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Higher Type 1 Interferon Levels in Plasma of Asymptomatic HIV-2 than in HIV-1 Individuals

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Abstract

A number of cytokines are secreted during HIV infection that enhances both innate and adaptive immune responses. Interferon-$\alpha/\beta/\gamma$, IL-12, IL-15 and IL-18 have been found to contribute to the development, maturation, differentiation and function of NK and other immune cells. The levels of IFN-$\alpha/\beta/\gamma$, IL-12, IL-15 and IL-18 were compared in the plasma of 90 HIV-1 infected and 90 HIV-2 infected subjects by ELISA or Cytometric Beads Array assays. The HIV-infected subjects were stratified according to CD4+ T cell counts into three groups: >500, 200 - 500 and <200 cells/ul, with 30 subjects in each group. Cytokine levels were also determined in the plasma of 50 HIV un-infected blood bank donors. Among the cytokines tested, IFN-α was found to be significantly increased in HIV-2 infected compared to HIV-1 infected subjects at high CD4+ T cell counts (p = 0.001). The levels of IFN-β were seen to differ between the two infections in patients from the category of medium CD4+ T cell counts: this was significantly increased in HIV-2 infected patients (p < 0.001) as well as compared to uninfected controls (p = 0.001). The levels of IFN-γ were similar at all the CD4+ T cell categories except for an increase in HIV-2 infected patients at low CD4+ T cell counts (p = 0.02). The levels of these cytokines were similar in all HIV-1 subjects. Also, the level of IL-12p70 was similar between the two infections but significantly higher in HIV-2 at low compared to medium CD4+ T cells categories (p = 0.047). Similar to IFN-γ and IL-12p70, the levels of both IL-18 and IL-15 were found to be significantly higher in HIV-2 infected patients compared to HIV-1 at low CD4+ T cell counts (p < 0.05). These data show that there is variability in the levels of innate cytokines at different stages of HIV infection but the finding of increased IFN-α in HIV-2 infected

asymptomatic subjects is consistent with the high innate NK responses previously noted at this stage of infection.

Keywords

Type I Interferon, HIV-I, HIV-2, Cytokines, Interleukins

1. Introduction

HIV infection results in activation of the immune system [1], which is associated with the production of cytokines that influence the outcome of the immune response to the virus. These cytokines are produced by both innate and adaptive immune cells and play a major role in coordinating the function of the cellular immune system [2]. Acute infection results in prominent Th1-like immune responses to activate cellular immunity but these switch over to Th2-like cytokine responses during chronic infection [3] [4]. However, during the course of the development of adaptive immunity, innate cytokines exert their roles early in infection for NK cell function.

The development and survival of NK cells start with innate type-1 cytokines, IFN-α/β by parachymal dendritic cells which also enhance the secretion of IL-12 and IL-15 for the homeostatic maintenance of cells of both the innate and adaptive immune systems [5] [6]. The maturation of NK cells is then mainly facilitated by IL-15 [7]-[9] which together with other cytokines such as IL-12 and IL-18, enhances NK cytotoxicity [10] and increases the production of IFN-γ [11] [12]. The subsequent secretion of IFN-α and γ by both NK and T cells represents the key effector functions in the control of viral infection [13] [14]. Therefore, the development, survival and function of NK cells in HIV infection depend upon the interaction between innate and adaptive cytokines. The role of cytokines for NK cell development and function in HIV infection was initially observed in the SIV model, where IFN-α was effective in blocking the attachment of the virus to infected cells as well as preventing reverse transcription in simian immunodeficiency virus infection of Rhesus Macaques [15] [16]. Later studies showed that protection of cells from HIV infection was associated with high levels of production of IFN-α [17]-[19] which was found to be very important in the maturation of antigen presenting cells [20]. Similar effects have been observed in other viruses such as MCMV, when *in vitro* replication was effectively neutralised by IFN-α/β [21] and these cytokines were found to effectively enhance MCMV-specific NK cell cytotoxity in mouse [22]. However, fluctuating levels of IFN-α and IL-8 in chronic hepatitis B infection were associated with TRAIL-mediated killing of hepatocytes by NK cells [23].

It is therefore not clear if the levels of these cytokines influence or have any impact on innate protective effector function during infection. With the observation of improved function of NK cells in HIV-2 compared to HIV-1 infection at high CD4$^+$ T cell counts [24], the *ex vivo* plasma levels of innate cytokines, IFN-α/β, IFN-γ, IL-12, IL-15 and IL-18, were determined and compared in the two infections.

2. Materials and Methods

2.1. The Study Subjects

Ninety HIV-1 and 90 HIV-2 subjects were recruited from a cohort of HIV infected subjects who attend clinic at the MRC Laboratories in Fajara, the Gambia [25]. The cohort were made up of female sex workers, cases of tuberculosis or suspected cases of sexually transmitted diseases and also clinically healthy HIV infected individuals. The patients visit the clinic every 3 months and samples are requested for their CD4 and viral load assessment every 6 months. Regular counselling and treatment for the opportunistic infection are offered them. Trained field workers to ascertain the vital status of any of them who failed to visit the clinic for the 3-month follow-up check up carry out home visit. The subjects who were asymptomatic were not on antiretroviral treatment. However, antiretroviral treatment is now available to HIV infected patients in the Gambia with acquired immunodeficiency syndrome. Samples were taken after obtaining a signed consent forms from of the subjects according to the regulation of Gambia Ethical Committee. The characteristic of the selected subjects and clinical outcome are shown in **Table 1** and **Table 2**.

Table 1. Profile of HIV-infected patients and healthy control.

Groups	CD4 counts/ul	n	Male	Female	Age Mean ± SE	CD4% Mean ± SE	CD8% Mean ± SE	Viral Load (log)
	>500	30	10	20	31.25 ± 1.46	28.58 ± 1.14	41.47 ± 2.12	4.3 ± 0.1 a
HIV-1	200 - 500	30	7	23	30.52 ± 1.33	21.03 ± 1.66	49.93 ± 1.89	4.6 ± 0.1 b
	<200	30	10	20	36.38 ± 1.85	6.03 ± 0.69	63.73 ± 2.42	5.2 ± 0.01 c
	>500	30	7	23	39.69 ± 2.44	33.80 ± 1.62	37.87 ± 2.08	3.2 ± 0.1 a
HIV-2	200 - 500	30	19	11	39.75 ± 1.34	23.30 ± 1.40	45.87 ± 2.35	3.4 ± 0.2 b
	<200	30	16	14	38.13 ± 2.16	9.17 ± 1.04	59.87 ± 3.13	4.9 ± 0.2 c
Healthy Control	-	50	36	1 4	33.02 ± 1.25	37.12 ± 0.99	26.80 ± 1.13	-

a: $p < 0.0001$, b: $p < 0.0001$, c: $p = 0.07$.

Table 2. Clinical data and opportunistic infections of subjects involved in the study.

	HIV-1			HIV-2		
CD4+ T cell category, cells/ml	>500	200 - 500	<200	>500	200 - 500	<200
Mean weight (Kg)	56.03	61.07	52.02	56.31	59.34	54.07
Acute primary retroviral infection	0	0	0	0	0	0
Persistent generalised lymphadenopathy	0	2	0	0	0	0
WHO, 1990, Performance Scale:						
(a)	21	21	15	25	20	17
(b)	5	4	10	3	3	6
(c)	0	0	2	0	2	2
Diarrhoea ≥ 1 mnth	0	3	3	1	1	2
Fever	5	7	8	6	3	6
Cryptococcal meningitis	0	0	0	0	0	0
Pulmonary tuberculosis	1	0	3	1	0	0
Extra-pulmonary tuberculosis	0	0	0	0	0	0
Kaposis sarcoma	0	0	0	0	0	0
Candidiasis (oral and/or esophagous)	1	1	5	1	1	5
Herpes zoster (shingles)	0	1	0	1	0	0
Weakness ≥ 2 weeks	0	0	4	0	2	2

(a) Asymptomatic, normal activity; (b) Symptomatic, normal activity; (c) Bedridden <50% of the time during the last month.

2.2. Blood Samples

Fifteen millilitres of blood were collected from the subjects during their usual clinic visits. The HIV infected subjects were classified into 3 categories according to their CD4-T cell count: high (>500 cells/ul), medium (200 - 500 cells/ul) and low (<200 cells/ul). Each category consisted of 30 subjects. Fifteen millilitres of blood were also requested from the 50 healthy HIV-negative subjects at the Royal Victoria Teaching Hospital Blood Bank, Banjul The Gambia. Peripheral blood mononuclear cells (PBMCs) were separated from blood by high density Ficoll separation technique.

2.3. ELISA for Estimation of Plasma IFN-α, IFN-β and IL-18

Interferon alpha, beta and IL-18 were measured in plasma samples from 90 HIV-1 and 90 HIV-2 infected subjects using ELISA (Antigenix America Inc, USA). Plasma samples from 50 HIV uninfected controls were also assayed. Briefly, plasma samples for the IFN assays were diluted at 1 in 10 and for IL-18 at 1 to 5 in sample diluents and used with their respective serially diluted standards on monoclonal antibody-coated 96-well plates. The plates were incubated for 1 hour at room temperature and then washed once with wash solution (diluted 1 in 20) to remove the unbound antigens from the coating antibodies. This was followed by addition of secondary antibody to bind to the antigen-antibody complex. Plates were washed following 1 hour incubation at room temperature. After 3 washes, horse-radish peroxidase was added, incubated for 1 hour, and then washed again four times before the substrate, tetramethyl-benzidine (TMB), was added. Development of a blue colour occurred within 15 mins, the intensity of which depended on the extent of the antigen-antibody complex formed. The reaction was stopped by addition of H_2SO_4 stop solution. The plates were read at 450 wavelength using a Multiskan Ascent (Thermo Labsystem, Helsinki, Finland) ELISA plate reader. The plates for IL-18 were read at dual wavelength of 450nm and the reference at 620 nm; the concentrations were determined with reference to the standard curve plotted from the serially diluted calibrator concentrations.

2.4. Cytokine Bead Array Bio-Plex Assay (CBA) for Estimation of IFN-γ, IL-12p70 and IL-15

Interferon-γ, IL-12p70 and IL-15 were analysed using a Cytokine Bead Array assay (CBA, BIORAD, UK). Samples were diluted at a ratio of 1 to 3 with sample diluent and 50 ul was incubated with 50 ul beads attached to antibodies for the respective cytokines in a 96-well plate for 2 hrs on a shaker at room temperature. Standard solutions with decreasing concentrations of the cytokines were also added to the plates. The plates were washed three times with Bio-Plex washing buffer and 25 ul of biotinylated detection antibodies was added for 30 mins incubation to bind to the cytokine-antibody-beads complex. After incubation, the plates were washed again and 50 ul streptavidin conjugated to PE (phycoerythrin) was added and further incubated for 10 mins on a shaker at room temperature. The plates were finally washed and 125 ul of Bio-Plex buffer was added to the wells before plates were read on Bio-Plex array machine (Bio Rad, France).

2.5. Statistical Analysis

Statistical analysis was carried out using Stata version 8 and p values of less than 0.05 were considered statistically significant. HIV status and CD4 categories were used as predictors of the cytokines levels (IFN-$\alpha/\beta/\gamma$, IL-12p70, IL-15 and IL-18) and their effects were considered using multivariate linear regression models after transforming the data. Where transformation was not possible logistic regression was used after dichotomising the data. Data were presented as medians with interquartile ranges.

3. Results

3.1. Higher Levels of Plasma IFN-α in Asymptomatic HIV-2 Infection

The comparison of plasma levels of IFN-α showed significantly higher levels in HIV-2 compared to HIV-1 infected individuals at high CD4^{+} T cell counts: 1.0 (0.0 - 19.5) pg/ml for HIV-1 versus 41.7 (0.0 - 53.6) pg/ml for HIV-2, p = 0.001 (**Figure 1**). The levels were similar at medium and low CD4^{+} T cell counts: 3.3 (0.0 - 31.1) pg/ml for HIV-1 versus 14.8 (0.0 - 35.4) pg/ml for HIV-2, p = 0.793 and 1.0 (0.0 - 3.04) pg/ml for HIV-1 versus 1.0 (0.0 - 11.4) g/ml for HIV-2, p = 0.838, respectively. At low CD4^{+} T cell counts, the levels of IFN-α in HIV-1 (p = 0.017) and HIV-2 (p = 0.017) infected individuals were significantly lower than in the uninfected controls, 6.5 (0.0 - 26.4) pg/ml. There were similar levels of IFN-α in HIV-1 infected subjects at all categories of CD4^{+} T cell counts, p > 0.05. In HIV-2 infected subjects, the levels decreased as CD4^{+} T cell counts decreased, but were similar between higher and medium CD4^{+} T cell counts (p > 0.05) and significantly decreased at low CD4^{+} T cell counts (p = 0.001).

3.2. The Levels of IFN-β and IFN-γ in the Plasma of HIV-1 and HIV-2 Infected Subjects and HIV Uninfected Controls

Unlike IFN-α, the levels of IFN-β showed no differences between the two infections at high CD4^{+} T cell counts:
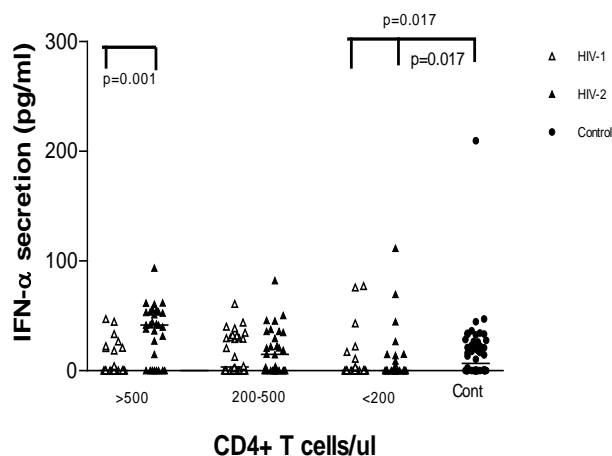
Figure 1. The level of IFN-α in the plasma of HIV-1 and HIV-2 infected subjects and HIV uninfected controls. The median concentration represented by a bar at >500, 200 - 500 and <200 cell/ml CD4$^+$ T cell categories. p values, when significant, are shown in the figure.

257.9 (0.0 - 1413) pg/ml for HIV-1 and 74.57 (0.0 - 511.8l) pg/ml for HIV-2, p = 0.437 (**Figure 2(a)**). However, there were significantly lower levels in HIV-1 infected compared to HIV-2 infected individuals at medium CD4$^+$ T cells counts: 10.0 (0.0 - 414.5) pg/ml for HIV-1 versus 436.5 (384.8 - 589.4) pg/ml for HIV-2, p < 0.001. The levels became similar again at low CD4$^+$ T cells counts: 39.4 (0.0 - 218.2) pg/ml for HIV-1 versus 176.6 (0.0 - 396.3) pg/ml for HIV-2, p = 0.247. Subjects with HIV-1 infection maintained similar levels of IFN-β at all CD4$^+$ T cell categories which were similar to uninfected controls whereas in HIV-2 infected subjects it was significantly elevated at medium CD4$^+$ T cell counts (p = 0.004) compared with uninfected controls. The levels of IFN-γ, assayed by multiplex beads rather than ELISA, showed a similar trend (**Figure 2(b)**): levels were similar between the two infections at all categories of CD4$^+$ T cell counts. At high CD4$^+$ T cell counts, levels were 2.5 (2.5 - 4.01) pg/ml for HIV-1 versus 2.5 (1.5 - 2.5) pg/ml for HIV-2, p = 0.897, at medium CD4$^+$ T cell counts, 0.5 (0.5 - 2.5) pg/ml for HIV-1 versus 0.5 (0.5 - 4.0) pg/ml for HIV-2, p = 0.06 and at low CD4$^+$ T cell counts, 3.6 (0.33 - 9.35) pg/ml for HIV-1 versus 6.5 (3.6 - 17.5) pg/ml for HIV-2, p = 0.09. In HIV-1 infected subjects, the levels of IFN-γ were similar at all the CD4$^+$ T cell count categories. However, in HIV-2 infected people there was significantly lower levels at high compared to low CD4$^+$ T cell counts (p = 0.02). The level of IFN-γ production in the plasma of HIV-2 subjects at low CD4$^+$ T cell counts was also significantly higher than in HIV uninfected controls, 2.5 (0.5 - 3.6) pg/ml, p = 0.01.

3.3. Plasma Levels of IL-12p70, IL-18 and IL-15 in HIV-1 and HIV-2 Infected Subjects and HIV Uninfected Controls

The levels of IL-12p70, IL-18 and IL-15 are shown in **Table 3**. The level of IL-12p70 was low in general and similar between the two infections at all the CD4$^+$ T cell categories. However, in HIV-2 subjects there was a significant increased in IL-12p70 at low compared with medium CD4$^+$ T cells counts, p = 0.047. As observed with IFN-γ, the levels of both IL-18 and IL-15 were found to be significantly increased in HIV-2 infected subjects compared with HIV-1 infected subjects at low CD4$^+$ T cell counts only (p = 0.014 and p = 0.031) respectively. The levels of these cytokines were similar at high and medium CD4$^+$ T cell counts and between the two infections. There was a significantly higher level of IL-15 in HIV-2 infected people with low CD4$^+$ T cell counts compared to that of uninfected controls (p = 0.01).

4. Discussion

Human Immunodeficiency Virus infection is associated with production of several cytokines as a result of immune activation. These cytokines are essential in the development and maturation of cells that exert subsequent effector functions in the attempt to control infection. In addition, these secreted molecules determine the cytokine polarization of cells that influence the level and type of function of the immune cells. This study showed
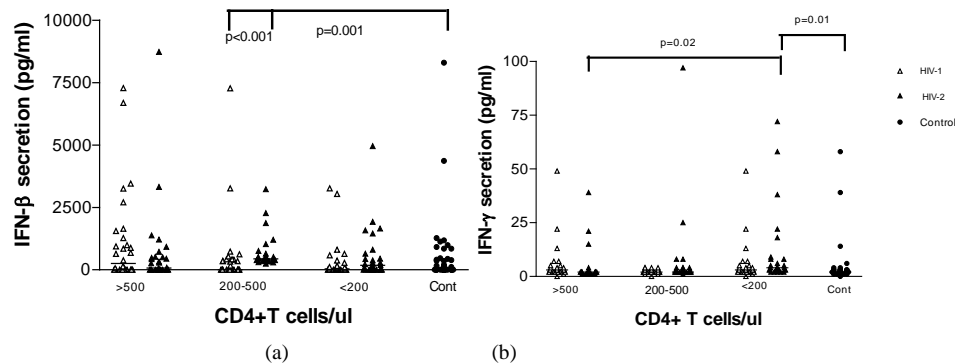
Figure 2. Plasma levels of (a) IFN-β and (b) IFN-γ in HIV-1and HIV-2 infected subjects and HIV uninfected controls. The median concentration represented by a bar at >500, 200 - 500 and <200 cell/ml CD4$^+$ T cell categories. P values, when significant, are shown in the figures. IFN-β was assayed by ELISA and IFN-γ by CBA.

Table 3. Plasma levels of IL-12p70, IL-18 and IL-15 in HIV-1 and HIV-2 infected subjects and HIV uninfected controls. IL-12p70 and IL-15 were assayed by CBA whereas IL-18 was assayed by ELISA.

	HIV-1	HIV-2	Healthy control
CD4	Median, pg/ml	Median, pg/ml	Median, pg/ml
Counts	Interquartile	Interquartile	Interquartile
IL-12p70			
>500	1.08 (1.01 - 1.1)	1.01 (1.01 - 1.08)	0.8 (0.02 - 1.01)
200 - 500	0.07 (0.0 - 0.1)	0.01 (0.0 - 0.1)$^{\theta}$	
<200	0.8 (0.02 - 1.3)	0.4 (0.02 - 0.9)$^{\theta}$	
IL-18			
>500	301.1 (129 - 616)	201.8 (97.7 - 426.2)	159.8 (58.3 - 285.2)
200 - 500	373.1 (225.6 - 973.3)	333.4 (162.0 - 656.8)	
<200	568.9 (418.0 - 777.4)$^{\alpha}$	1173.0 (559.6 - 2139.0)$^{\alpha}$	
IL-15			
>500	55.0 (45.5 - 72.5)	48.0 (40.0 - 61.0)	52.5(43.0 - 59.5)$^{\varepsilon}$
200 - 500	52.0 (44.0 - 67.0)	53.5 (46.0 - 66.0)	
<200	63.0 (53.5 - 91.0)$^{\delta}$	76.5 (62.0 - 76.5)$^{\delta\varepsilon}$	

$^{\theta}$p = 0.047, $^{\alpha}$p = 0.014, $^{\delta}$p = 0.031, $^{\varepsilon}$p = 0.01.

that innate and proinflammatory cytokine levels of IL-12, IL-15, IL-18, IFN-$\alpha/\beta/\gamma$ in the plasma of both HIV-1 and HIV-2 infected subjects are variable and exhibit different patterns at different stages of infections. However, for type 1 interferon, there was a higher level of IFN-α in the plasma of HIV-2 infected compared with HIV-1 infected subjects at high CD4$^+$ T cell counts. Type 1 interferon is produced in the early immune response to infection and it acts as an effective regulator of adaptive immunity [26] making the most potent natural signal in viral infections. Our results suggest that the levels of IFN-α but not IFN-β are crucial for control of HIV infection, as increased NK cell function was found in long-term non-progressive HIV-2 infection [24]. Besides regulating adaptive immunity and increasing the lytic potential of NK cells, type 1 interferon has other biological functions such as inhibiting viral replication [27] through the increased synthesis of enzymes, oligoadenylate synthetases, that collectively interfere with the replication and proliferation of virus and modulate MHC-I expression [28]. Early in infection, the secretion of induce type 1 interferons dendritic cells to produce IL-12 and IL-15 which are very important in enhancing the activity of both NK and cytotoxic T cells [5]. In this study, the levels of IFN-β and IFN-α showed a different pattern: the levels of IFN-β were generally high but a significantly higher level was found in HIV-2 subjects with medium CD4$^+$ T cell counts. The impact of IFN-β on the control of infection may not necessarily be dependent on plasma levels but perhaps this cytokine works in concert with

other cytokines such as IFN-α. However, the two cytokines come from different cell sources, for the major source of IFN-α is mononuclear phagocytes (leukocyte IFN) whilst that of IFN-β is fibroblast interferon [29] and this may be the reason why they exhibit a different pattern of secretion. Interferon-α has, however, been noted to be elevated in chronic HIV-1 infection [27] and therefore the decreased level of IFN-α in HIV-2 infected subjects at low CD4$^+$ T cell counts further indicates its likely role in primary infection for the support of an effective immune response. HIV-2 infection is characterised by prolonged stable CD4$^+$ T cell counts and indeed IFN-α assists in preventing apoptosis of both CD4$^+$ and CD8$^+$ T cells in HIV infection [19] [29] [30]. Therefore, the high levels of type 1 interferons may be supporting the persistently high CD4$^+$ T cell numbers in HIV-2 infection from an early stage of infection.

The secretion of cytokines IFN-γ, IL-12p70, IL-15 and IL-18 were found to be low and similar between the two infections but elevated in subjects with decreased CD4$^+$ T cell counts. As with IFN-β, these cytokines may require minimal levels of secretion for an effective function in infection. Although, the reason for increased levels of these cytokines at low CD4$^+$ T cell counts is not clear, it is likely they may be a means to drive Th1 type immune response as in the case of IL-18 [31] [32]. Both normal and decreased levels of IL-12 have been reported in primary HIV-1 infection [33]. IL-18 enhances IFN-γ production in the presence of IL-12 [34] and has been implicated in increased antiviral activity in some animal models [35] [36]. However, an elevated level of IL-18 has been noted in chronic HIV-1 infection as proviral cytokine which may disrupt the expression of NK receptors and induce apoptosis [37] [38]. Under normal signal transduction, these cytokines act synergistically with IFN-α/β to enhance the development and activation of both NK and T cells [5] [39] [40] which then results in the production of IFN-γ to activate both innate and adaptive immune responses. Not only is the differentiation and maturation process of NK cells regulated by IL-12, IL-15, IL-18 and IFN-α [21] [41] but combinations of cytokines such as IL-2 and IL-15 enhance the expression of NKp44 on NK cells [42]. Therefore the balance of the levels of these cytokines *in vivo* at different stage of the developmental process may influence NK and T cells in controlling viral replication. It is therefore possible that the effectiveness of cytokines in HIV infection may depend not only on the levels of production but also on their ability to work synergistically. This hypothesis is supported by the fact that the levels of IL-12, IL-15 and IL-18 in both HIV-1 and HIV-2 infected subjects at high CD4$^+$ T cell counts were found to be similar, even though our previous studies showed significantly increased functional NK cytolytic activity, together with secretion of MIP-1β, RANTES, IFN-γ and TNF-α in HIV-2 compared with HIV-1 infected subjects with a similar frequency of NK cells [24]. This may therefore suggest that an efficient coordinated production and activity of these cytokines on NK cells in early HIV-2 infection promotes stable CD4$^+$ T cell counts. This further supports the similar clinical outcome observed in the subjects from both infections according to CD4$^+$ T cell groupings (**Table 2**) although primary infections were not known. Thus effective immune response occurs in early stage of HIV infection and sustained for a longer period in HIV-2 subjects with stable CD4$^+$ T cells counts. Further studies involving determination of cytokine levels in isolated and cultured NK cells *in vitro* may support these observations.

5. Conclusion

In conclusion, production of innate cytokines is variable in intensity during HIV-1 and HIV-2 infections. Whereas high levels of IFN-α may be associated with the asymptomatic HIV-2 non-progressive state, the impact of others such as IL-12, IL-15, IL-18 and IFN-β on infection may not so much be dependent on their absolute levels but more on their synergistic effect in the control of viral replication during primary infection.

Acknowledgements

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