Polymorphisms in the CD14 and TLR4 genes independently predict CD4+ T-cell recovery in HIV-infected individuals on antiretroviral therapy

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Type of article: Major article

Running title: *TLR4* and *CD14* SNP in CD4 count recovery post-ART

Keywords: C-260T *CD14* SNP, HIV, CD4 recovery, microbial translocation, LPS, sCD14

Abstract: 200 words

Main text: 3137 words

Note:

Data was presented as poster at the 7th IAS Conference of HIV Pathogenesis, Treatment and Prevention 30 June - 3 July 2013 Kuala Lumpur, Malaysia. (Poster Exhibition -- WEPE453)

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Abstract

Background. Chronic HIV infection leads to marked depletion of CD4+ T cells in the gastrointestinal (GI) tract and increased microbial translocation measured by an increase in circulating lipopolysaccharide (LPS) levels. Here, we hypothesised that single-nucleotide polymorphisms (SNPs) in genes encoding the Toll-like receptor 4 (TLR4) and CD14, the principal receptors for LPS, were associated with CD4+ T-cell recovery post-antiretroviral therapy (ART).

Methods. Prospective study of predominantly Caucasian HIV-infected subjects receiving suppressive ART for at least 12 months. We analysed the CD14 SNPs C-260T and the TLR4 SNPs A+896G, C+1196T. We also determined the levels of LPS and soluble CD14 (sCD14) in plasma samples collected pre- and post-ART initiation. CD4+ T-cell recovery was assessed by linear mixed models.

Results. Following ART, individuals with a TT genotype compared to a CT or CC genotype for CD14 C-260T SNP showed higher levels of sCD14 (p=0.008 and 0.003 respectively). The CC genotype for the CD14 C-260T SNP, compared to CT or TT and the TLR4 SNP (AC/GT) compared to the homozygous genotype (AA/CC) were both independently associated with enhanced long-term CD4+ T-cell recovery (>3 months; p<0.001).

Conclusions. Polymorphisms in CD14 and TLR4 are independently associated with long-term CD4+ T-cell recovery in HIV-infected individuals post-ART.

INTRODUCTION

Combination antiretroviral therapy (ART) has led to a dramatic reduction in morbidity and mortality of individuals infected with human immunodeficiency virus (HIV). Successful clinical outcomes are dependent on adequate recovery of CD4+ T cells following ART [1]. Several studies have shown that ~30% of patients fail to restore CD4+ T-cell counts >500cells/µL despite years of suppressive therapy [2]. There is now increasing evidence to show that patients failing to achieve CD4+ T-cell counts >500 cells/µL are at increased risk of developing serious non-AIDS events [3], including cardiovascular disease (CVD), hypertension, liver disease, non-AIDS malignancies and neurocognitive impairment [4]. Recent studies suggest that normalization of CD4+ T-cell counts should be defined as >900cell/µL [5].

HIV disease progression is associated with increased adaptive and innate immune activation (IA) [6], which is multi-factorial including increased expansion of CMV-specific T cells [7] and persistent HIV RNA at low levels [8]. However, a major contributing factor to immune activation is the depletion of CD4+ T cells in gut-associated lymphoid tissue (GALT) during the acute HIV infection [9-11] leading to increased microbial translocation (MT). Upon initiation of ART, MT is reduced as measured by reduction in the circulating levels of lipopolysaccharide (LPS), but this rarely reaches the levels of HIV uninfected controls [9, 10, 12, 13]. A case-control study that compared CD4+ T-cell non-responders (CD4+ T-cell counts <200cells/µL after 24 months of suppressive ART) versus full responders (>400cells/µL after 24 months of suppressive ART), showed that LPS was significantly increased in non-responders [10].

Toll-like receptor 4 (TLR4) and CD14 expressed on macrophages, monocytes, dendritic cells (DCs) and neutrophils [14], are the main cellular sensors of LPS [15]. Binding of LPS to these receptors induces a signaling cascade leading to activation of NF-κB, and eventual secretion of pro-inflammatory cytokines [16]. CD14 can also be found in plasma in a soluble form [17] and soluble CD14 (sCD14) is elevated in HIV-infected individuals despite long-term suppressive ART [18]. Elevated sCD14 has also been associated with CVD, neurological abnormalities, lymphoma and mortality in ART-treated HIV-infected individuals [4]. Collectively, these data demonstrate that host responses to elevated LPS levels could influence the rate of CD4+ T-cell recovery post-ART, and consequently to development of important clinical end-points in HIV disease.

Polymorphisms in the genes encoding for CD14 and TLR4 have been shown to determine responsiveness to LPS [19]. A common single-nucleotide polymorphism (SNP) found at position -260 of the *CD14* promoter (where a C→T change occurs) enhances the transcriptional activity of *CD14,* which is associated with an increase in high-density membrane-bound CD14 on monocytes as well as elevated levels of sCD14 in tissue culture supernatants along with other pro-inflammatory cytokines such as TNF-α and IL-6 [20, 21]. Others have shown evidence for an association between the CD14 159TT genotype and inflammatory conditions including chronic periodontitis [22], myocardial infarction [23], Crohn's disease [24], and susceptibility to brucellosis [25].

The main TLR4 SNPs that have been studied include A+896G (rs4986790) and C+1196T (rs4986791) [26]. Compared to non-carriers, carriers of A+896G/C+1196T showed blunted inflammatory responses to inhaled LPS [26] as well as reduced plasma IL-6, IL-1β and C-reactive protein (CRP) levels in response to LPS administration *ex vivo* [27]. Others have

shown that both these TLR4 SNPs were associated with reduced prevalence of diabetic neuropathy in type 2 diabetes [28], and that carriers of these SNPs were more susceptible to severe bacterial infections [29, 30].

In this study, we hypothesized that SNPs in the TLR4 and CD14 genes will have an impact on CD4+ T-cell recovery in HIV-infected individuals following initiation of ART secondary to a change in response to LPS. Using multivariate analysis, we examined the clinical and biological factors associated with restoration of CD4+ T cells and found that both TLR4 and CD14 SNPs were independently associated with enhanced long-term CD4+ T-cell recovery.

METHODS

Patients

Patients were recruited from the Alfred Hospital and the Melbourne Sexual Health Centre (Melbourne, Australia) based on previously published inclusion criteria [13]. All plasma and peripheral blood mononuclear cells (PBMCs) were collected at pre-ART, and one time-point at least 12 months after the initiation of ART. The clinical specimens were processed by standard protocols within 24hrs of collection. Demographic and clinical parameters such as date of birth, gender, HIV exposure category, diagnosis of AIDS-defining illnesses (ADIs) before initiation of ART, co-infection with hepatitis B and C viruses, and initial ART treatment regimen were obtained from the Victorian HIV Service Database of the Alfred Hospital. All CD4+ T-cell counts and plasma HIV RNA results were also collected longitudinally from ART initiation to the most recent data available. All participants provided written informed consent and the protocols were approved by the Research and Ethics Committee of Alfred Health.

CD14 and TLR4 genotyping

DNA was obtained from cryopreserved PBMCs. Genotyping of a common polymorphism in *CD14*, C-260T (rs2569190) and two common polymorphisms in the TLR4 gene, A+896G (rs4986790) and C+1196T (rs4986791) was performed using the Sequenom MassARRAY iPLEX platform at the Australian Genome Research Facility.

Plasma LPS and sCD14

MT was estimated by measuring LPS levels using a commercial chromogenic limulus amebocyte lysate (LAL) assay (Lonza Walkersville Inc., Walkersville, MD), and sCD14 levels using a Quantikine sCD14 enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) as previously described [13]. All samples were assayed in duplicate, and samples from the same patient (i.e. pre- and post-ART samples) were always assayed on the same plate to minimise interassay variation. Additionally, two samples were used as internal quality control (QC) for each assay. The results of each plate were only included if the coefficient of variance (CV) of the two QC samples was <15%.

Statistical analysis

Genotype frequencies were tested for Hardy-Weinberg equilibrium by chi-square test. The association between TLR4 and CD14 SNPs, LPS and sCD14 was analysed using the Kruskal-Wallis test with a post-hoc two-tailed Mann-Whitney U test. Linear mixed model (LMM) regression analysis was used to adjust for differences in the LPS levels across the genotypes tested. Plasma levels of LPS and sCD14 of a paired-sample (pre- and post-ART) were compared using the Wilcoxon signed-rank test. Piecewise regression by LMM was used to determine the association between TLR4 and CD14 SNPs with the rate of CD4+ T-cell recovery at two different time-periods separately (i.e. baseline to 3 months, and 3 months

onwards) after ART initiation. The association between TLR4 and CD14 SNPs and other clinical parameters to CD4+ T-cell counts post-ART were first assessed using univariate LMM. Variables with $p<0.2$ were considered as candidates and included in the multivariate analysis. Variables with $p<0.05$ were considered as independent predictors. As CD4+ T-cell count was an outcome variable that was significantly skewed and was resistant to sensible transformation (log, square-root transformation) the use of maximum likelihood estimation was precluded. In this case generalised estimating equation (GEE) was used in the LMM analysis.

RESULTS

Patient demography and clinical characteristics

We recruited 156 Caucasian patients, and DNA was available from 125 patients. Cohort demographics and clinical details of the patients are described in **Table 1**. After initiation of ART, all patients had viral suppression (plasma HIV RNA <400copies/mL) throughout the follow-up period except three patients who had a single episode of virological failure (plasma HIV RNA >400copies/mL). The success rate of amplification to determine the genotype was 100%. Of the 125 patients examined, 54 were heterozygous and 34 homozygous for the *CD14* -260T allele; and 12 heterozygous for the *TLR-4* SNPs. There were no patients homozygous for the *TLR4* SNPs. This resulted in an allele frequency of 0.49 for *CD14* SNP (χ^2 test $p=0.13$) and 0.048 for *TLR4* SNPs (χ^2 test $p=0.573$) that was in HWE. Both TLR4 SNPs A+896G and C+1196T were co-segregated.

Suppressive ART for 12 months resulted in significant decrease of plasma LPS and sCD14 levels

The effects of long-term suppressive ART on HIV viral load, CD4+ T-cell count MT and innate immune activation were assessed in plasma samples of patients before and at one time-point after at least 12 months of suppressive ART. We showed that both LPS and sCD14 levels were significantly reduced following ART (both *p*<0.0001) (Wilcoxon signed rank test, data not shown), consistent with multiple prior studies [9, 13, 31]. We found a weak correlation between sCD14 and LPS levels (r=0.2044; p=0.0222) pre-ART (**Figure 1A**), and also between pre-ART CD4+ T-cell count with LPS (r=-0.195; p=0.029) and sCD14 (r=-0.404; p<0.0001) (**Figure 1B-C**). Pre-ART HIV viral load was not correlated with plasma levels of LPS; whilst it correlated with the levels of sCD14 (r=0.329; p=0.0002), and inversely with CD4+ T-cell count (r=-0.268; p=0.003) (**Figure 1D-F**).

SNPs in CD14 but not TLR4 were associated with increased sCD14 levels post-ART

We next compared the levels of CD4+ T-cell count, LPS, sCD14 and HIV viral load in plasma among different CD14 and TLR4 genotypes. We found that CD4+ T-cell count and sCD14 in the post-ART samples was significantly different amongst the different CD14 genotypes (Kruskal-Wallis test, *p*=0.024 and *p*=0.006 respectively) but not in the pre-ART samples. A post-hoc analysisshowed that genotype CC was associated with significant higher level of CD4 T-cell count compared to CT (*p*=0.013; Mann Whiteny U test) and a trend of higher CD4 T-cell count compared to TT genotype (*p*=0.058) (**Figure 2A, bottom left**).

Genotype CC and CT were associated with lower levels of plasma sCD14 as compared to TT (*p*=0.0026 and *p*=0.083 respectively); whilst the difference between CC and CT were not significant (*p*=0.6) (**Figure 2A**). Using a regression model adjusted for LPS levels post-ART

and years on ART, the CC genotype compared to the CC and CT genotypes was independently associated with lower sCD14 levels (hazard ratio [HR] -0.11, 95% confidence interval [CI] -0.13 – -0.077; p<0.001).

The CD14 genotypes were not associated with CD4+ T-cell count, plasma sCD14 and HIV viral load pre-ART, and LPS pre- and post-ART (**Figure 2A, B**). The TLR4 SNPs, A+896G and C+1196T were not associated with these four variables pre- or post-ART (**Figure 2A, B**). Together, we concluded that only the presence of CD14 CC genotype was associated with lower sCD14 levels post-ART.

The correlation of sCD14, LPS levels and CD4+ T-cell counts depends on the CD4 and plasma HIV viral load

As the plasma levels of LPS, sCD14 and CD4+ T-cell count were poorly correlated, we speculated if this could be influenced by the degree of immune recovery. We therefore performed a post-hoc analysis stratified by baseline CD4+ T-cell levels and plasma HIV viral load and showed that the levels of LPS and sCD14 were more strongly correlated in individuals with baseline CD4+ T-cell count <100 cells/μL (r=0.622; p=0.0009) and baseline HIV viral load >5log/mL (r=0.385; p=0.002). In this cohort, a similar correlation was not observed among individuals with baseline CD4 >100 cells/μL and HIV viral load <5log/mL. We also found that the levels of LPS and sCD14 were more strongly correlated in individuals with CD14 -260TT (r=0.484; p=0.0037) and TLR4 +896AA (r=0.246; p=0.008) the two genotypes that were associated with an increased responsiveness towards LPS stimulation (**Figure 2C, left panel**)

SNPs in CD14 and TLR4 were independently associated with enhanced CD4+ T-cell recovery

In order to assess the independent influence of CD14 and TLR4 SNPs on CD4+ T-cell recovery following suppressive ART, we performed multivariate piece-wise regression by LMM, controlling for clinical and biological factors that have previously been shown to be associated with CD4+ T-cell reconstitution. In general, recovery of CD4+ T cells during viral suppression occurs in two phases [32]. During the first 3 months of ART, the number of CD4+ T cells rapidly increases due to T-cell homeostasis [33, 34], improved thymic output [35] and redistribution of T cells into peripheral circulation [36], followed by a slower phase of T cell repopulation that takes up to several years post-ART and is largely driven by T-cell proliferation and enhanced thymic production [37]. Considering the biphasic pattern of CD4+ T-cell recovery, LMM was performed at two different time periods following initiation of ART i.e. from baseline to 3 months; and 4 months onwards as we have previously reported [38].

In unadjusted analyses from baseline to 3 months, multiple factors were significantly associated with early CD4+ T-cell recovery (**Table 2**). In adjusted analyses, baseline CD4+ T-cell counts (β-coefficient=0.87; 95% CI=0.779–0.966; *p*<0.001) was associated with enhanced early CD4+ T-cell recovery; whilst receiving a PI regimen (β-coefficient=-38.81; 95% CI=-67.39 – -10.23; *p*=0.008) was associated with decreased early CD4+ T-cell recovery (**Table 2**).

Next, we investigated the determinants associated with late CD4+ T-cell recovery i.e. four months and beyond. Unadjusted analyses are shown in **Table 3**. In an adjusted model, multiple clinical parameters remained significant including baseline CD4+ T-cell count, age

at ART initiation, receiving trimethoprim and sulfamethoxazole (TMP-SMX) therapy at the time of initiation of ART, post-ART LPS level, pre- and post-ART sCD14 levels, previous ADIs, gender, positive for HBV antigen, mode of HIV transmission as well as both CD14 and TLR4 genotype. Our preliminary analysis showed that individuals with *CD14* -260CC compared to CT and TT had a higher CD4+ T-cell count (β-coefficient 117 (*p*=0.002) and 87 (*p*=0.0026) respectively). Therefore, TT and CT were combined and compared with CC in subsequent analyses. The pooled β-coefficient of *CD14* -206CC genotype compared to CT/TT was independently associated with enhanced late CD4+ T-cell recovery (β-coefficient=65.71; 95% CI=42.51–88.92; *p*<0.001). In the same model, the TLR4 SNPs (AG/CT) as compared to the genotype AA/CC were also independently associated with enhanced late CD4+ T-cell recovery (β-coefficient =111.87; 95% CI=78.33–159.41; *p*<0.001) (**Table 3**).

DISCUSSION

In the present study, we sought to investigate the relationship of TLR4 and CD14 SNPs with LPS and sCD14 and CD4+ T-cell recovery in HIV-infected individuals who were on suppressive ART. We found significant associations between TLR4 and CD14 SNPs with the trajectory of late recovery of CD4+ T-cell counts following ART using LMM. In addition, using a multivariate analysis, we found that the TLR4 genotype +896AG/+1196CT and CD14 genotype -260CC were independently associated with enhanced late CD4+ T-cell recovery post-ART. Finally, we showed that plasma LPS and sCD14 were significantly correlated in HIV-infected participants with a CD4 T-cell count <100cells/μL, HIV viral load > 5 log/mL and in individuals carrying the CD14 -260TT and TLR4 +896AA SNPs, the genotypes associated with an increased responsiveness or sensitivity towards LPS stimulation.

In this study, we assessed the changes in CD4+ T-cell counts over time after initiation of ART as a continuous variable using GEE analysis, controlling for time varying covariates. Although several others have used Cox regression to study CD4+ T-cell recovery [39, 40], we believe that the LMM approach is more robust because it takes into account the heterogeneity in both the rate and magnitude of CD4+ T-cell count increases following ART [2, 41].

The TLR4 SNPS in exon 3 - A+896G and C+1196T - are common non-synonymous SNPs with replacement of an aspartic acid (Asp) residue with glycine (Gly) at amino acid position 299 (Asp299Gly); and threonine with an isoleucine at position 399 (Thr399Ile), respectively [42]. Both SNPs are known to affect the extracellular domain of TLR4. Nonetheless, the precise mechanism whereby these SNPs affect LPS signaling is not clearly understood. A recent study suggested that the SNPs could interfere with TLR4 dimerization, and therefore prevent the assembly of intracellular docking platforms for adapter recruitment leading to the hyporesponsiveness to LPS stimulation [43]. In line with this, we demonstrated in a multivariate model that carriage of *TLR4* +896AG/+1196CT was associated with greater late CD4+ T-cell reconstitution, independent of LPS levels. However, since TLR4 +896AG was less prevalent (9.6%), we also felt that the SNP effect might have been masked by the effect of the more prevalent SNP i.e. *CD14 -260CC* (29.6%) in the univariate analysis. Therefore, the *TLR4 +896AG* SNP only showed its effect after the adjustment in multivariate model.

The SNP in the CD14 gene, C-260T (also known as C-159T) is a common genetic polymorphism, which occurs in the promoter region of the *CD14* [44] and is reported to occur in ~48% of Caucasians [45]. This SNP (C→T) reportedly reduces the binding affinity of suppressor proteins (Sp) to its binding domain and hence enhances the promoter activity of

CD14 [46]. Consistent with this finding, we found that the CC genotype was associated with lower plasma levels of sCD14 (and TT genotype with higher sCD14), independent of LPS. Interestingly, we only observed this in plasma collected post-ART. This potentially could be due the high level of immune activation pre-ART which may have masked the effects of CD14 C-260T SNPs.

We have recently examined the relationship of these same polymorphisms and cardiovascular disease in a cohort of individuals on ART recruited in Malaysia [47]. We are currently examining the relationship of these polymorphisms to CD4 recovery but given that this cohort is entirely Asian and they initiated ART late (median CD4 = 63 cells/µL compared to median CD4= 202 cells/µL in this manuscript), we anticipate that the relationship between the polymorphisms and CD4 recovery that we describe here may be quite different. Ideally, the relationship between the polymorphisms described here and CD4 recovery should be examined in a replicate cohort of similar ethnicity which we are currently planning.

There were a number of limitations in our study. First, although this was a longitudinal study, patients were enrolled post-ART and therefore samples were obtained at varying time-points following ART initiation. We adjusted for time between initiating ART and sample collection in the multivariate model, but our results could have been confounded by loss to follow up or death, although death is now significantly reduced in HIV-infected individuals on ART [48]. Second, only a small fraction of our patients were carriers of the rare allele for TLR4 SNPs, and therefore this analysis had less statistical power. Although there is a plausible biological basis to support our findings of the genetic association between TLR4 and CD14 SNPs and recovery of CD4+ T cells following ART, given the small sample size, these findings require confirmation in an independent cohort of similar ethnicity. Lastly, the CD4/CD8 T-cell ratio