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Impaired interleukin-5 (IL-5) production by T cells as a prognostic marker of disease progression in human immunodeficiency virus type 1 (HIV-1)-infected children

Abstract

We studied cytokine production by stimulated peripheral blood mononuclear cells from 55 HIV-infected children born to HIV-infected mothers, and compared it to that of exposed, but uninfected, age-matched children. Cytokine production was quantified using a commercially available specific ELISA kit. Cell

proliferation was evaluated by incorporation of (3H) thymidine. A significant defect in type 1 cytokine production of IFN-g and IL-2 in HIV-infected children compared to controls was observed, but without a concomitant increase in type 2 cytokines. Indeed, IL-5 production was even lower in HIV-infected children than in controls, the IL-5 decrease being the best predictive marker of immunodeficiency. Furthermore, IL-5 levels were decreased from the early phases of HIV infection, being significantly lower in the clinical category B with respect to controls, and in AIDS with respect to both controls and children in category A. Such a strong correlation with the stage of infection has not been previously described in HIV-infected children. In addition, we found a correlation between SI/X4 viral phenotype and lower IL-5 levels. Our data suggest a dysfunctional cytokine production by PBMC from HIV-infected children as regards both Th1 and Th2 cytokines resulting from quantitative as well as qualitative defects induced by HIV-1.

Full text

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) has multiple effects on cytokine production *in vivo* as well as *in vitro*. These effects could be direct, due to infection of cells or binding of virus to the cell surface, or induced by soluble viral proteins [1], or indirect effects mediated by the response to viral infection [2].

HIV isolates have been classified as T cell-line-tropic or syncytium-inducing (SI) viruses and macrophage-tropic or non-SI (NSI) viruses according to their biological characteristics [3-6]. NSI viruses are present early after infection in nearly all HIV-infected individuals [3, 7, 8]. In contrast, SI variants are usually present in the latter stages of infection in more than half but not all of HIV-infected individuals [3, 8]. The emergence of SI variants is presumably a sign of poor prognosis and often correlates with faster $CD4^+$ cell depletion and rapid disease progression [3, 4, 7, 9, 10].

There are two types of Th lymphocytes that secrete different cytokines. Th1 lymphocytes produce high levels of interferon-gamma (IFN-gamma), IL-2, and tumour necrosis factor-alpha (TNF-alpha), but neither IL-4 nor IL-10. In contrast, Th2 cells secrete preferentially IL-4, IL-5 and IL-10, but no IFN-gamma [11].

Besides being secreted by T CD4 cells it is now well known that some of these cytokines are also secreted by T CD8 cells and non T cells. Thus, it is more appropriate to refer to them as type 1/type 2 cytokines. The initial cytokine scenario of any infection determines the development of one or other type of response [12]. Many studies have focused on the type 1/type 2 cytokine balance during HIV-1 infection [11, 13-16]. Enhanced IL-4 and IL-10 production and concomitant suppression of IL-2 and IFN-gamma production by PBMC, in response to PHA have been reported in HIV-infected adults [14] and children [16]. On the basis of these studies, a switch from a type 1 to type 2 cytokine response have been proposed to identify the subjects at risk of rapid progression of HIV-1 infection [11, 14, 15, 17]. However, other authors did not find a clear correlation between a type 1 to type 2 switch with disease progression [18-20]. The impact of understanding the cytokine response in HIV infection is underscored by the fact that certain cytokines affect viral replication either positively or negatively [21] and are crucial for immunity.

Studies of cytokine production by PBMC in children are scarce although some information concerning cytokine production in HIV-infected children does exist [13, 16, 22]. The divergent findings on the nature of the imbalance in cytokine production during HIV infection in adults, have prompted us to analyse this issue in children.

Our results indicate that IL-5, a type 2 cytokine, production by PBMC is depressed in HIV-infected children as infection progresses. Moreover, IL-5 levels could be used as a marker of disease progression in children.

METHODS

Patients and control subjects

Fifty-five HIV-infected children born to HIV-infected mothers were studied from December 1994 to February 1999, at the General University Hospital Gregorio Marañón of Madrid, Spain. None of these 55 children were breastfed, neither had a history of haemophilia or blood transfusion. Forty HIV-1 children had received combination antiretroviral therapy (ART) for 23.67 ± 3.15 months (range, 5-85 months), before entry into the study. Drugs were prescribed by the treating physician according to CDCP guidelines [23]. As a control group, 16 age-matched, uninfected children born from HIV-infected mothers were studied.

Written informed consent was obtained for all the paediatric patients. The local Ethics Committee of the Hospital also gave consent. Clinical classification was based on the 1994 revised guidelines of the Centre for Disease Control and Prevention (CDCP) [24].

Cytokine production assays and proliferation

PBMC were isolated from blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). Total PBMC were seeded in 96-well, U-bottomed microtitre plates (10^5/100 mul per well), and stimulated with PHA (Difco Laboratories, Inc, Detroit, Michigam, USA) for 72 hours at 37° C. After 72 hours of culture, supernatants were harvested, and their cytokine (IL-2, IFN-gamma and IL-5) content quantified using commercially available specific ELISA kits. These kits were used according to the manufacturer's instructions (IL-2, R&D Systems, Minneapolis, MN, USA; IFN-gamma, IL-4 and IL-5 Endogen Inc., Cambridge, MA, USA). Concentrations were assayed in duplicate.

Cell proliferation was evaluated by incorporation of (^{3}H) thymidine and cells were harvested on glass fibre filters using an automatic cell harvester (Skatron, Norway). Radioactive incorporation was measured in a liquid scintillation spectrometer (1450 Microbeta Trilux, Wallac, Turku, Finland). The assay was carried out in triplicate.

Quantification of T cell subsets in peripheral blood

T lymphocyte subsets in peripheral blood were quantified by direct immunofluorescence using monoclonal antibodies of the T series and flow cytometry (FACScan, Becton-Dickinson) as previously described [3].

Quantitative HIV-1 RNA assay

Blood samples were collected in EDTA-coated tubes, separated within 4 hours and plasma stored at - 70° C. The plasma viral load (VL) was measured in 200 mul plasma using a quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay (Amplicor monitor kit, Roche Diagnostic Systems, Brandenburg, New Jersey, USA). The lower limit of detection was 200 copies/ml.

Virus isolation

HIV-1 was isolated using a microculture technique. Briefly, PBMC (2×10^6), from patients, isolated from blood by Ficoll-hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden) [3] were cultured with an equal number of PBMC from healthy donors. Donor PBMC were pre-treated for 48 hours with PHA (Difco Laboratories, Inc, Detroit, MI, USA) in RPMI-1640 (Gibco, Paisley, Scotland, UK) supplemented with 20% heat-inactivated, foetal bovine serum, 5% IL-2 (Boehringer Mannheim), 100 UI/ml penicillin, and 100 mug/ml streptomycin (Gibco). Fresh PBMC (2×10^6) were added to the culture once a week. Supernatants were collected twice a week and tested for the presence of p24 HIV-1 antigen (Ag) with the use of a commercially available kit (INNOTEST HIV Antigen mAB, INNOGENETICS N.V. Haven, Zwijnaarde-Bélgica). The criteria for a positive coculture, and determination of the days to first positive were based on HIV p24 Ag results obtained from biweekly culture samples [3].

Determination of the viral phenotype

PHA-stimulated PBMC were infected with cell-free supernatant collected from each virus isolate, and after 7-10 days, the infected PBMC were cocultured with the MT-2 cell line. Cultures were maintained in the same medium, as described for the PBMC cultures, but without IL-2. Half of the cultures were replaced with fresh culture medium twice a week. Twice a week, the MT-2 cells were observed for SI formation under light microscopy [3], and the culture supernatants were tested for HIV-1 p24 Ag production [3]. An isolate was considered as SI if both formation of syncytia under light microscopy and p24 Ag production in the culture supernatants were detected. Moreover, as the MT-2 cell line expresses CD4 receptor and CXCR4 coreceptor, but not CCR5, this cell line can be useful to differentiate CXCR4-using or SI from CCR5-using or NSI strains. R5 strains did not infect the MT-2 cells. X4 strains infected the MT-2 cells, with p24 Ag production in the culture supernatants and sincytium formation. BaL (R5 or NSI) and pNL3 (X4 or SI) were used as controls.

Statistical analysis

$CD4^+$ and $CD8^+$ T lymphocyte counts are expressed as percentages. VL is recorded as standardised $\log_{10}$ VL (copies/ml). Cytokine concentration levels were normalised in the cultures of each patient according to control group levels. Comparisons of cytokine dynamics among different groups disclosed a

quite asymmetric distribution. Thus, a central tendency and dispersion were conveyed using median interquartilic range levels, respectively.

Non-parametric tests were used because of the small sample size and the free distribution of data (the data did not come from normalised populations and with the same variances). The Kruskal-Wallis "H" test, a non-parametric analogue of the variance analysis of one factor that compares overall differences between the groups studied was used. When the differences were significant, the Mann-Whitney "U" test was used to analyse which were the groups that showed significant differences. Spearman correlation analysis, a variant of the Pearson correlation coefficient, was also used. The sign of the R coefficient indicates the sense of the association, being direct when there is a positive sign and inverse when negative. The Fisher exact test was applied for qualitative variables.

RESULTS

Characteristics of the HIV-infected children

Clinical, immunological and virological characteristics of the 55 HIV-infected children are illustrated in Table 1. As expected, HIV-infected children had overall lower $CD4^+$ ($p < 0.01$) and higher $CD8^+$ T cells ($p < 0.01$) than the control group [25]. HIV-infected children were categorised according to the clinical category and the cut-off levels of 15% $CD4^+$ and 25% $CD8^+$ (Table 1). Children with $CD4^+$ ¾ 15% had higher $\log_{10}$ VL ($p < 0.05$) and higher proportion of viral isolates with the SI/X4 phenotype than children with $CD4^+$ >= 15%. Significant differences between % $CD4^+$ in clinical categories A and B
($p = 0.046$) and C ($p = 0.001$) were observed (Table 1).

Cytokine production by PBMC of HIV-infected children without stratification

In agreement with previous results in adults, HIV-infected children had lower proliferative responses than the control group in response to PHA ($p < 0.01$) (Table 2). This correlates with a lower production of the T cell growth factor IL-2. More interestingly, PHA-stimulated PBMC showed significantly decreased levels of IL-5 production. IFN-gamma was also decreased but this did not reach statistical significance (Table 2). Production by PHA-stimulated PBMC in HIV-

infected children was found to be below the sensitivity of the ELISA (data not shown).

Cytokine production by PBMC according to clinical categories and percentages of $CD4^+$ and $CD8^+$ T-lymphocytes

Interestingly, in PHA-stimulated PBMC, both IL-5 and IL-2 production decreased as infection progressed, their production being significantly lower in children in clinical category B and C than in the control group (Table 2). Asymptomatic children (clinical category A), did not have significant IL-5 and IL-2 changes. By contrast, IFN-gamma production was not significantly different in any of the three clinical categories with respect to controls. PHA-proliferative responses were lower in children with AIDS and in category A, but surprisingly not in B, with respect to the control group (Table 2).

PHA-stimulated PBMC from HIV-infected children with > 15% $CD4^+$ and ¾ 15% $CD4^+$ produced significantly lower levels of IL-2 and IL-5 than the control group (Table 2). Significant differences in the PHA-stimulated production of IL-2 and IL-5 were observed between HIV-infected children with $CD4^+$ ¾ 15% and $CD4^+$ > 15% ($p < 0.01$), being always higher in children with $CD4^+$ > 15% (Table 2). Proliferation and production of IFN-gamma were also higher in the latter group (Table 2).

IL-2 and IL-5 production by PHA-stimulated PBMC were lower in HIV^+ children with $CD8^+$ > 25% than in the control group (Table 2). The levels of IL-2, IL-5 and IFN-gamma were significantly lower in the group with $CD8^+$ ¾ 25% than the control group ($p < 0.05$) (Table 2). Proliferative responses to PHA were lower in children with $CD8^+$ > 25% than in the control group (Table 2).

Predictive value of immunological and virological markers

A univariate logistic regression analysis for the production of different cytokines, and proliferation assays with immunological and virological markers were performed, disclosing a significant association with the presence of moderate immunodeficiency ($CD4^+$ ² 25%) [24] and severe immunodeficiency ($CD4^+$ ² 15%) [24] (Table 3). The logistic regression analysis was applied by taking the independent variables with their continuous levels or by dichotomising them according to the 25 percentile (P25). Statistically

significant OR > 1 levels indicate a positive association with immunodeficiency, while levels of OR < 1 indicate a negative association.

In the case of immunodeficiency ($CD4^+$ ≤ 25%), a significant OR < 1 for the proliferative response to PHA and production of IL-5 by PHA-stimulated PBMC was observed (Table 3). The P25 of IL-5 showed the lowest OR value {0.08 (CI 95%: 0.02; 0.35) p < 0.001}. These data indicate that levels below the P25 of IL-5 production were strongly associated with immunodeficiency. When calculating the 1/OR value for IL-5, a value of 12 (CI 95%: 2.86; 50.21) was obtained, giving a clear idea of its predictive value. With respect to continuous variables, a significant value of OR for only IL-5 was found {0.90 (CI 95%: 0.83; 0.97) p = 0.003}.

When assessing the association between severe immunodeficiency ($CD4^+$ ≤ 15%) and dichotomised markers, a significant OR < 1 for the proliferative response and for production of IL-5 by PBMC after PHA-activation was found (Table 3). In all cases, the OR value was lower than 0.2 and the production of IL-5 in response to PHA showed the highest predictive power {0.05 (CI 95%: 0.01; 0.31) p = 0.006}. The 1/OR value for IL-5 was 18.84 (CI 95%: 3.27; 108.5), clearly indicating its importance. A significant OR > 1 for the cut-off level of 4.5 $\log_{10}$ VL copies/ml and for the biological phenotype SI/X4 of virus isolates were found (Table 3). These data indicated a strong association of those variables with severe immunodeficiency ($CD4^+$ ≤ 15%). Interestingly, an association between low IL-5 levels and isolation of virus with the SI/X4 phenotype was also found. VL and IL-5 were the continuous independent variables with significant association with severe immunodeficiency and OR < 1 (Table 3). The VL was the marker with the highest predictive power, with a value of 2.78 (95% CI: 1.27; 5.88) (p = 0.01) (Table 3). Low levels of IL-5 and high $\log_{10}$ VL were found to be associated with severe immunodeficiency.

DISCUSSION

The HIV-specific cytotoxic response by $CD8^+$ T-lymphocytes (CTLs) has been identified early in healthy seropositive subjects and seems to have a crucial role in inhibiting HIV replication during this phase [26]. However, this specific-CTL activity, which is dependent on IFN-gamma secretion by memory but not naïve cells, is less potent in children [27, 28], and may be explained by a delay in the production of IFN-gamma. In agreement with this hypothesis, activated PBMC of

HIV-infected children produced lower levels of IFN-gamma than the control group, even when children were rated according to $CD4^+$ and $CD8^+$ T cell percentages. On the other hand, the stable levels of IFN-gamma production by PHA-stimulated PBMC as the infection progress, could be compensated for by the increased numbers of CD8 T cells in these children.

We found lower IL-2 production in all groups of HIV-infected children studied compared to the control group. This decrease in IL-2 production was associated with disease progression. Defective production of IL-2 by PBMC in HIV-infected children may be a consequence of the lower percentage of $CD4^+$ T cell in the PBMC. In agreement with this hypothesis, HIV-infected children with more than 15% of $CD4^+$ T-lymphocytes, secreted more IL-2 than children below this threshold. Thus, the decline of $CD4^+$ T-lymphocytes and therefore disease progression is responsible, at least in part, for the lower IL-2 production. We have previously reported that the immune response is preserved in non-progressors, while in progressors HIV-infected children we observed diminished IL-2 production even after antiretroviral therapy [29, 30]. Altogether, our data clearly indicate that IL-2 production may be considered to be a very sensitive index of early immune defects in children.

While data on IFN-gamma and IL-2 would be in agreement with a switch from type 1 to type 2 cytokine pattern, a concomitant increase of type 2 cytokines was not observed in HIV-infected children at any clinical or immunological stage of the disease. Thus, no increase, but even a decrease, in IL-5 production, a typical type 2 cytokine, by PBMC was observed. Contrary to other results [31], we could not detect IL-4 production in PHA-stimulated PBMC from children. We have found decreased type 2 cytokines in HIV-infected children since the early phases of the infection, with mild or moderate symptoms and preserved immunological status, in contrast with previous reports that describe defects in the PBMC response to mitogens relatively late during HIV infection [26]. Furthermore, PBMC of HIV-infected children with $CD4^+$ ¾ 15% produced significantly lower IL-5 than those with $CD4^+$ › 15%. Children with higher $CD4^+$ T cell counts had higher IL-5 levels. Our data suggest that the loss of type 1 cytokine production in HIV infection is insufficient to induce an increase in type 2 cytokine production, as reported in adults by some authors [20, 32] and in children [33], by measuring IL-4, as a type 2 cytokine. Thus, our results suggest that the cytokine profile in HIV-infected children does not fulfil the hypothesis of a type 1/type 2 switch. Most importantly, our results confirm that, in addition to a

quantitative defect in total $CD4^+$ T cells, qualitative defects also take place in AIDS [34]. Thus, we found a significant OR < 1 value for the proliferative response and PHA-induced IL-5 production in HIV-infected children with moderate ($CD4^+$ < 25%) or severe ($CD4^+$ < 15%) immunodeficiency. These results indicate a strong correlation between lower IL-5 levels and the degree of immunodeficiency in HIV-infected children, showing a strong predictive power. Previous studies have addressed the production of IL-5 in HIV-1-infected children, with contradictory results [22, 35, 36]. Thus, in one study IL-5 production by PBMC was associated with higher IL-5 production [22], whereas in another study, no changes in IL-5 mRNA was found [36]. However, none of these studies stratified HIV+ children according to clinical and immunological categories, and therefore no clear correlation between IL-5 production and clinical or immunological status has been previously reported. Our data are in agreement with recent data suggesting the impairment in IL-5 production as one of the factors associated with the "paradoxal" eosinopenia observed in HIV-infected patients from tropical areas [37], since IL-5 is the major cytokine affecting eosinophil differentiation.

On the other hand, increased apoptosis has been described in HIV-infected PBMC [38, 39], which may explain the lower type 1 (IL-2) and type 2 (IL-5) cytokine production in PHA-stimulated PBMC from HIV-infected children. In our study, this possibility was ruled out since the viability of cultures at 72 hours assessed using trypan blue staining of PBMC from HIV-infected children in PHA-stimulated cultures, was always greater than 90%.

In addition, we have further confirmed a significant association between high VL, SI/X4 viral phenotype and severe immunodeficiency [9]. We have also found a correlation between SI/X4 viral phenotype and lower IL-5 levels. Thus, HIV-infected children with lower CD4 T cell levels showed a correlation with SI/X4 viral phenotype and low IL-5 production. This seems to be apparently in contrast with another report that found a positive correlation between another type 2 cytokine, IL-4, and the SI/X4 phenotype [34]. However, in this case the correlation may be due to the fact that IL-4 downregulates CCR5 and upregulates CXCR4 [40], and not to the levels of Th2 cells. Besides, IL-4 can be secreted by other non-T cell populations [41], whereas IL-5 is mostly secreted by Th2 cells [42].

CONCLUSION

In conclusion, our results indicate a decrease in both type 1 and type 2 cytokine production in stimulated PBMC from HIV-infected children. Interestingly, the decrease of IL-5, a type 2 cytokine, seems to have a good predictive value for the immunodeficiency status.

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Figures

Table 1
Clinical, immunological and virological characteristics of the HIV-infected children in the PBMC cytokine production study

	No. HIV-1 Children	Age (years)*	% CD4+*	% CD8+*	Log_{10}* VL	Phenotype SI/X4	Phenotype NSI/R5
HIV-1+	55	6.9 ± 0.5	29.4 ± 2.25	38.3 ± 2.0	4.21 ± 0.14	22 (42.3%)	30 (57.7%)
Clinical categories							
A	16	6.2 ± 1.05	40.1 ± 2.72	35.2 ± 2.82	4.09 ± 0.24	4 (26.7%)	11 (73.3%)
B	18	7.8 ± 0.83	28.0 ±4.34	40.8 ± 4.53	4.05 ± 0.21	7 (41.2%)	10 (58.8%)
C (AIDS)	21	6.8 ± 0.85	22.4 ± 3.3**	38.5 ± 2.84	4.45 ± 0.28	11 (55.0%)	9 (45.0%)
% CD4+ T cells							
CD4+ > 15%	42	6.7 ± 0.6	36.6 ± 1.78	37.2 ± 1.76	3.98 ± 0.14	13 (32.5%)	27 (67.5%)
CD4+ ≤ 15%	13	7.9 ± 0.79	6.1 ± 1.41††	41.7 ± 6.35	4.98 ± 0.30††	9 (75.0%)†	3 (25.0%)†
% CD8+ T cells							
CD8+ > 25%	45	6.94 ± 0.56	28.8 ± 2.3	43.0 ± 1.86	4.20 ± 0.15	15 (34.1%)	29 (65.9%)
CD8+ ≤ 25%	10	6.11 ± 1.00	32.1 ± 7.2	18.3 ± 1.76‡‡	4.24 ± 0.41	7 (87.5%)‡	1 (12.5%)‡

(*) Values expressed as mean ± s.e.e. (min; max). VL: viral load; SI/X4: syncytia formation; NSI/R5I: non-syncytia formation; NA: nucleoside analogue; X4: viral phenotype; R5: viral phenotype; Differences with A-category (*: p < 0,05; **: p < 0,01). Differences with CD4+ >15% (†: p < 0,05; ††: p < 0,01). Differences with CD8+ > 25% (‡: p < 0,05; ‡‡: p < 0,01).

Table 2
Summary of PBMC activation status and cytokine production in culture, in HIV-infected children

	IL-2	IFN-γ	IL-5	Proliferative response
Control group	69.0 (161.88) 18-1,773	194 (101.67) 65-337	36.6 (74.45) 1-238	95,667 (55,152) 54,336-156,698
HIV-1+	30.6 (67)* 1-1,775	90.1 (2,630) 1-10,044	19.9 (23)** 1-181.68	59,999 (84,063)* 2,603-269,010
Clinical categories				
A	35.7 (153) 2-1,775	75.7 (4,708) 8-9,270	26.3 (42) 1-130	38,691 (43782)** 6,121-155,670
B	30.0 (124) * 1-1,093	102 (3,270) 1-9,400	19.4 (23) ** 1-36	98,120 (75263) 4,731-269,010
C	29.8 (40)* 1-12,56	90.1 (200) 1-10,044	13.2 (193)**† 1-182	45,657 (96,190)* 2,603-178,870
% CD4+ T cells				
CD4+ > 15%	34.8 (162)** 2-1,775	94.3 (2,154) 1-9,270	23.8 (18)* 1-182	64,477 (82,824) 2,603-269,010
CD4+ ≤ 15% 1-84	15.0 (39)**‡‡ 1-10,044	50 (5,397) 1-15	1.4 (11)**‡‡ 4,731-116,150	16,000 (80,032)
% CD8+ T cells				
CD8+ > 25%	30.6 (128)* 1-1,775	140.8 (4,172) 1-10,044	19.4 (24) ** 1-182	598,86 (78,665) * 2,603-269,010
CD8+ ≤ 25%	30.7 (20)* 6-203	48.3 (330)* 1-8,047	23.7 (26)* 1-130	96,156 (153,817) 4,731-178,870

Median (IQR) Min-Max. Cytokine levels are expressed in pg/ml and proliferative responses in c.p.m. Differences with control (*: p < 0.05; **: p < 0.01) by U-test of Mann-Whitney. Differences with A-group (†: p < 0.05). Differences with CD4+ > 15% (‡: p < 0.05; ‡‡: p < 0.01).

Table 3
Predictive value of immunological and virological markers of immunodeficiency ($CD4^+ < 25\%$) and severe immunodeficiency ($CD4^+ < 15\%$) by logistic regression analysis

		Dichotomous				Continuous		
		OR	95% CI	*p*	*P25	OR	95% CI	*p*
Moderate immunodeficiency ($CD4^+ < 25\%$)	**PHA response**							
	Proliferation	0.26	0.08; 0.85	*0.025*	10,979	1.00	1.00; 1.00	0.294
	IL-2	0.63	0.20; 1.96	0.419	25.62	1.00	1.00; 1.01	0.143
	IFN-γ	0.76	0.23; 2.44	0.637	46.96	1.01	1.00; 1.01	0.133
	IL-5	0.08	0.02; 0.35	*< 0.001*	12.08	0.90	0.83; 0.97	*0.003*
	Virological markers							
	Log_{10} VL	1.75	0.55; 5.56	0.348	4.5	1.45	0.82; 2.50	0.20
	Phenotype SI/X4	2.33	0.71; 7.69	0.163	–	–	–	–
Severe immunodeficiency ($CD4^+ < 15\%$)	**PHA response**							
	Proliferation	0.10	0.02; 0.52	*0.006*	10,979	1.00	0.99; 1.01	0.219
	IL-2	0.31	0.09; 1.14	0.077	25.62	0.98	0.96; 1.01	0.138
	IFN-γ	0.47	0.13; 1.67	0.243	46.96	1.00	1.00; 1.01	0.432
	IL-5	0.05	0.01; 0.31	*0.001*	12.08	0.85	0.76; 0.94	*0.004*
	Virological markers							
	Log_{10} VL	5.56	1.41; 20.00	*0.014*	4.5	2.78	1.27; 5.88	*0.010*
	Phenotype SI/X4	6.25	1.45; 25.00	*0.014*	–	–	–	–

Continuous markers were dichotomised by percentile 25 (P25). (*) P25 values of the markers used as cut-off levels.

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