

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

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**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<sub>Ba-L</sub> strain. Seven days post HIV-1 infection we evaluated: 1) p24 production (ELISA); 2) CD4<sup>+</sup>/IL-21<sup>+</sup> and CD4<sup>+</sup>/IL-17<sup>+</sup> 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<sup>+</sup> T lymphocytes as well as perforin and granzyme by peripheral blood mononuclear cells (qPCR). The same analyses were performed on the 15 HIV-positive 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<sup>+</sup>/IL21<sup>+</sup> and CD4<sup>+</sup>/IL17<sup>+</sup> 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.

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## Introduction

Interleukin 21 (IL-21) is a pleiotropic cytokine composed of four  $\alpha$  helical bundles and produced by natural killer T

(NKT) cells, CD4<sup>+</sup>, T lymphocytes, T follicular helper (TFH) cells and TH17 lymphocytes, with lower levels of production by several other populations of lymphohaematopoietic cells [1,2].

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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<sup>+</sup> T-cell subsets and stimulates CD8<sup>+</sup> 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- $\gamma$ t (ROR $\gamma$ t), 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-positive (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<sup>+</sup> 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<sup>+</sup> T cells [9]. Notably, stimulation of HIV-1 specific CD8<sup>+</sup> T cells in the presence of IL-21 leads to enhanced degranulation and cytotoxic effector function [10]. Moreover, CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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  $\pm$  SD: 44.5  $\pm$  10.4 for HESN; 43.3  $\pm$  12 for HIV+, and 46.7  $\pm$  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<sup>+</sup> cell counts were 617 cells/ $\mu$ l (224–1007 cells/ $\mu$ l), 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 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 ethylene diamine tetra acetic acid (EDTA) (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<sup>+</sup> T cells

PBMCs were cultured for two hours in treated plates at 37°C to allow the adhesion of CD4-expressing monocytes. CD4<sup>+</sup> 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<sup>+</sup> T-cell purity, assessed by flow cytometry, ranged between 92 and 97%.

### HIV-1 strain

The R5 tropic HIV-1<sub>BaL</sub> (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  $\times 10^6$  cells HIV-1<sub>BaL</sub> virus with a cellular density of 2  $\times 10^6$ /ml, and incubated for 24 h at 37°C and 5% CO<sub>2</sub>. Cells were then washed and resuspended in medium containing IL-2 (15 ng/ml) (R&D Systems, Minneapolis, Minnesota, USA) and PHA (1  $\mu$ g/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<sub>2</sub>. Seven days post-HIV-1 infection 1  $\times 10^6$  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<sup>+</sup> 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 Bio Rad, 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  $\times 10^6$  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<sup>+</sup> cells, using a FC500 flow cytometer (Beckman-Coulter, San Jose, California, USA).

### Gene expression analysis

RNA was extracted from basal and in-vitro HIV-1 infection CD4<sup>+</sup> 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- $\mu$ l final volume containing 1  $\mu$ mol/l random hexanucleotide primers, 1  $\mu$ mol/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 Connect<sup>TM</sup> real-time PCR system (Bio Rad) 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 ( $\beta$ -actin)

reference genes calculated by the  $2^{-\Delta\Delta C_t}$  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<sup>+</sup> 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  $2^{-\Delta\Delta C_t}$ . Data were analyzed using Student's *t* or ANOVA test by GRAPHPAD PRISM version 5 (Graphpad Software, La Jolla, California, USA), and *P* values of 0.05 or less were considered to be significant.

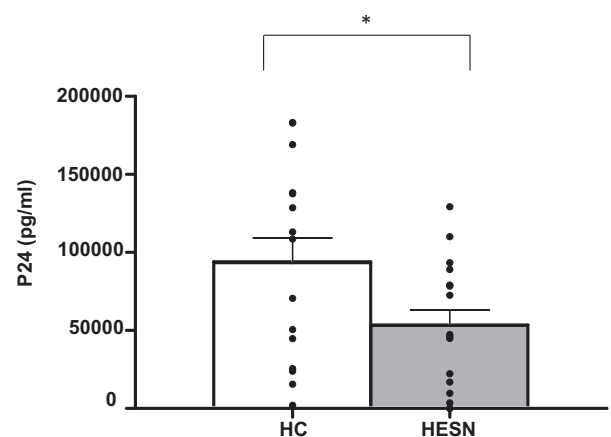
## Results

### 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<sub>Ba-L</sub> infection by measuring p24 levels in supernatants seven days after in-vitro HIV-1 infection. The results confirmed a reduced susceptibility to HIV-1<sub>Ba-L</sub> 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<sup>+</sup> 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<sup>+</sup> T cells, both in basal condition and 7 days post in-vitro HIV-1 infection. In basal condition, IL-21 mRNA expression 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<sup>+</sup> T cells but in the HESN cohort it was



**Fig. 1. HIV-1-exposed seronegative individual (HESN) peripheral blood mononuclear cells (PBMCs) were less susceptible to in-vitro HIV-1<sub>Ba-L</sub> infection compared to healthy controls.** PBMCs from 15 HESNs (light grey bar) and healthy controls (white bar), were in-vitro infected with a R5 HIV-1<sub>Ba-L</sub>. 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.

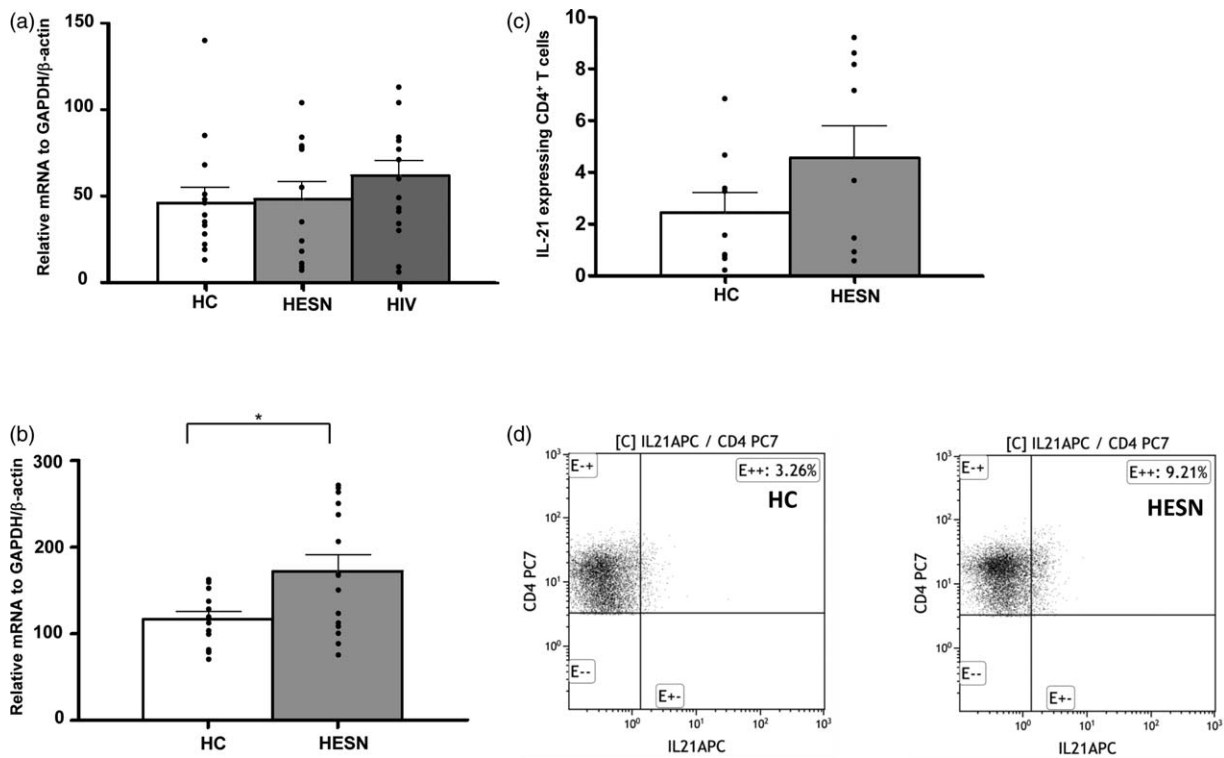
twice as high than the uninfected conditions; these differences were statistically significant (*P* < 0.05) (Fig. 2b). Similarly, 7-days post HIV-1 infection the percentage of IL-21 producing CD4<sup>+</sup> T cells was higher in HESN in comparison with healthy controls, although these differences approached but did not reach statistical significance (Fig. 2c).

### Interleukin-6 expression is increased in basal and in-vitro HIV-1-infected CD4<sup>+</sup> T cells from HIV-1-exposed seronegative individual

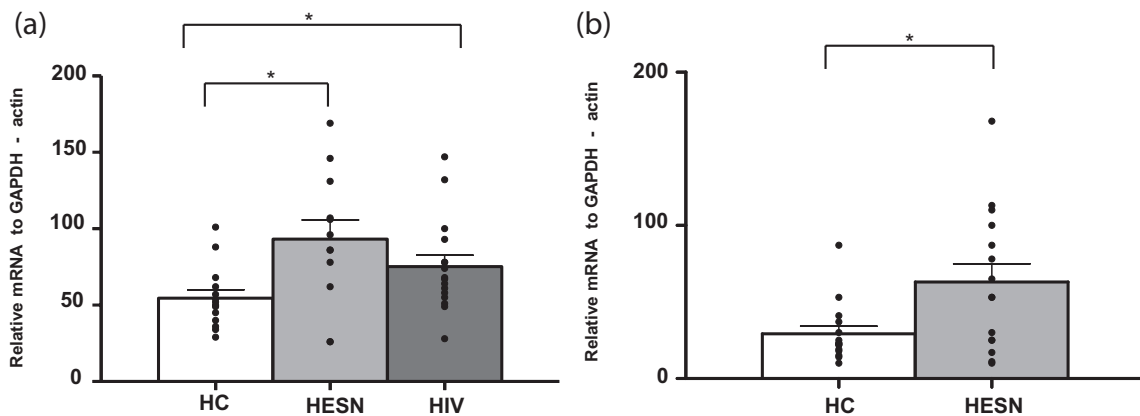
As the antiviral effects of IL-21 are at least partially mediated by the activation of the transcription factor STAT3 [12], we investigated the expression level of other STAT3-inducing factors. We first took into consideration IL-6, a pro-inflammatory cytokine whose expression level was shown to increase in HESN individuals following Toll-like receptor stimulation [18]. In basal condition, IL-6 expression was similar in CD4<sup>+</sup> 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).

### IL-17 expression in CD4<sup>+</sup> 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



**Fig. 2. Interleukin-21 (IL-21) expression was higher in HIV-1 infected CD4<sup>+</sup> T cells from HIV-1-exposed seronegative individual (HESN).** (a) IL-21 mRNA expression in unstimulated (baseline) CD4<sup>+</sup> T cells isolated from healthy controls (white bars), HESN (light grey bars) and HIV+ (grey bars). (b) IL-21 mRNA expression in CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells from HIV-1-exposed seronegative individual (HESN).** A) IL-6 mRNA expression in unstimulated (baseline) CD4<sup>+</sup> T cells isolated from healthy controls (white bars), HESN (light grey bars) and HIV+ (grey bars). B) IL-6 mRNA expression in CD4<sup>+</sup> 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.

the observed increases in IL-21 production in HIV-1-infected HESN CD4<sup>+</sup> 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<sup>+</sup> 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).

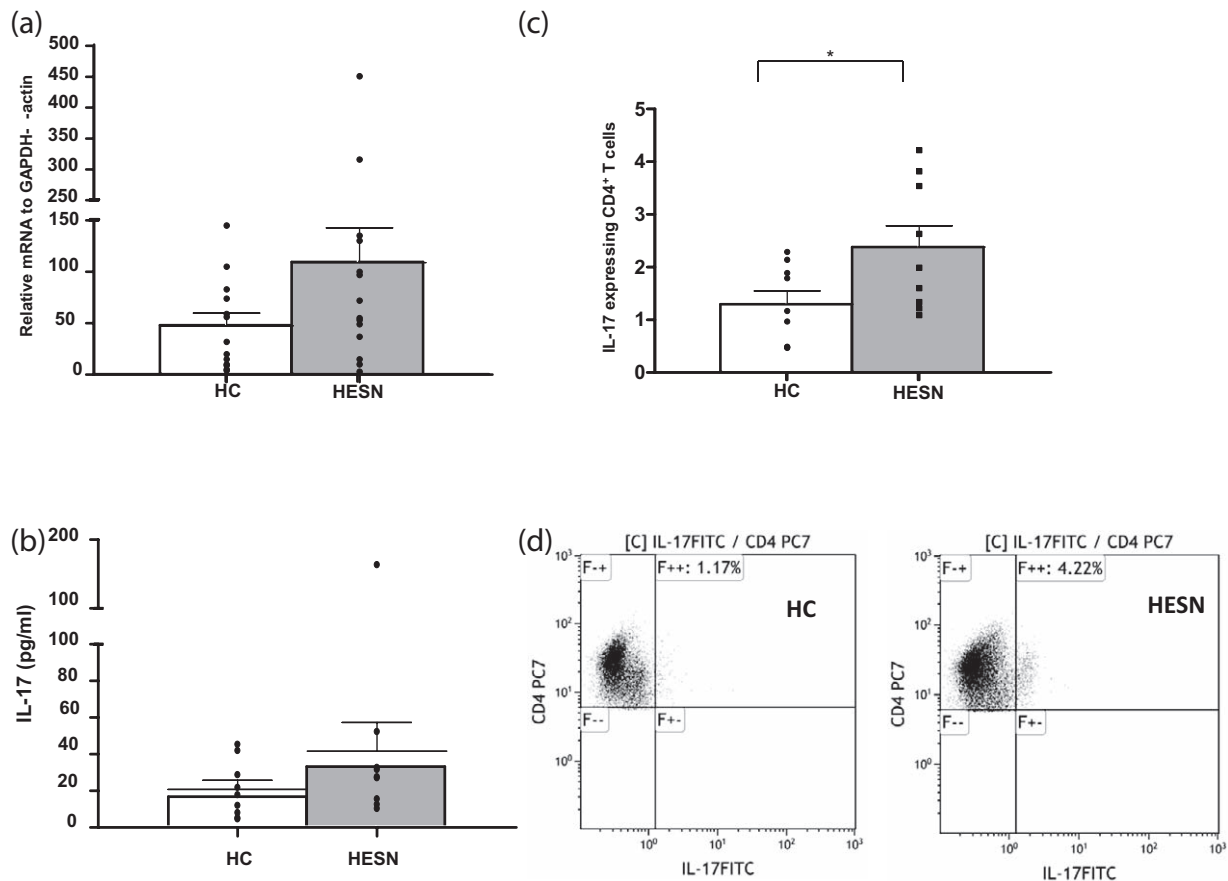
### MiRNA-29 (miR-29) family expression is increased in CD4<sup>+</sup> T cells from HIV-1-exposed seronegative individual following in-vitro HIV-1 infection

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

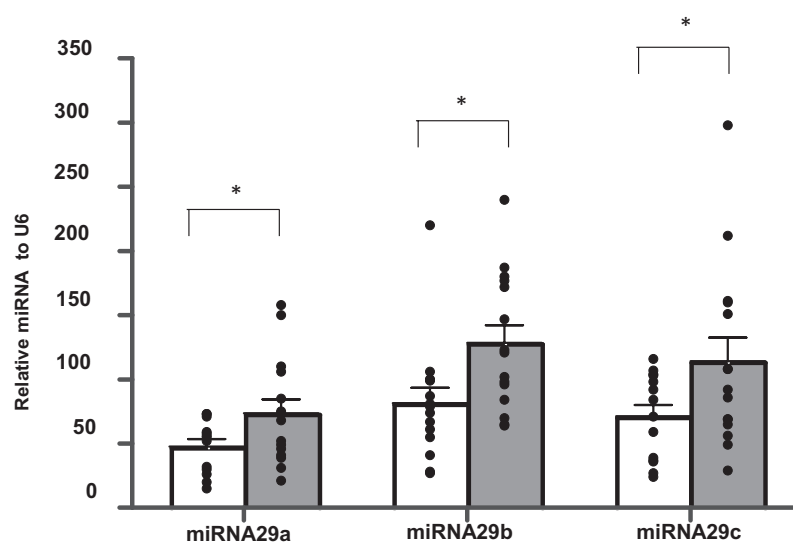
HIV-restricted miR-29 family. Interestingly, 7-days post in-vitro HIV-1 infection, the expression of all the miR-29 members was significantly increased in CD4<sup>+</sup> T cells from HESN compared to healthy controls (miR-29a, miR-29b, and miR-29c:  $P < 0.05$ ) (Fig. 5).

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



**Fig. 4. IL-17 expression was increased in in-vitro HIV-1 infected CD4<sup>+</sup> T cells from HIV-1-exposed seronegative individual (HESN).** (a) IL-17 mRNA expression in CD4<sup>+</sup> 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<sup>+</sup> 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. \* =  $P < 0.05$ .



**Fig. 5. MiR-29a, b, c expression was augmented in 7-days post HIV-1 infection CD4<sup>+</sup> T cells from HIV-1-exposed seronegative individual (HESN).** MiR-29 a, b, c expression was analysed in CD4<sup>+</sup> 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. \* $P < 0.05$ .

## 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 [46,47].

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<sup>+</sup> 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.

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## Conflicts of interest

The authors have no conflicts of interest.

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