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Possible Involvement of Nuclear Factor Kappa B in Rapid Disease Progression in ART Naive HIV-1 Infected Remunerated Blood Donors with History of Oral Iron Intake

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Abstract

A retrospective analysis on the assessment of the level of p65 component of the transcription factor Nuclear Factor Kappa B (NF-kB p65) in the nuclear extract of lipopolysaccharide stimulated peripheral blood mononuclear cells (PBMCs) of remunerated blood donors with HIV-1 infection revealed NF-kB p65 level to be significantly higher in the subgroup with history of oral iron intake compared to the HIV-1 infected subgroup without such history. The level of NF-kB p65 in iron consuming subgroup of HIV-1 positive donors showed positive correlation with the serum ferritin level and with the rate of increase in viral load. The NF-kB p65 level also showed positive correlation with the level of superoxide produced by cultured Monocyte derived macrophages (MDMs) as well as with the levels of the immune activation markers viz. Tumour necrosis factor alpha (TNF-α) and two of its soluble markers *i.e.* Tumour necrosis factor receptor types one and two (TNFRI and TNFRII) reported in earlier studies in the same subgroup. The opposing roles of NF-kB in situation of iron overload in HIV-1 infection *i.e.* disease enhancement on one hand and facilitation of effective antiretroviral therapy through activation of HIV-1 in the latently infected cells on other hand suggest the need for further research to weigh benefits and risks of iron therapy in situations where iron deficiency in HIV-1 infection may be a serious consideration.

Keywords

NF-kB p65, Viral Load, Immune Activation Markers, Iron Overload

1. Introduction

There are numerous reports indicating enhancing effect of iron on disease progression in Human Immunodeficiency Virus type-1 (HIV-1) infection [1] [2] [3]. In one of our previous study on an asymptomatic group of remunerated blood donors at early stage of HIV-1 infection, it was found that the subgroup with history of oral iron intake and biochemical evidence of serum iron overload had significantly faster disease progression with development of high incidence of pulmonary tuberculosis on follow up compared to the similar subgroup but without any history of iron intake [4]. However, at the same time, in the second part of documentation on the same subgroups of donors highlighting different set of investigations, iron consuming subgroup demonstrated high level of superoxide production by cultured monocyte derived macrophages (MDMs) [5] that was an apparently paradoxical observation since superoxide anion is considered an important bactericidal tool of macrophages against *Mycobacterium tuberculosis* (M.tb). Various reactive oxygen species (ROS) including superoxide facilitate multiplication of HIV-1 via activation of the transcription factor viz. nuclear factor kappa B (NF-κB), of which the main 65kDa transactivation domain, *i.e.* NF-κB p65 or RelA migrates (translocates) to nucleus on activation where it plays a pivotal role in transactivation of viral genes, including that of HIV-1 [6] [7]. Thus it may be possible that any potentially beneficial bactericidal effect of superoxide against M.tb is offset by its contribution in multiplication of HIV-1 in high iron status. Further, in the first part of the report, the iron consuming subgroup of HIV-1 positive donors showed high state of immune activation as evident from elevated serum levels of Tumor Necrosis Factor alpha (TNF-α) and two of its receptors viz. TNFRI (p55) and TNFRII (p75) although the probable mechanism for such high immune activation was not explored [4]. The present report provides retrospective analysis of NF-κB p65 level in nuclear extracts of peripheral blood mononuclear cells (PBMCs) stimulated by Lipopolysaccharide (LPS) in the subgroups of asymptomatic HIV-1 positive blood donor with history of oral iron intake, in relation to reported levels of serum ferritin, rate of increase in HIV-1 viral load, superoxide produced by MDM culture and serum levels of the three immune activation markers viz. TNF-α, TNFRI and TNFRII [4] [5].

2. Materials and Methods

2.1. Study Subjects and Protocol of the Study

The present report includes a total of 118 HIV-1 seropositive remunerated blood donors comprising of 60 with and 58 without history of iron intake (Subgroups I and II respectively) enrolled in our earlier study with the demographic and epidemiological characteristics described in the same study [4]. Briefly the subjects, seropositive for HIV-1 infection by screening ELISA and confirmatory western blot, were at their initial asymptomatic stage of HIV-1 infection as evident by duration of infection and unimpaired peripheral CD4 lymphocyte counts (*i.e.* >

500/cu mm) and did not have serological evidence of co-infections by Hepatitis B virus (HBV), Hepatitis C virus (HCV) and Syphilis. The enrolled blood donors were subjected to estimation of NF-kB p65 in nuclear extracts of LPS stimulated PBMCs at enrolment, details of which previously unreported, is presented in the present report. Further, the NF-kB p65 level in PBMC nuclear extract was assessed in iron consuming subgroup of donors in relation to serum levels of ferritin and immune activation markers *i.e.* TNF-*α*, TNFRI and TNFRII as well as superoxide production by cultured MDMs observed earlier on the same group of donors [4] [5]. The present report also analyses correlation between NF-kB p65 level in nuclear extract of LPS stimulated PBMCs with the pattern of changes in viral load following viral set point in Subgroup I, not reported in our previous studies. Data on 40 healthy non-donor community controls included in the previous two studies [4] [5] are included for analysis.

2.2. Estimation of NF-κB p65 in Nuclear Extract of LPS Stimulated PBMC Culture

2.2.1. Isolation of PBMCs

About 6 ml of blood collected in EDTA was centrifuged at 1000 g × 20 minutes to obtain cellular fraction in the pellet. The supernatant representing the plasma was removed gently and the pellet representing the cellular fraction was suspended in equal volume of phosphate buffered saline (PBS, pH 7.4), mixed with wide mouth pipette and gently layered on Ficoll-hypaque (sp.gr 1.007) followed by centrifugation at 1200 g for 20 minutes. The buffy coat, rich in PBMCs, was washed 3 times in isotonic PBS. The PBMC suspension was found to contain >95% of viable cells by trypan blue exclusion method and >97% pure PBMCs by flow cytometric analysis using monoclonal anti-CD3, anti-CD19 and anti-CD14 antibodies. The PBMC suspension was adjusted to ~1×10^6 cells/ml.

2.2.2. Estimation of NF-κB p65 in Nuclear Extracts of LPS Stimulated PBMCs

This was carried out following broadly the original protocol by Zheng *et al.* [8] with minor modifications adapted by Yu *et al.* [9]. The protocol involved three steps viz. 1) treatment of PBMCs with mitogen LPS to induce translocation of NF-kB p65 from cytoplasm into the nucleus 2) extraction of nuclear proteins from LPS stimulated PBMCs 3) estimation of NF-kB p65 in nuclear protein extracts of LPS stimulated PBMCs.

1) Treatment of PBMCs with LPS: Three ml of PBMC suspension(~3×10^6 cells) was subjected to treatment with LPS (derived from *Escherichia coli*, serotype 0111:B4, Sigma chemicals, USA), 10 ng/ml for 10 minutes of incubation at 37˚C in a humidified CO_2 chamber to facilitate translocation of NF-kB p65 from cytoplasm into nucleus. One ml of PBMC suspension (~1×10^6 cells) was included without any mitogen treatment as control and incubated under identical condition.

2) Extraction of nuclear proteins from LPS stimulated PBMC nuclei: This

procedure was carried out in presence of protease inhibitor cocktail to prevent loss of extracted protein.

a) Reagents used:

The reagents for extraction of proteins viz. Nonidet P-40, HEPES, DTT (DL-Dithiothreitol), $MgCl_2$, KCL, Tris-HCl, Na-EDTA and Glycerol were all procured from local supplier (Hi-Media Laboratories, India). The reagents for preparation of protease inhibitor cocktail as per concentrations mentioned were all procured from a different source (Sigma chemicals USA) viz. Phenyl-methyl-sulfonyl fluoride (PMSF) (Cat No P7626) 0.5 mM, Leupeptine (Cat No L8511) 1 mg/ml, Pepstatin (Cat No P 4625) 0.5 mg/ml, Aprotinin (Cat No A1153) 1 mg/ml, EGTA (Cat No E8145) 1mM, Sodium orthovanadate (Na_3VO_4, Cat No S6508) 1mM. PMSF was prepared fresh each time before use.

b) Extraction of nuclear protein from LPS stimulated and unstimulated (control) PBMCs:

LPS treated PBMC suspension was centrifuged at 750 g at 4°C. The pellet was washed twice with cold HEPES buffer followed by suspension in 20 - 40 μl ice cold hypotonic HEPES buffer composed of HEPES (10 mM, pH 7.8), $MgCl_2$ (1.5 mM), KCL (10 mM), DTT (0.5 mM) containing 0.1% Nonidet P-40. The suspension was kept on ice for 10 minutes followed by centrifugation at 10,000 g for 10 minutes. The supernatant was designated as cytoplasmic fraction. The pellet representing nuclear fraction was re-suspended in 35 - 40 μl ice cold high salt buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25% Glycerol) containing 10 μl of protease inhibitor cocktail, followed by incubation at 4°C for 15 minutes with intermittent shaking. The suspension containing lysed nuclei was vortexed and centrifuged at 10,000 g at 4°C for 10 minutes for settling the nuclear debris. The supernatant representing the nuclear extract was collected and stored at −80°C with addition of 75 - 100 μl of HEPES buffer (pH 7.9).

The protein concentration of the extract was determined by Bradford's method in 96-well microplate using Coomassie dye reagent with bovine serum albumin as calibration standards [10]. The concentration of protein in nuclear extract suspension was adjusted to 100 μg/ml.

3) Estimation of NF-kB p65 in nuclear fraction: This procedure was carried out by commercial ELISA kit as per manufacturer's protocol (Cusabio, Wuhan, China; sensitivity 0.078 ng/ml, intra-assay and inter-assay coefficient of variation <10%). Briefly, 100 μl (10 μg) of nuclear protein extracts in triplicate and 100 μl of dilutions of standards (supplied with the kit) were charged in the ELISA plate wells coated with monoclonal anti-NF-kB p65 antibody followed by incubation at 37°C for 2 hr. The liquid was aspirated from the wells and 100 μl of biotin-conjugated anti-NF-kB p65 antibody was added to each well. The plate was incubated at 37°C for 1 hr followed by washing 3 times. One hundred microliter of HRP-avidin conjugate was added onto the wells and the plate was incubated at 37°C for 1 hr.The plate was then washed 5 times. Ninety microliter of TMB

was added and incubated at 37°C for 15 - 30 minutes in dark followed by addition of 100 μl of 0.18 M H_2SO_4 as stop solution. The plate was read at 450 nm with correction at wavelength of 540 nm. Concentration of NF-kB p65 in the nuclear extract was expressed as nanogram (ng) of NF-kB p65 per microgram (μg) of nuclear protein extract after taking the average of three wells [11].

3. Statistical Analysis

Statistical analysis was carried out by Stata, version 11 (Stata-Corp., 2010). Comparison of levels of NF-kB p65 in PBMC Nuclear extracts in subgroup I, subgroup II and controls was carried out by student's t test, with ANOVA performed for overall comparison. The correlation between laboratory parameters was evaluated by Spearman's rank coefficient. Linear regression analysis was used to assess the relationship between the laboratory parameters.

4. Results

The level of NF-kB p65 in LPS stimulated PBMC nuclear extract, expressed as nanogram per microgram of protein in nuclear extract, was significantly higher with mean and standard deviation as 4.01 and 1.07 respectively in the subgroup of HIV-1 positive blood donors with history of oral iron intake (*i.e.* subgroup I) compared to the subgroup without any such history (subgroup II) with mean and standard deviation as 1.02 and 0.43 respectively ($p < 0.001$). The corresponding level of NF-kB p65 in the healthy control group was much lower compared to both the subgroups of HIV-1 positive donors ($p < 0.001$) with mean and standard deviation as 0.48 and 0.08 (Figure 1). The level of NF-kB p65 in
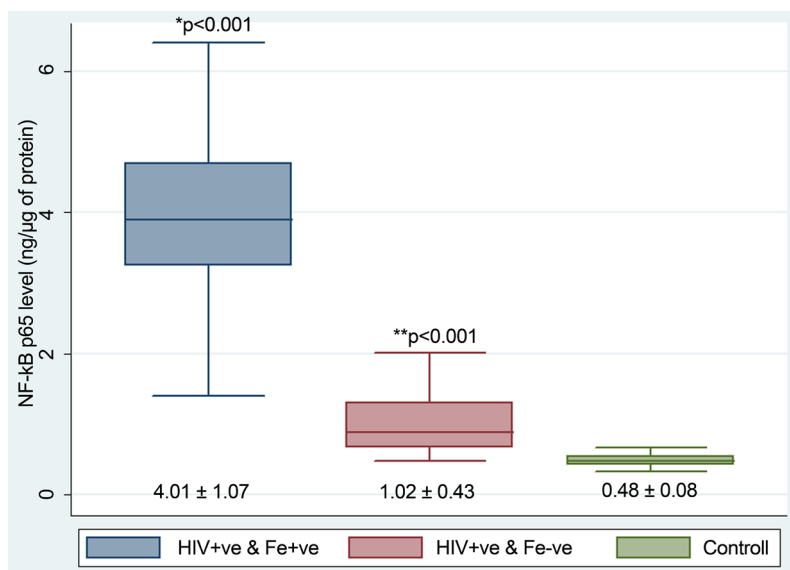

Figure 1. Box plot showing levels of NF-kB p65 in PBMC derived nuclear extracts in the two subgroups of HIV-1 infected blood donors with and without history of oral iron intake (indicated as HIV+ & Fe+ and HIV+ & Fe− respectively) as well as in controls. * symbolizes significant difference compared to subgroup II as well as to controls while ** symbolizes significant difference compared to controls.

subgroup I donors showed positive correlation with the serum ferritin level (mean ± SD as 328 ± 136 µg/L) (**Figure 2**) and with the rate of increase (per month) in HIV-1 viral load over 6 months following viral set point (mean ± SD of $\log_{10}$ HIV-1 viral load at viral set point as 4.35 ± 0.68) (**Figure 3**). The NF-kB p65 level also showed positive correlation with the level of superoxide produced by recombinant human gamma interferon (rhIFN-γ) primed MDM culture (mean ± SD as 17.67 ± 4.97 nmol/mg protein/min) (**Figure 4**) as well as with the levels of all the three immune activation markers *i.e.* TNF-α (mean ± SD as 24.6 ± 10.5 pg/ml),TNFRI (mean ± SD as 3.8 ± 2.5 ng/ml) and TNFRII (mean ± SD as 10.2 ± 3.8 ng/ml) (**Figures 5(a)-(c)**).
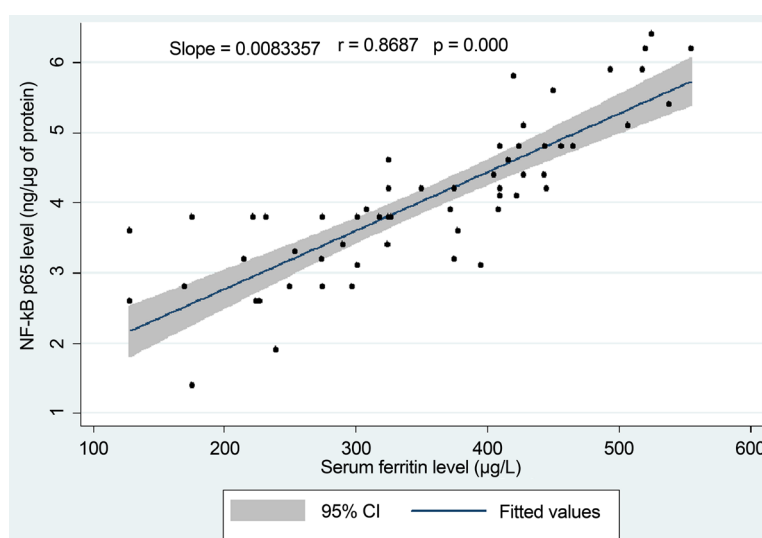

Figure 2. Correlation between serum ferritin level and PBMC derived NF-kB p65 level in the subgroup of HIV-1 infected blood donors with history of oral iron intake.
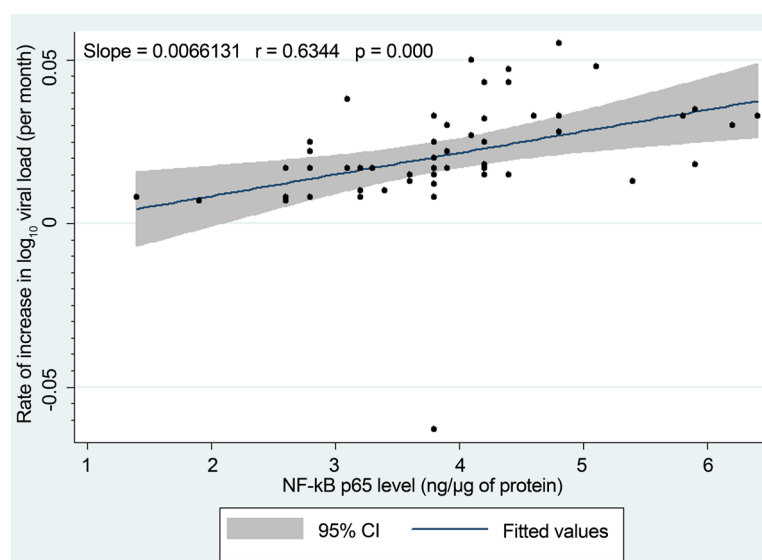

Figure 3. Correlation between PBMC derived NF-kB p65 level and rate of increase in viral load during 6 months period following viral set point in the subgroup of HIV-1 infected blood donors with history of oral iron intake.
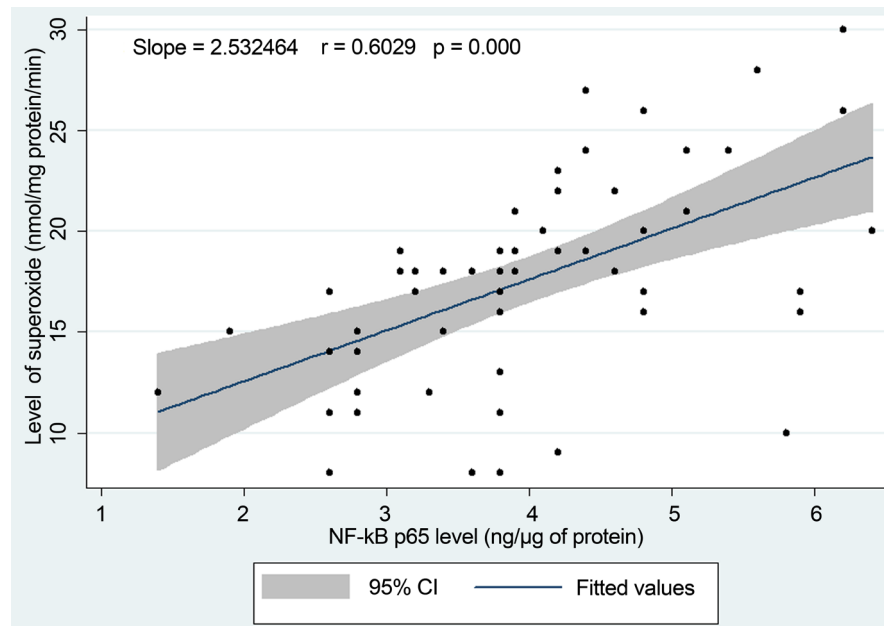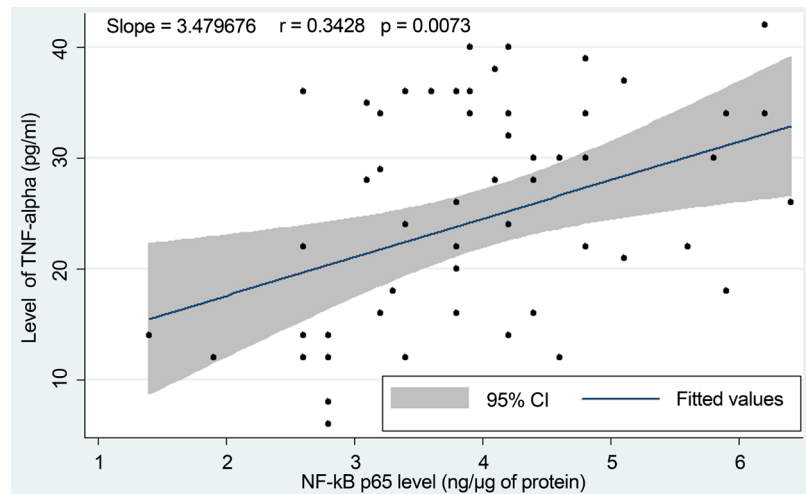
Figure 4. Correlation between PBMC derived NF-kB p65 level and quantity of superoxide produced by MDM culture in the subgroup of HIV-1 infected blood donors with history of oral iron intake.

5. Discussion

Effect of iron on enhancement of disease progression in HIV-1 infection has been substantially established (reviewed by Gordeuk *et al.* [2] [12]). They are broadly based on several observations. First, in HIV-1 positive thalassemia major patients, the rate of progression of HIV disease was significantly faster in patients with lower doses of desferrioxamine and higher serum ferritin concentrations. Second, the inadvertent simultaneous administration of low doses of oral iron with dapsone for the prophylaxis of *Pneumocystis carinii* (now known as *Pneumocystis jiroveci*) pneumonia in HIV-positive patients may have been associated with excess mortality. Third, study of haptoglobin polymorphisms in HIV-positive subjects indicated that the haptoglobin 2-2 polymorphism is associated with higher iron stores and shortened survival as compared with the haptoglobin 1-1 or 2-1 phenotypes. Fourth, retrospective study of bone marrow macrophage iron in HIV-positive patients suggested that survival is shorter with high iron stores. Additional evidence suggesting iron overload as a risk factor for rapid progression of the disease includes slowing down of the disease course following iron chelation therapy in HIV-positive patients with thalassemia major [13].

The progression of HIV-1 infection correlates closely with the replication efficiency of the virus [14]. Replication of HIV-1 in turn is coupled with the transcription of the viral genome that is initiated from the long-term repeat (LTR) region of the viral genome containing adjacent NF-kB binding sites [15]. This was further supported by the observation that mutants of IkBa that block NF-kB induction also inhibit HIV-1 infection in T cells by interfering with viral
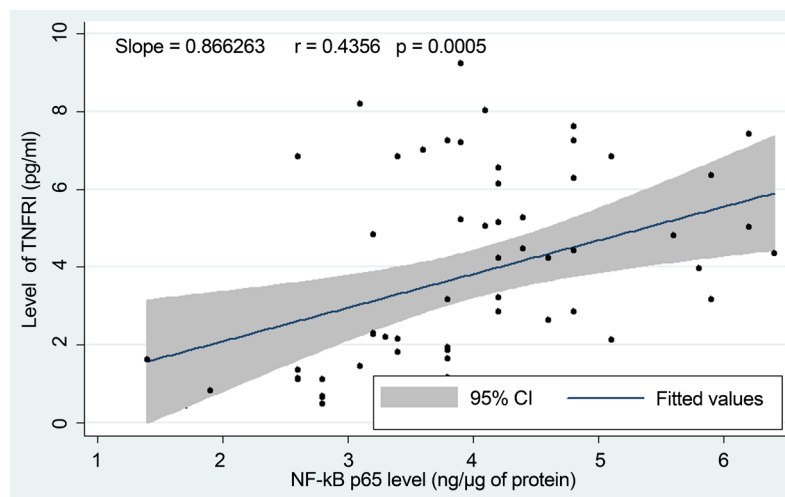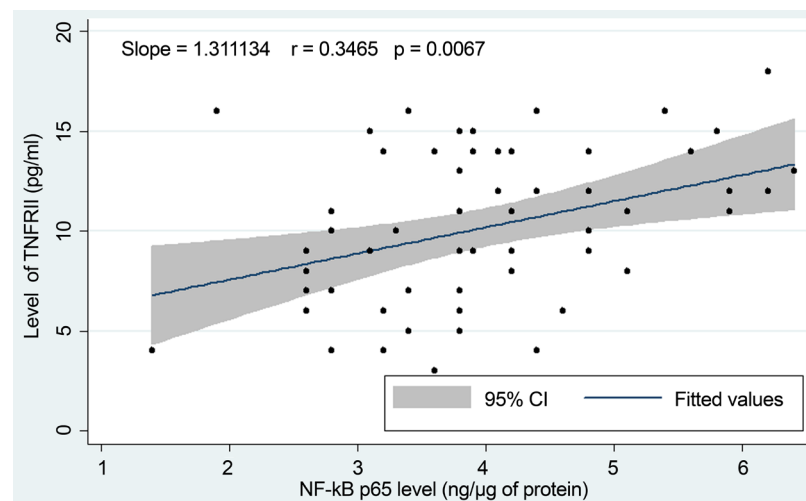
Figure 5. Correlations between PBMC derived NF-kB p65 levels and serum levels of (a) TNF-α, (b) TNF RI and (c) TNF RII in the subgroup of HIV-1 infected blood donors with history of oral iron intake.

transcription [16]. The transcriptional activity of LTR region is dependent on the number of NF-kB binding sites. The LTR from HIV-1 subtype C, the predominant subtype in India [17] contains three NF-kB binding sites [18]. Activation of NF-kB by iron is an important element in the life cycle of HIV-1 [3]. In addition, reverse transcription of HIV RNA into DNA by the enzyme reverse transcriptase requires the iron dependent protein Ribonucleotide Reductase [19]. In cultured cells, excess of iron is associated with an increased HIV-1 viral replication while iron chelators inhibit HIV-1 transcription [20]. Thus, elevated level of NF-kB p65 in stimulated PBMC observed in the iron consuming subgroup of blood donors with its level showing positive correlations with serum ferritin level and with rate of increase in plasma viral load substantiates the possible interrelationship between iron overload and disease progression in HIV-1 infected blood donors reported earlier [4].

High level generation of reactive oxygen species (ROS) including superoxide has been reported in situations of iron overload e.g. in thalassemia children [21] which support the high superoxide production by MDMs reported earlier in the iron consuming subgroup [5]. Reactive oxygen species (ROS) are known to be potent stimulators of NF-kB [22] raising the possibility of an alternate or additional pathway of high viral replication in high iron state that is reflected by the positive correlation between level of NF-kB p65 in the present report and superoxide level in cultured MDMs reported in the earlier study.

Macrophage is the largest producer of TNF-α [23]. High iron level has not been reported to influence TNF-α production by macrophages. In fact, the mean concentrations of immunoreactive tumor necrosis factor-alpha (TNF-alpha) in the supernatants were recorded to be lower in the hereditary hemochromatosis patients compared to healthy controls [24]. Thus it is more likely that increased levels of TNF-α, TNFRI and TNFRII encountered in iron consuming blood donors are mediated by NF-kB as evident by the positive correlation between NF-kB p65 level and the levels of three immune activation markers [25].

Persistence of a latent pool of HIV-1 provirus poses a barrier for effective anti-retroviral therapy as integrated HIV-1 is not affected by the anti-retroviral drugs until viral transcription is activated [26]. From this point of view, iron with its role of activating NF-kB, should be a beneficial adjunct towards effective antiretroviral therapy. At the same time, numerous possible adverse outcomes of HIV-1 infection due to iron overload reported so far are likely to outweigh such benefit. Therefore, further research is needed to judge merits and demerits of oral iron therapy that is not uncommonly considered for treatment of anemia in HIV-1 infection.

Acknowledgements

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