (grey bars). (b) IL-21 mRNA expression in CD4$^+$ T cells magnetically isolated from in-vitro HIV-1 infected healthy controls (white bars) and HESN (light grey bars) peripheral blood mononuclear cells (PBMCs). Analyses were performed 7 days post in-vitro HIV-1 infection. (c) Percentage of IL-21-expressing CD4$^+$ T cells from healthy controls (white bars) and HESN (light grey bars) 7 days post in-vitro HIV-1 infection. (d) Dot-plots of one healthy controls and one HESN representative of their groups. Mean values and SE are shown. $^*P < 0.05$.

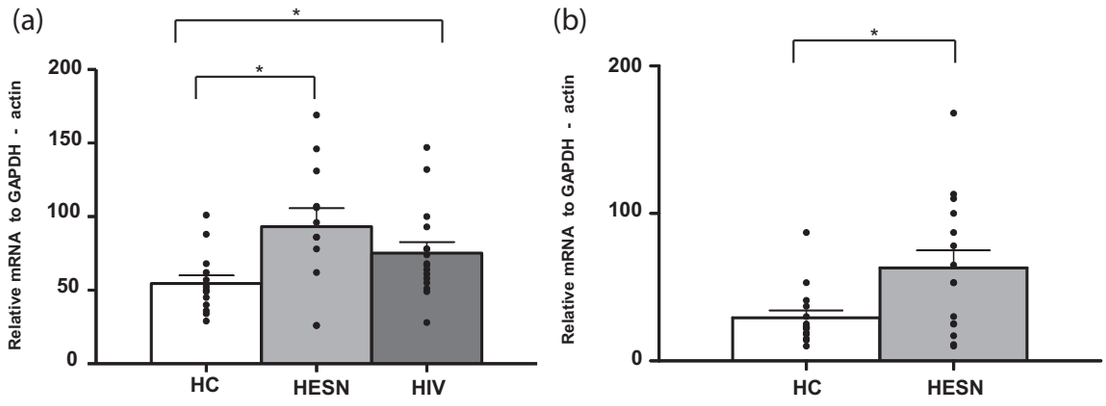

Fig. 3. Interleukin-6 (IL-6) expression was higher in basal and in-vitro HIV-1 infected CD4$^+$ T cells from HIV-1-exposed seronegative individual (HESN). A) IL-6 mRNA expression in unstimulated (baseline) CD4$^+$ T cells isolated from healthy controls (white bars), HESN (light grey bars) and HIV+ (grey bars). B) IL-6 mRNA expression in CD4$^+$ T cells magnetically isolated from in-vitro HIV-1 infected healthy controls (white bars) and HESN (light grey bars) peripheral blood mononuclear cells (PBMCs). Analyses were performed 7-days post in-vitro HIV-1 infection. Mean values and S.E. are shown. $^*= P < 0.05$.

IL-17 expression in CD4$^+$ T cells from HIV-1-exposed seronegative individual is augmented following in-vitro HIV-1 infection

IL-21 production by TH17 cells stabilizes and enlarges this cellular population, we therefore analysed whether the observed increases in IL-21 production in HIV-1-infected HESN CD4$^+$ T cells could result in an augmented production of other Th17-produced cytokines. Results showed this to be the case as IL-17 mRNA and protein expression were increased in HESN compared to healthy controls, although these differences did not reach statistical significance (Fig. 4a and b). Notably, even IL-17-producing CD4$^+$ T cells were significantly increased in HESN compared to healthy controls following in-vitro HIV-1 infection (HESN vs. healthy controls: $P < 0.04$) (Fig. 4c).

MiRNA-29 (miR-29) family expression is increased in CD4$^+$ T cells from HIV-1-exposed seronegative individual following in-vitro HIV-1 infection

IL-21 induces the expression of the miR-29 family members to limit HIV-1 replication. We, therefore, verified if the early anti-HIV-1 response promoted by IL-21 in HESN, is mediated by the up-regulation of the HIV-restricted miR-29 family. Interestingly, 7-days post in-vitro HIV-1 infection, the expression of all the miR-29 members was significantly increased in CD4$^+$ T cells from HESN compared to healthy controls (miR-29a, miR-29b, and miR-29c: $P < 0.05$) (Fig. 5).

Perforin and Granzyme expression was comparable in HIV-1-exposed seronegative individual and healthy controls both in basal and in-vitro HIV-1 infected peripheral blood mononuclear cells

Ex-vivo assays suggested, that IL-21 can stimulate perforin and granzyme expression in HIV-1-specific cytotoxic T cells. Therefore, we quantified perforin and granzyme expression in both basal and 7-days post in-vitro HIV-1 infected PBMCs. However, no differences were observed either in unstimulated (Supplementary Figure 1A, http://links.lww.com/QAD/B321) or in

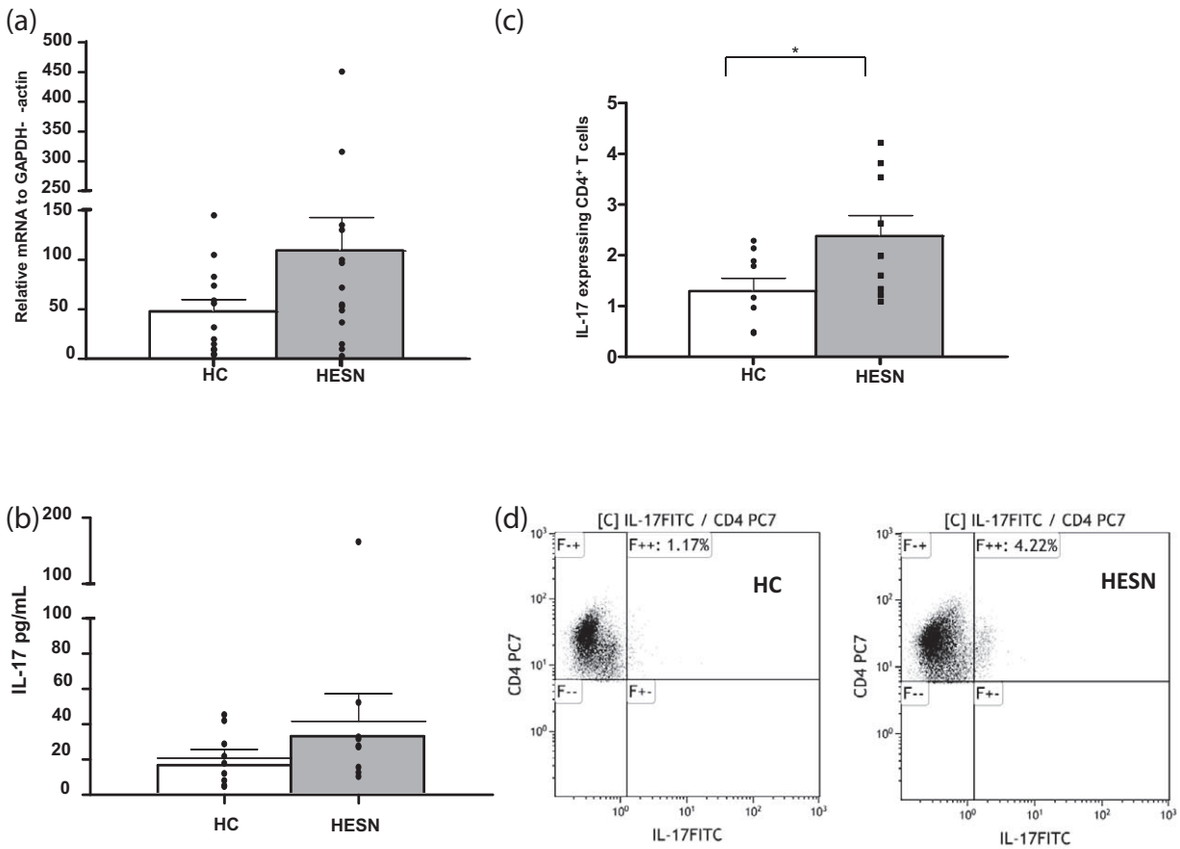

Fig. 4. IL-17 expression was increased in in-vitro HIV-1 infected CD4$^+$ T cells from HIV-1-exposed seronegative individual (HESN). (a) IL-17 mRNA expression in CD4$^+$ T cells magnetically isolated from in-vitro HIV-1 infected healthy controls (white bars) and HESN (light grey bars) PBMCs. Analyses were performed 7-days post in-vitro HIV-1 infection. B) IL-17 protein expression detected in supernatant from 7-day post in-vitro HIV-1 infected PBMCs from healthy controls (white bars) and HESN (light grey bars). C) Percentage of IL-17-expressing CD4$^+$ T cells from healthy controls (white bars) and HESN (light grey bars) in 7-day post in-vitro HIV-1 infected PBMCs. D) Dot-Plots of one healthy controls and one HESN representative of their groups. Mean values and S.E. are shown. *= $P < 0.05$.

CE: Tripti; AIDS-D-18-00025; Total nos of Pages: 11;
AIDS-D-18-00025

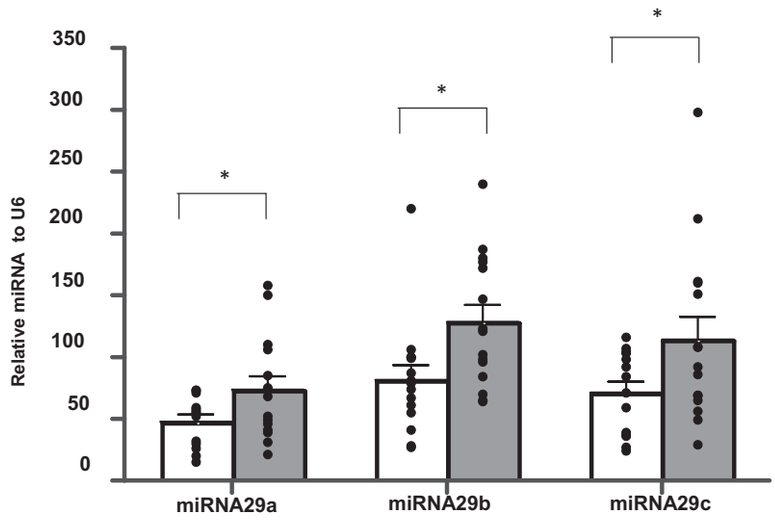

Fig. 5. MiR-29a, b, c expression was augmented in 7-days post HIV-1 infection CD4$^+$ T cells from HIV-1-exposed seronegative individual (HESN). Mir-29 a, b, c expression was analysed in CD4$^+$ T cells magnetically isolated from in-vitro HIV-1 infected peripheral blood mononuclear cells (PBMCs) from healthy controls (white bars) and HESN (light grey bars). Analyses were performed 7-days post in-vitro HIV-1 infection. Mean values and S.E. are shown. $*P < 0.05$.

HIV-1-infected PBMCs (Supplementary Figure 1B, http://links.lww.com/QAD/B321).

Discussion

The study of natural resistance to HIV-1 infection in HESN offers an opportunity to identify mechanisms of 'innate' protection that could be exploited in the setting up of preventive or therapeutic strategies. IL-21 is a potent immune modulator functionally linking innate and adaptive immunity. In the field of HIV-1-infection an association between IL-21 and progression of infection in elite controllers has already been established [10,24], and serum IL-21 levels are associated to both progression of HIV-1 infection and the response to antiretroviral therapy [12]. Nonetheless, thus far its role in the field of natural resistance to HIV-1 infection has not been investigated. In the attempt to address this issue, we analysed the expression of this cytokine in a cohort of well characterized HESN both in basal condition and in in-vitro HIV-1-infected cells. As a mixed cell culture more accurately reflects what might happen *in vivo*, we performed the HIV-1-infection assays on PBMCs isolated from HESN and healthy controls but cytokine expression was analysed only on CD4$^+$ T cells isolated from these cultures for two main reasons: they are the main producers of IL-21 as well as the main target of HIV-1.

Results showed that IL-21 expression in HESN is significantly increased following HIV-1 exposure and is accompanied by a substantial reduced viral replication. The molecular mechanisms responsible for IL-21 antiviral activity are numerous. First, IL-21 induces the expression of the cytotoxic molecules granzyme B and perforin in-vitro in CD8$^+$ T cells and NK cells of mice chronically infected with lymphocytic choriomeningitis virus (LCMV) [7,23–26]. Second, IL-21 triggers antiviral humoral response by enhancing B cell functions in both chronically SIV-infected rhesus macaques [27] and humans [2,28–30]. Third, IL-21 elicits the STAT3-dependent expression of miR-29 [13], a miRNA family which plays a crucial role in thwarting HIV-1 replication [15] and whose expression is significantly augmented in HESN PBMCs [19]. In our experimental setting, no significant difference were detected in perforin and granzyme expression in any of the analysed conditions. This result is consistent with the observation that protective virus-specific cellular responses promoted by IL-21 develops several weeks after HIV-1 exposure, therefore this protective mechanism would not be involved in the initial days after exposure [31]. Conversely, our findings show that the expression of all the miR-29 family members was significantly increased in HIV-1-infected HESN CD4$^+$ T cells, thus strengthening the association between IL-21 and miRNA-29.

Nevertheless, the mechanism responsible for IL-21 upregulation in HESN remains elusive and an in-depth

analysis of the molecular scheme through which IL-21/miR-29 axis could control susceptibility to HIV-1 infection is mandatory.

As IL-21 serves as an autocrine factor secreted by Th17 cells that promotes and sustains Th17 lineage commitment [32] we verified if IL-21 increase was paired with the release of other cytokines by this cellular subset. Notably, the percentage of IL-17-expressing $CD4^+$ T cells was significantly increased in HESN and a similar trend was observed for both IL-17 mRNA and protein expression in supernatant from 7-days post HIV-1 infected PBMCs. Based on the recent acquisition of TH17 role in HIV-1 replication [32,33]. Wacleche *et al*. reported an extremely detailed phenotyping of Th17 cells in ART-treated HIV-1-infected patients. In particular, she identified a CCR6(+) subset lacking CXCR3 and CCR4 that shares Th17 features and is a major source of IL-21 [34].The analyses of this and other Th17 cellular subsets would be essential to gain new insights in the understanding of IL-21 role in controlling susceptibility to HIV-1 infection.

IL-6 was increased as well, even in basal conditions, in HESN. This observation is interesting as IL-6 induces IL-21 production in $CD4^+$ T cells [34–36]. It is thus possible to speculate that the high production of IL-21 and, in turn, the augmented expression of miR-29 seen in HESN, could be ascribable to this cytokine. It is important to underline that, while IL-6 expression was significantly increased in HESN $CD4^+$ T cells both in unstimulated conditions and upon HIV-1 infection, many studies report a reduced expression of this cytokine as well as of IL-17 [37–39], in other HESN cohorts [40,41]. Once more, these contrasting results recall the unresolved issue of the role played by immune-activation and immune-quiescence in the HESN phenomenon. Overall the increased expression of IL-6 and IL-17 described herein support the concept that an inflammatory response is critical to contain exposure to (limited) amounts of HIV-1, hampering viral replication and dissemination, thus lowering the likelihood of infection, as previously reported [42–45]. Additionally, an inflammatory profile could boost the activation of a more vigorous adaptive antiviral immune response and might result in a virus exposure-prompted natural immune defensive phenotype against HIV-1. Though this idea is confirmed by several findings this is just one of the theories currently being investigated. A possible way to reconcile these discrepant results is the difference in the rate of HIV-1-exposure in the analysed cohorts. Sporadic contacts with the virus, as is the case of our HESN, would result in an immune activation profile; recurrent contact with HIV-1 would trigger immune quiescence, as observed in the sex workers from the Nairobi cohort [46].

Certainly, this study present some limitations, such as the reduced number of individuals enrolled. However, the observation that miR-29 expression is increased in HIV-1-infected $CD4^+$ T cells confirms previous findings on PBMCs from our HESN cohort and corroborate the possibility that these miRNAs play a role in limiting HIV-1-replication.

Furthermore, the results obtained by Paiardini's group [48] suggest that exogenous IL-21 administration, during early SIV infection, improved the maintenance of Th17 cells and the integrity of the intestinal mucosal barrier, thus reducing the onset of immune dysfunctions and chronic activation. These results support our findings and provide a plausible explanation on how IL-21 could exert a protective effect in the very first phases of HIV-1 exposure.

Taken together these findings suggest that the antiviral IL-21/miR-29 axis is one of the antiviral host-factors able to modulate susceptibility to HIV-1 infection in HESN. This being the case, approaches that exogenously increase IL-21 or prompt preexisting cellular reservoir of IL-21 might be useful in stimulating immune resistance against initial HIV-1 infection.

Acknowledgements

$HIV\text{-}1_{Ba\text{-}L}$ was provided through the EU programme EVA centre for AIDS Reagents NIBSC, UK.

M.C. and M.B, conceived the study; P.A.S.O. and I.S. wrote the paper; C.F. revised the paper; S.V.I., V.M., E.M.L., M.M. and D.T. performed the experiments and analysed the data; S.L.C., F.M. and F.V. selected and enrolled the subjects included in the study.

Conflicts of interest

This project was supported by Fondazione Banca del monte di Lombardia FBML (FON_NAZ17CFENI_01).

The authors have no conflicts of interest.

References

1. Pène J, Gauchat JF, Lécart S, Drouet E, Guglielmi P, Boulay V, *et al*. **Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells.** *J Immunol Baltim Md 1950* 2004; **172**:5154–5157.
2. Avery DT, Bryant VL, Ma CS, Malefyt R, de W, Tangye SG. **IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4.** *J Immunol* 2008; **181**:1767–1779.
3. Mittal A, Murugaiyan G, Beynon V, Hu D, Weiner HL. **IL-27 induction of IL-21 from human $CD8^+$ T cells induces granzyme B in an autocrine manner.** *Immunol Cell Biol* 2012; **90**:831–835.

4. Mehta DS, Wurster AL, Whitters MJ, Young DA, Collins M, Grusby MJ. **IL-21 induces the apoptosis of resting and activated primary B cells.** *J Immunol* 2003; **170**:4111–4118.
5. Korn T, Bettelli E, Gao W, Awasthi A, Jäger A, Strom TB, *et al*. **IL-21 initiates an alternative pathway to induce proinflammatory TH17 cells.** *Nature* 2007; **448**:484–487.
6. Veldhoen M. **Interleukin 17 is a chief orchestrator of immunity.** *Nat Immunol* 2017; **18**:612.
7. Elsaesser H, Sauer K, Brooks DG. **IL-21 is required to control chronic viral infection.** *Science* 2009; **324**:1569–1572.
8. Iannello A, Boulassel M-R, Samarani S, Debbeche O, Tremblay C, Toma E, *et al*. **Dynamics and consequences of IL-21 production in HIV-infected individuals: a longitudinal and cross-sectional study.** *J Immunol* 2010; **184**:114–126.
9. Chevalier MF, Jülg B, Pyo A, Flanders M, Ranasinghe S, Soghoian DZ, *et al*. **HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T Cell Function.** *J Virol* 2011; **85**:733–741.
10. Leonard WJ. **Cytokines and immunodeficiency diseases.** *Nat Rev Immunol* 2001; **1**:200.
11. Williams LD, Bansal A, Sabbaj S, Heath SL, Song W, Tang J, *et al*. **Interleukin-21-producing HIV-1-specific CD8 T cells are preferentially seen in elite controllers.** *J Virol* 2011; **85**:2316–2324.
12. Tian Y, Zajac AJ. **IL-21 and T cell differentiation: consider the context.** *Trends Immunol* 2016; **37**:557–568.
13. Adoro S, Cubillos-Ruiz JR, Chen X, Deruaz M, Vrbanac VD, Song M, *et al*. **IL-21 induces antiviral microRNA-29 in CD4 T cells to limit HIV-1 infection.** *Nat Commun* 2015; **6**:7562.
14. Bartel DP, Chen C-Z. **Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs.** *Nat Rev Genet* 2004; **5**:396.
15. Ahluwalia JK, Khan SZ, Soni K, Rawat P, Gupta A, Hariharan M, *et al*. **Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication.** *Retrovirology* 2008; **5**:117.
16. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. **The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury.** *Physiol Genomics* 2012; **44**:237–244.
17. Chiang K, Sung T-L, Rice AP. **Regulation of cyclin T1 and HIV-1 replication by MicroRNAs in resting CD4+ T lymphocytes.** *J Virol* 2012; **86**:3244–3252.
18. Sun G, Li H, Wu X, Covarrubias M, Scherer L, Meinking K, *et al*. **Interplay between HIV-1 infection and host microRNAs.** *Nucleic Acids Res* 2012; **40**:2181–2196.
19. Yahyaei S, Biasin M, Saulle I, Gnudi F, De Luca M, Tasca KI, *et al*. **Identification of a specific miRNA profile in HIV-exposed seronegative individuals.** *J Acquir Immune Defic Syndr* 2016; **73**:11–19.
20. Mazzoli S, Trabattoni D, Lo Caputo S, Piconi S, Blé C, Meacci F, *et al*. **HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals.** *Nat Med* 1997; **3**:1250–1257.
21. Sung T-L, Rice AP. **miR-198 inhibits HIV-1 gene expression and replication in monocytes and its mechanism of action appears to involve repression of cyclin T1.** *PLOS Pathog* 2009; **5**:e1000263.
22. Egaña-Gorroño L, Escribà T, Boulanger N, Guardo AC, León A, Bargalló ME, *et al*. **Differential MicroRNA expression profile between stimulated PBMCs from HIV-1 infected elite controllers and viremic progressors.** *PLOS ONE* 2014; **9**:e106360.
23. Pallikkuth S, Rogers K, Villinger F, Vaccari M, DosterII M, Franchini G, *et al*. **Interleukin-21 administration to Rhesus macaques chronically infected with Simian Immunodeficiency Virus increases cytotoxic effector molecules in T cells and NK cells and enhances B cell function without increasing immune activation or viral replication.** *Vaccine* 2011; **29**:9229–9238.
24. Yi JS, Du M, Zajac AJ. **A vital role For IL-21 in the control of a chronic viral infection.** *Science* 2009; **324**:1572–1576.
25. Fröhlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, *et al*. **IL-21R on T cells is critical for sustained functionality and control of chronic viral infection.** *Science* 2009; **324**:1576–1580.
26. White L, Krishnan S, Strbo N, Liu H, Kolber MA, Lichtenheld MG, *et al*. **Differential effects of IL-21 and IL-15 on perforin expression, lysosomal degranulation, and proliferation in CD8 T cells of patients with human immunodeficiency virus-1 (HIV).** *Blood* 2007; **109**:3873–3880.
27. Pallikkuth S, Kanthikeel SP, Silva SY, Fischl M, Pahwa R, Pahwa S. **Upregulation of IL-21 receptor on B cells and IL-21 secretion distinguishes novel 2009 H1N1 vaccine responders from non-responders among HIV-infected persons on combination antiretroviral therapy.** *J Immunol* 2011; **186**:6173–6181.
28. Borte S, Pan-Hammarström Q, Liu C, Sack U, Borte M, Wagner U, *et al*. **Interleukin-21 restores immunoglobulin production ex vivo in patients with common variable immunodeficiency and selective IgA deficiency.** *Blood* 2009; **114**:4089–4098.
29. Konforte D, Simard N, Paige CJ. **IL-21: an executor of B cell fate.** *J Immunol* 2009; **182**:1781–1787.
30. Streeck H, Nixon DF. **T cell immunity in acute HIV-1 infection.** *J Infect Dis* 2010; **202 (Suppl 2)**:S302–S308.
31. Wei L, Laurence A, Elias KM, O'Shea JJ. **IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner.** *J Biol Chem* 2007; **282**:34605–34610.
32. Planas D, Zhang Y, Monteiro P, Goulet JP, Gosselin A, Grandvaux N, *et al*. HIV-1 selectively targets gut-homing CCR6+CD4+ T cells via mTOR-dependent mechanisms. JCI Insight [Internet]. 2(15). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5543920/. [Accessed 29 March 2018]
33. Sun H, Kim D, Li X, Kiselinova M, Ouyang Z, Vandekerckhove L, *et al*. **Th1/17 polarization of CD4 T cells supports HIV-1 persistence during antiretroviral therapy.** *J Virol* 2015; **89**:11284–11293.
34. Wacleche VS, Goulet J-P, Gosselin A, Monteiro P, Soudeyns H, Fromentin R, *et al*. **New insights into the heterogeneity of Th17 subsets contributing to HIV-1 persistence during antiretroviral therapy.** *Retrovirology* 2016[cited 2018 Mar 29];13(1). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4995622/.
35. Suto A, Kashiwakuma D, Kagami S, Hirose K, Watanabe N, Yokote K, *et al*. **Development and characterization of IL-21–producing CD4+ T cells.** *J Exp Med* 2008; **205**:1369–1379.
36. Dienz O, Eaton SM, Bond JP, Neveu W, Moquin D, Noubade R, *et al*. **The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells.** *J Exp Med* 2009; **206**:69–78.
37. Diehl SA, Schmidlin H, Nagasawa M, Blom B, Spits H. **IL-6 triggers IL-21 production by human CD4+ T cells to drive STAT3-dependent plasma cell differentiation in B cells.** *Immunol Cell Biol* 2012; **90**:802–811.
38. Chege D, Chai Y, Huibner S, Kain T, Wachihi C, Kimani M, *et al*. **Blunted IL17/IL22 and pro-inflammatory cytokine responses in the genital tract and blood of HIV-exposed, seronegative female sex workers in Kenya.** *PLoS One [Internet]* 2012; **7**:e43670.
39. Thibodeau V, Fourcade L, Labbé A-C, Alary M, Guédou F, Poudrier J, *et al*. **Highly-exposed HIV-1 seronegative female commercial sex workers sustain in their genital mucosa increased frequencies of tolerogenic myeloid and regulatory T-cells.** *Sci Rep* 2017; **7**:43857.
40. Card CM, Ball TB, Fowke KR. **Immune quiescence: a model of protection against HIV infection.** *Retrovirology* 2013; **10**:141.
41. Lajoie J, Kimani M, Plummer FA, Nyamiobo F, Kaul R, Kimani J, *et al*. **Association of sex work with reduced activation of the mucosal immune system.** *J Infect Dis* 2014; **210**:319–329.
42. Hernandez JC, Giraldo DM, Paul S, Urcuqui-Inchima S. **Involvement of neutrophil hyporesponse and the role of toll-like receptors in human immunodeficiency virus 1 protection.** *PLOS ONE* 2015; **10**:e0119844.
43. Saulle I, Biasin M, Gnudi F, Rainone V, Ibba SV, Lo Caputo S, *et al*. **Short communication: immune activation is present in HIV-1-exposed seronegative individuals and is independent of microbial translocation.** *AIDS Res Hum Retroviruses* 2016; **32**:129–133.
44. Biasin M, Piacentini L, Caputo SL, Naddeo V, Pierotti P, Borelli M, *et al*. **TLR activation pathways in HIV-1–exposed seronegative individuals.** *J Immunol* 2010; **184**:2710–2717.
45. Tran HK, Chartier L, Troung LX, Nguyen NN, Fontanet A, Barré-Sinoussi FE, *et al*. **Systemic immune activation in HIV-1-exposed uninfected vietnamese intravascular drug users.** *AIDS Res Hum Retroviruses* 2006; **22**:255–261.

AQ8

46. Naranbhai V, Abdool Karim SS, Altfeld M, Samsunder N, Durgiah R, Sibeko S, *et al*. **Innate immune activation enhances HIV acquisition in women, diminishing the effectiveness of tenofovir microbicide gel.** *J Infect Dis* 2012; **206**:993–1001.
47. Kuebler PJ, Mehrotra ML, Shaw BI, Leadabrand KS, Milush JM, York VA, *et al*. **Persistent HIV type 1 seronegative status is associated with lower CD8+ T-cell activation.** *J Infect Dis* 2016; **213**:569–573.
48. Pallikkuth S, Micci L, Ende ZS, Iriele RI, Cervasi B, Lawson B, *et al*. **Maintenance of intestinal Th17 cells and reduced microbial translocation in SIV-infected Rhesus macaques treated with interleukin (IL)-21.** *PLoS Pathog* 2013; **9**:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3701718/. [Accessed 29 March 2018]

AQ9

Uncited reference

[47].

AQ7

QAD

Manuscript No. AIDS-D-18-00025

AIDS
Typeset by Thomson Digital
for Wolters Kluwer

Dear Author,

During the preparation of your manuscript for typesetting, some queries have arisen. These are listed below. Please check your typeset proof carefully and mark any corrections in the margin as neatly as possible or compile them as a separate list. This form should then be returned with your marked proof/ list of corrections to the Production Editor.

QUERIES: to be answered by AUTHOR/EDITOR?

QUERY NO.	QUERY DETAILS	RESPONSE
<AQ1>	Article title has been slightly modified as per style. Please review and approve.	
<AQ2>	Please confirm whether surnames/family names (red) have been identified correctly in the author byline.	
<AQ3>	Affiliations have been set as per style. Please check for accuracy of information.	
<AQ4>	Please check and confirm the corresponding author's name and relevant details for correctness.	
<AQ5>	Please check all section headings for appropriateness.	
<AQ6>	Please provide city for "Beckman-Coulter".	
<AQ7>	Please provide citation for ref 47.	
<AQ8>	References 23 and 27 are identical. Hence 23 has been removed and subsequent references have been renumbered in the text and in the bibliographic list. Please check.	
<AQ9>	Please update ref 48 with volume number and page range.	