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HIV-1 co-infection increases relapse rate and shortens survival in patients with visceral leishmaniasis

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

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ABSTRACT

Patients co-infected with visceral leishmaniasis (VL) and HIV-1 (VL/HIV patients) suffer from recurrent VL relapses and increased mortality. The aim of our study was to test the hypothesis that HIV patients who present with their first episode of VL (primary VL/HIV patients) experience less relapses and lower mortality as compared to VL/HIV patients who have a previous history of VL relapses (recurrent VL/HIV patients). Our results show that primary VL/HIV patients have a lower parasite load and that their relapse-free survival is significantly longer. Relapses in both groups of patients occur independently of HIV viral load. Our clinical and immunological analyses of these patients at the time of diagnosis and during follow-up show that the poorer prognosis of recurrent VL/HIV patients is accompanied by lower weight gain and lower recovery of all blood cell lineages, as well as lower production of antigen-specific IFNγ, lower $CD4^+$ T cell counts and higher expression levels of the inhibitory receptor PD1 on $CD4^+$ and $CD8^+$ T cells.

We propose that in addition to the current treatments, novel interventions should be considered at the time of VL diagnosis in VL/HIV patients and suggest that improved anti-leishmanial and antiretroviral treatments, as well as immune therapy, through PD1/PDL-1 blockade and/or through IFNγ administration, could result in more efficient parasite killing and thereby reduce the relapse rate and improve survival.

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INTRODUCTION

Visceral leishmaniasis (VL), also named kala-azar, is a potentially fatal neglected tropical disease, caused by parasites of the genus *Leishmania*. An estimated 50,000 to 90,000 new cases of VL occur worldwide annually, but only 17,082 new cases of VL were reported in 2018, with Brazil, Ethiopia, India, South Sudan and Sudan, each reported >1000 VL cases, together representing 83% of all cases globally [1]. Because of the remote location of VL endemic areas and the lack of surveillance, it is widely accepted that this is a vast underestimation of the real burden of VL in endemic areas. VL imposes a huge pressure on the developing countries and delays economic growth, with an approximate annual loss of 2.3 million disability-adjusted life years [2].

In Ethiopia, where this study took place, VL is one of the most significant vector-borne diseases: over 3.2 million people are at risk of infection [3]. VL is caused by infections with parasites of the *Leishmania* (*L.*) *donovani* species complex. Not all infected individuals will develop the disease: some will stay asymptomatic, but in those who develop VL, the disease is characterised by hepatosplenomegaly, fever, pancytopenia and severe weight loss; this stage of the disease is generally fatal if left untreated [4,5]. Following the HIV-1 pandemic, VL has emerged as an opportunistic infection: HIV infection increases the risk of developing symptomatic VL [6,7] and VL accelerates the progression of HIV infection to AIDS. Ethiopia has the highest rate of VL/HIV co-infections in Africa, with HIV present in up to 30% of VL cases [8].

HIV co-infection presents a significant challenge in the prevention and control of VL [9,10]: VL/HIV co-infected patients experience increased rates of VL relapses, mortality and treatment failure compared to patients with VL alone [10-12].

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We have recently shown that 78.1% of VL/HIV patients experience at least one relapse [12]. Furthermore, antigen-specific IFNγ production by whole blood cells from VL/HIV patients was impaired throughout the entire study period, $CD4^+$ T cell counts remained persistently low and PD1 expression on $CD4^+$ T cells remained high. Our results suggest that in VL/HIV patients, the inability to restore the $CD4^+$ T cell counts to normal levels and the sustained high expression levels of PD1 on $CD4^+$ T cells results in the impaired ability of $CD4^+$ T cells to produce antigen-specific IFNγ and therefore plays a major role in the poor control of parasite replication [12].

Reliable data on both clinical and immunological parameters from VL/HIV patients who relapse are scarce: it has been shown that previous VL episodes in VL/HIV patients are associated with increased risk of VL relapse [13]. Based on this evidence and our previous work [12], we hypothesise that the rate of VL relapse is significantly lower in VL/HIV patients who present with their first episode of VL (primary VL/HIV patients), as compared to those with a previous history of VL (recurrent VL/HIV patients). To test his hypothesis, we performed the most extensive follow-up study of VL/HIV patients to date, to determine the frequency of VL relapses in primary and recurrent VL/HIV co-infected patients and identify clinical and immunological markers associated with VL relapse in these two cohorts.

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METHODS

Patient recruitment

For this cross-sectional study, we followed the cohort of 49 VL/HIV patients (median age 33.5±1.0 years) that was described in [12]. Twenty-one patients presented with their first episode of VL (primary VL/HIV, P VL/HIV) and 28 with at least one previous episode of VL (Table 1). The diagnosis of VL was based on positive serology (rK39) and the presence of *Leishmania* amastigotes in spleen or bone marrow aspirates [14]. The diagnosis of HIV was done in accordance with the Ethiopian National HIV Screening Test Guidelines [15]. Forty-six VL/HIV patients were on anti-retroviral therapy (ART) at the time of VL diagnosis; the remaining three started ART at the end of the anti-leishmanial treatment. All treatments were administered according to the Guideline for Diagnosis, Treatment and Prevention of Leishmaniasis in Ethiopia [16] (Table 2). At the end of treatment, a test of cure (TOC) was used for VL/HIV patients to decide if they could be discharged from hospital; a negative TOC is defined as follows: patients look improved, afebrile, and usually have a smaller spleen size, parasitological cure (absence of amastigotes in splenic aspirates) and an improved haematological profile. When the TOC was still positive (i.e. incomplete cure), treatment was continued until TOC became negative [16].

The definitions of no relapse and relapse are defined in the "Guidelines for diagnosis, treatment and prevention of leishmaniasis in Ethiopia" [16] as follows:

- no relapse: "*absence of clinical features of the disease 6 months after completion of the recommended dose and duration for VL treatment".*
- Relapse: "*patient with VL treatment history presenting with clinical visceral Leishmaniasis symptoms and is diagnosed with positive parasitology (LD bodies) after successful completion of the treatment".*

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Antiretroviral therapy (ART) was provided according to the National Guidelines for Comprehensive HIV Prevention, Care and Treatment [15, Table 3]).

Patients were recruited at four different time points [12]: time of diagnosis (ToD); end of treatment; 3 months after the end of treatment (3m); and 6-12 months after the end of treatment (6-12m). At the end of this 3-year study, all VL/HIV patients who had not relapsed during the 12 months follow-up were contacted by phone to find out if they had had any subsequent relapse.

Sample collection and processing

Venous blood (13ml) samples were taken and distributed as follows:

- 2.5ml in PAXgene tubes for RNA sequencing
- 8 ml in heparinised tubes: 3ml for the whole blood assay (WBA), 5ml to purify PBMC [17] (flowcytometry) and plasma (cytokines)
- 2.5ml in EDTA tubes (whole blood for: $CD4^+$ T cell count, white and red blood cell and platelet counts and plasma for viral load)

- WBA: soluble leishmania antigen (SLA) was prepared as described in [17].

- Flow cytometry: the following antibodies were used: $CD4^{FITC}$, $CD8^{PE\ CY7}$ and $PD1^{PE}$ (eBioscience). The data on PD1 expression are shown as the integrated Median Fluorescent Intensity (iMFI) [18].

- $CD4^+$ and $CD8^+$ T cell counts: 100µl of blood was stained with $CD4^{FITC}$, $CD8^{PE\ CY7}$ and $CD3^{PerCP\text{-}eFluor®\ 710}$ (eBioscience) for 15min at 4°C; red blood cells were lysed using BD FACS™ Lysing Solution for 5min at room temperature.

Acquisition was performed using BD Accuri™ C6 flow cytometry and data were analysed using BD Accuri C6 analysis software.

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- IFNγ and IL-10 levels were measured in the supernatant of the WBA using IFN gamma and IL-10 Human ELISA Kit (Invitrogen) according to the manufacturer's instructions. The optical densities obtained with the unstimulated whole blood cells were subtracted from the optical densities obtained with whole blood cells stimulated with PHA or SLA.

- HIV viral load: Plasma was isolated by centrifuging 2ml of blood and frozen at -80^{o}C. HIV viral load was measured in the Central Laboratory of the Amhara Public Health Institute, Bahir Dar, by using Abbott RealTime HIV-1 Qualitative (m2000sp), according to the manufacturer's instructions.

- White and red blood cell, and platelet counts were measured using a Sysmex XP-300T^{M} automated haematology analyser, (USA) following the manufacturer's instruction.

Relapse rate

Log-rank tests for duration of follow-up at event end points provided two-sided *p*-values; Kaplan-Meier curves are presented for visual interpretation. The primary outcome survival until three years of follow-up was completed; relapse was the only censoring event. Censoring events were reported at the pre-planned follow-up period (3, 6-12 and after 3 years) at which they were identified. Cox proportional-hazards regression analysis was used to estimate hazard ratios and 95% confidence intervals.

mRNA

RNA was extracted from 2.5 ml whole blood using the PAXgene 96 blood RNA kit (Qiagen) and globin mRNA was depleted using the GLOBINclear kit (Ambion). Sequencing libraries were prepared using the KAPA Stranded mRNA-Seq Kit (Roche) with 10 PCR cycles, then sequenced as 75bp paired-end reads on the

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Illumina HiSeq 4000 platform. Sequencing reads were mapped with Salmon v.1.30 against concatenated sequence of human gencode transcriptome release 34, transcripts for *L. donovani* LV9 from TriTrypDB release 46 and transcript data for an Ethiopian HIV type 1C virus (Genbank Accession U46016) - type 1C represents over 90% of HIV infections in Ethiopia. Pseudo-counts were imported into R v4.0.3 using the tximport v1.18.0 and transformed into lengthscaledTPM. Total HIV expression was quantified as the sum of counts across HIV transcripts, and total *Leishmania* expression was quantified as the sum across all LV9 transcripts with the exception of feature LdLV9.27.2.206410, an 18S rRNA gene to which human transcripts also map.

Statistical analysis

Statistical tests are specified in the legend of each figure. The following were used: Mann-Whitney, Kruskal-Wallis and Fisher's exact tests. Differences were considered statistically significant at $p<0.05$. *=p<0.05, **=p<0.01, ***=p<0.001 and ****=p<0.0001. Unless otherwise specified, results are expressed as median±SEM.

Study approval

This study was approved by the Institutional Review Board of the University of Gondar (IRB, reference O/V/P/RCS/05/1572/2017), the National Research Ethics Review Committee (NRERC, reference 310/130/2018) and Imperial College Research Ethics Committee (ICREC 17SM480). Informed written consent was obtained from each patient and control.

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RESULTS

CLINICAL DATA

Frequency of VL relapse in VL/HIV patients with primary VL and multiple relapses

We previously reported that during a 3-year follow-up, 78.1% of VL/HIV patients relapsed at least once [12]. In the current study, we divided that VL/HIV cohort into 2 groups, those who presented with VL for the first time (primary VL/HIV patients, P VL/HIV, n=21) and those who had a history of previous VL episodes (recurrent VL, R VL/HIV, n=28).

As presented in Figure 1 the relapse-free survival was significantly lower in R VL/HIV (p=0.0327). We then compared the mortality rate: over the 3-year follow-up, the number of deaths was higher in the R VL/HIV patients: 10/28 (35.7%) vs 4/21 (19%) deaths in P VL/HIV patients, but this did not reach statistical significance (Fisher's exact test: p=0.3882). As shown in Table 1, the number of relapses in the cohort of R VL/HIV ranged from 1 to 14. Overall, 17/20 (85%) of R and 8/12 (66.7%) of P VL/HIV patients experienced one or more VL relapse, this was also not significantly different (Fisher's exact test: p=0.3793). R VL/HIV relapsed faster as compared to P VL/HIV: 47.1% vs 25.0% at 3m, 29.4% vs 0% at 6m, 11.8% vs 25.8% at 12m and 11.7% vs 50% after 3 years, respectively.

Parasite grades and viral load

Next, we compared parasite grades in splenic aspirates of P and R VL/HIV patients at ToD. As shown in Figure 2A, parasite grades were significantly higher in the R than in the P VL/HIV cohort (p<0.0001). Parasite grade can only be measured when the spleen is palpable and >3cm below the costal margin; it is therefore mainly

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measured at ToD. We have previously shown that RNAseq can be used to measure the total expression of *L. donovani* mRNAs (*Ld* mRNA) in blood [12]. The median *Ld* mRNA was higher at ToD in R VL/HIV patients, this was not statistically significant (Table 4). However, at the end of treatment and at 3m, there was significantly more *Ld* mRNA in R VL/HIV (Figure 2B, Table 4). Then, despite an increased median *Ld* mRNA in R HIV/VL patients at 6-12m, the differences between the 2 groups were not significantly different (Table 4). In R VL/HIV patients, the levels of *Ld* mRNA increased at 3 and 6-12m to levels similar to those observed at ToD (data not shown).

Despite being on ART, the majority of patients with P VL/HIV and just under half of the R VL/HIV cohort had detectable plasma viral load (Table 5A). There were no significant differences in plasma viral load (Table 5B) and blood total expression of HIV mRNA (Table 5C) between P and R VL/HIV patients at each time point and over time.

Clinical presentations

Next, we assessed whether P and R VL/HIV patients presented with different clinical manifestations. At ToD, fever was significantly higher in P VL/HIV patients as compared to R VL/HIV patients (Figure 3A) but normalised thereafter to levels similar to controls (controls: 36.0±0.1°C, $p>0.05$, data not shown).

The median BMI of each group of VL/HIV patients was below the normal value of 18.5 [19] throughout follow-up and was significantly higher in R than P VL/HIV at ToD and EoT ($p=0.0173$ and $p=0.0192$ respectively, Figure 3B). The BMI of P HIV/VL, but not R VL/HIV, increased significantly over time ($p=0.0010$).

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Since hepatosplenomegaly is a typical sign of VL/HIV patients, spleen and liver sizes as measured below the costal margin are systematically recorded when patients present to the clinic. As shown in Figure 3C, spleen sizes were similar in both groups at ToD, EoT and 3m and decreased throughout follow-up, but were significantly higher at 6-12m in R VL/HIV (p=0.0061). The liver was also clearly measurable below the costal margin at ToD in both groups of patients and decreased throughout follow-up (Table 6).

Haematological profiles

White blood cell counts (WBCs) were similar in both groups of VL/HIV patients and increased at EoT, however, at 6-12m R VL/HIV patients had significantly lower WBCs than P VL/HIV patients (Table 7A). RBCs increased significantly in P HIV/VL patients until the 3m time point and plateaued at 6-12m; however, no significant improvement in RBCs was observed in the blood of R VL/HIV patients throughout the follow-up (Table 7B). Similarly, platelet (PLT) counts increased at EoT in P VL/HIV patients, plateaued thereafter and were higher than in R VL/HIV patients at 3 and 6-12 months (Table 7C); PLT counts of R VL/HIV patients did not change significantly throughout the follow up (Table 7C). Despite an increase in WBC, RBC and PLT counts in the blood of P VL/HIV, these values still remained significantly lower than those of healthy controls ($p<0.0001$, data not shown).

Antigen-specific production of IFNγ and IL-10 by whole blood cells from P and R VL/HIV patients

One of the hallmarks of VL/HIV patients is the inefficiency of whole blood cells to produce IFNγ in response to *Leishmania* antigen-specific stimulation at time of

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diagnosis and throughout follow-up [12]. Here we compared antigen-specific IFNγ production by WB cells from P and R HIV/VL patients. Results presented in Figure 4A show that whereas the levels of IFNγ did not significantly change over time in both groups, WB cells from R HIV/VL patients produced significantly less IFNγ than WB cells from P HIV/VL patients throughout follow-up. The production of IFNγ in response to PHA remained similar in both groups throughout follow-up (Table 8A), with values comparable or lower than those obtained in response to SLA (data not shown).

We have previously shown that antigen-specific production of IL-10 is impaired in the WBA in VL/HIV patients [12] and that it was not associated with disease severity. Our results presented in Figure 4B show that antigen-specific production of IL-10 by R VL/HIV WB cells was significantly lower at ToD but similar at EoT, 3 and 6-12m. The production of IL-10 in response to PHA increased significantly over time in both groups but was lower at 6-12m in R VL/HIV patients (Table 8B).

$CD4^+$ and $CD8^+$ T cell counts and PD1 expression

Our previous results showed that in VL/HIV patients, the failure to restore antigen-specific production of IFNγ correlated with persistently low $CD4^+$ T cell counts and high expression of PD1 on $CD4^+$ T cells. Results presented in Figure 5A show that despite low $CD4^+$ T cell counts in both P and R VL/HIV patients throughout follow-up as compared to controls ($p<0.0001$), $CD4^+$ T cell counts increased significantly in the P VL/HIV group, but not in the R VL/HIV group (Figure 5A). In contrast, $CD8^+$ T cell counts were similar between both groups of VL/HIV patients at all time points and were restored to levels similar to those of controls at EoT ($p>0.05$).

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We have previously shown that the expression level of PD1, an inhibitory receptor that can be associated with impaired effector functions, remained high on $CD4^+$ T cells in VL/HIV patients [12]. Results presented in Figure 5C show that CD4 PD1 iMFI in both P and R VL/HIV patients were higher throughout follow-up as compared to controls ($p<0.0001$), and that CD4 PD1 iMFI levels decreased significantly in the P VL/HIV group, but not in the R VL/HIV group. Similarly, although CD8 PD1 MFI remained significantly higher in both VL/HIV groups as compared to controls throughout follow-up (Figure 5D, $p<0.0001$), CD8 PD1 iMFI decreased over time in P VL/HIV, but not R VL/HIV.

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DISCUSSION

We have previously shown that over a period of 3 years, VL/HIV patients experience a high rate of relapse [12]. Here we analysed this cohort of patients further by comparing clinical and immunological parameters in VL/HIV patients who presented with their first episode of VL and those with a previous history of VL relapse.

Our results show that there was a poorer recovery in weight gain and blood cell counts, higher spleen size and parasite load in R VL/HIV patients, as compared to P VL/HIV patients:

- The median BMI of both groups of patients remained below 18.5 throughout the follow-up, however the BMI increased significantly in the P, but not the R VL/HIV cohort. Malnutrition plays a crucial role in increased susceptibility to infection and/or disease severity by weakening both innate and acquired immunity [20,21]. It is therefore possible that the lower BMI observed in R VL/HIV patients at 6-12 months contributes to their poorer prognosis. Better management of malnutrition in both groups of VL/HIV patients could improve their ability to mount an effective immune response.
- Splenomegaly increased in R VL/HIV patients during the follow-up period, consistent with the observed increase in the parasite load over time in this group of patients.
- Although both groups of patients remained pancytopenic, the increase in WBC, RBC and PLT counts following treatment was less efficient in R VL/HIV patients. Bone marrow suppression can contribute to pancytopenia, and both VL and HIV infection have been associated with bone marrow failure [22-24]. Both pathogens can infect hematopoietic stem/progenitor cells (HSPCs), and this can impair haematopoiesis. The higher parasite load observed in R

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VL/HIV patients might contribute to the poorer recovery of all blood cell lineages.

- Although the parasite load decreased at EoT, the load as measured in blood remained higher from EoT onwards in R VL/HIV patients; at 3 and 6-12m it was similar to the load at ToD. These results are consistent with the higher relapse rate in these patients, 85% in the R VL/HIV versus 65% in the P VL/HIV cohort, indicating that R VL/HIV patients have a poorer ability to control parasite replication.

To identify possible mechanisms responsible for this inability to efficiently control parasite replication, we analysed immunological parameters. It has been shown that in splenic aspirates, IFNγ produced by $CD4^+$ T cells contribute to parasite killing [25]. We and others have speculated that in VL/HIV patients, the impairment of antigen-specific IFNγ production by $CD4^+$ T cells plays a key role in the inefficient control of parasite replication [12,25]. Here we show that the levels of antigen-specific IFNγ produced by WB cells were even further reduced in R VL/HIV as compared to P VL/HIV patients. This reduction in IFNγ might explain the higher parasite load detected in splenic aspirates and blood of R VL/HIV patients. Antigen-specific IFNγ is mainly produced by $CD4^+$ T cells in the whole blood assay [25]. It is therefore plausible that low IFNγ levels are a consequence of low $CD4^+$ T cell counts. Here we show that both factors are likely to be involved: the $CD4^+$ T cell counts are even lower in R VL/HIV and are accompanied with significantly lower production of antigen-specific IFNγ in the WBA, as compared to P VL/HIV.

Low $CD4^+$ T cell counts could be due to poor HIV control: in our study, many patients still had detectable viral loads throughout the follow-up despite being on ART. The

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recovery of $CD4^+$ cells is often stunted in individuals who started with low $CD4^+$ T cell counts [26-28]; but there is no ART regimen that has been shown to boost $CD4^+$ T cell recovery. Inclusion of dolutegravir in first line treatment could help to improve $CD4^+$ T cell recovery through more efficient suppression of viral replication [29]. It is also possible that there is some resistance to HIV drugs in this population: this has been reported to both first and second-line treatments [30]. Poor HIV control may also be due to difficulties accessing ART for the population of migrant workers during the agricultural season [12]; indeed, this population is highly mobile and frequently lack access to health facilities where they can get ART. Poor adherence to ART is also likely to play a role: whereas adherence counselling is available to HIV patients, a recent study about ART adherence in the hospital in Gondar showed that adherence to ART was negatively associated with rural residence, lack of knowledge about HIV and ART, undisclosed HIV status to partners, and low CD4 count [31].

T cell exhaustion is another factor that might account for the low antigen-specific IFNγ level observed in VL/HIV patients. T cell exhaustion is characterised by a gradual loss of effector functions and co-expression of inhibitory receptors [32-34]. PD1 is upregulated on T cells by signals such as IL-2, IL-7, type I IFNs and signalling via the TCR: it is therefore a marker of T cell activation [35]. However, during chronic infection, the levels of PD1 remain high and are associated with T cell dysfunction [34]. It is therefore plausible that in VL/HIV patients, persistent antigenic stimulation due to *Leishmania* and HIV contributes to T cell exhaustion. T cells responding to chronic infection undergo progressive loss of functions; since R VL/HIV patients have had a more intense and longer exposure to both pathogens, this could have resulted in higher PD1 CD4 iMFI and lower antigen-specific IFNγ production as compared to P VL/HIV. Our results also show that whereas $CD8^+$ T cell counts are restored at EoT,

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CD8 PD1 iMFI decreased significantly in P VL/HIV but not in R VL/HIV patients, suggesting that $CD8^+$ T cells may also have an exhausted phenotype. It has been previously shown that $CD8^+$ T cells from the blood of VL patients have an anergic/exhausted phenotype, as shown by high levels of CTLA4 and PD1 [36]. However, we did not detect CTLA4 on T cells in VL/HIV patients [12]. In the study by Gautam *et al.*, the authors show that $CD8^+$ T cells contribute to the basal levels of IFNγ in whole blood, but not to the antigen-specific IFNγ production [36]; similarly the study by Kumar *et al.* that showed that $CD4^+$ T cells but not $CD8^+$ T cells produce IFNγ in the WBA [25]. We have previously discussed fundamental differences between VL patients in India and in Ethiopia: whereas WB cells from VL patients from Northwest Ethiopia have an impaired ability to produce antigen-specific IFNγ at ToD, this is not altered in VL patients from India [12,17,37]; we hypothesised that these differences were associated with the less severe VL symptoms in Indian patients as compared to patients from Northwest Ethiopia [17]. In view of these differences, since we did not determine the phenotype of IFNγ producing cells in our WBA, we cannot exclude the possibility that $CD8^+$ T cells produce antigen-specific IFNγ. It is also possible that $CD8^+$ T cells contribute to the elevated levels of IFNγ detected in the plasma of VL/HIV patients [12].

VL/HIV patients are likely to play a major role in the transmission of VL. A recent study by Singh et al. showed that patients with active VL, but not asymptomatic or successfully treated VL patients, can transmit the parasites to sand flies [38]. Another study showed that VL/HIV co-infected individuals transmitted the parasites most efficiently to the sand fly vectors [39]. Since VL/HIV patients harbour higher parasite loads than VL patients, these co-infected individuals are likely to be a significant reservoir for *L. donovani* and have a high potential for parasite transmission; thereby

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preventing the elimination of visceral leishmaniasis. From a public health perspective, it is important to note not only the high parasite burden in these patients but also the potential for drug resistance to emerge. Given the importance of parasite load to transmission, the contribution of treatment failure in VL/HIV patients to the reservoir in the community needs to be determined and the cost to the health service as well as the health implications to the individual considered when determining management.

In summary our results show that VL/HIV patients who have a history of previous VL episodes relapse sooner and more often and are more likely to die than those presenting with their first episode of VL. The poorer prognosis of R as compared to P VL/HIV patients is accompanied by lower weight gain and recovery of WBC, RBC and PLT counts; and lower production of antigen specific IFNγ, lower $CD4^+$ T cell counts and higher CD4 and CD8 PD1 iMFI. Given the poor outcome predicted by VL/HIV coinfection and a history of relapse, specific measures should be tried to improve the long-term prognosis of these patients: an improvement of their ART treatment, such as inclusion of dolutegravir; more ART adherence counselling; a better follow-up of their nutritional status; and longer anti-leishmanial treatment. Additional molecular monitoring to inform the duration of anti-leishmanial treatment should be explored in future studies. Immune therapy, through PD1/PDL-1 blockade, that might improve the impaired production of antigen-specific IFNγ and/or through IFNγ administration [40] could result in more efficient parasite killing in these patients. Such interventions might contribute to the prevention of further relapse.

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AUTHOR CONTRIBUTIONS

All authors discussed the results and contributed to the final manuscript. Study conception and design: PK, YT, IM; Acquisition of data: YT, EA, TM, PK; Analysis and interpretation of data: YT, EA, RW, MK, ML, SUF, GPT, IM, JAC, PK; Drafting of manuscript: PK, YT, GPT, JAC, IM

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TABLES

Table 1: recurrent VL/HIV patients: number of relapses at ToD

Number of relapses	Number of patients
1	4
2	7
3	5
4	2
5	1
6	1
7	1
9	2
10	2
12	1
14	2

Number of previous relapses in R VL/HIV patients (n=28) at ToD when this study was started. ToD=time of diagnosis

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Table 2: Type and duration of anti-leishmanial treatments

Number of P VL/HIV patients	Treatment	Duration of treatment (days)
1	SSG+PM	61
3	SSG	28
1	AmBisome	65
4	AmBisome	28
7	AmBisome + Miltefosine	28
1	AmBisome + Miltefosine	30
1	AmBisome + Miltefosine	40
1	AmBisome + Miltefosine	45
1	AmBisome + Miltefosine	62
1	AmBisome + pentamidine	30
Number of R VL/HIV patients	**Treatments**	**Duration of treatment (days)**
1	SSG+PM	61
1	SSG	28
1	SSG	71
2	AmBisome	28
1	AmBisome	65
10	AmBisome + Miltefosine	28
1	AmBisome + Miltefosine	45
1	AmBisome + Miltefosine	62
1	AmBisome + Miltefosine	90

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1	AmBisome + Miltefosine	52
1	AmBisome + Miltefosine	101
1	AmBisome + pentamidine	30
1	AmBisome + pentamidine	62
5		Treatment not completed

SSG= Sodium stibogluconate, PM= Paramomycin and AmBisome= Liposomal amphotericin B.

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Table 3: ART

Number of P VL/HIV	**ART at ToD**	**ART after EoT**
18	TDF, 3TC and EFV	
1	AZT, 3TC and EFV	
2	Not on ART	TDF, 3TC and EFV
Number of R VL/HIV		
25	TDF, 3TC and EFV	
2	TDF 3TC and ATV	
1	Not on ART	TDF, 3TC and EFV

TDF: Tenofovir disoproxil fumarate, 3TC: Lamivudine, EFV: Efavirenz, AZT: azidothymidine, ATV: atazanavir. ToD=Time of Diagnosis; EoT=End of Treatment.

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Table 4: *L. donovani* mRNAs

	P VL/HIV	**R VL/HIV**	***p* values**
ToD	136±608	1786±1229	0.1068
EoT	0.1±0.4	1.5±341.4	0.0007
3m	0.1±149.3	1047±1188	0.0099
6-12m	1.7±83.6	791±2847	0.0822
***p* values**	<0.0001	0.0056	

Quantification of the total expression of *L. donovani* mRNA in blood from P (ToD: n=12, EoT: n=16, 3m: n=8, 6-12m: n=7) and R VL/HIVVL/HIV (ToD: n=23, EoT: n=14, 3m: n=16, 6-12m: n=18)

Statistical differences between P and R VL/HIV patients at each time point were determined using a Mann-Whitney test; statistical differences between the 4 different time points for each cohort of patients were determined by Kruskal-Wallis test.

LD mRNA= *L. donovani* mRNA. ToD=Time of Diagnosis; EoT=End of Treatment; 3m=3 months post EoT; 6-12m=6-12 months post EoT.

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Table 5A: % of VL/HIV patients with detectable viral load

	P VL/HIV	R VL/HIV
ToD	66.7%	47.6%
EoT	80.0%	44.4%
3m	27.3%	50%
6-12m	66.7%	38.1%

Table 5B: HIV-1 viral load in plasma (copies/ml of plasma)

	P VL/HIV	R VL/HIV	*p* values
ToD	381±553679	200±581955	0.8452
EoT	150±50305	0.1±208331	0.4946
3m	542.5±160.9	2,873±99923	0.0799
6-12m	1,341±136928	629.5±199439	0.2758

Table 5C: HIV mRNA in whole blood (total length-scale TPM)

	P VL/HIV	P VL/HIV	*p* values
ToD	1,119±764,364	437±660,554	0.9743
EoT	150±53763	0.1±122616	0.1813
3m	0.1±28.1	14,635±120,216	0.0511
6-12m	1,341±273,690	0.1±173,035	0.1136

A. % of P VL/HIV (ToD: n=18, EoT: n=15, 3m: n=11, 6-12m: n=7) and R VL/HIV (ToD: n=21, EoT: n=14, 3m: n=16, 6-12m: n=6) patients who had detectable viral loads at ToD, EoT, 3 and 6-12m. **B.** HIV-1 viral load in plasma from P VL/HIV (ToD: n=18, EoT: n=15, 3m: n=11, 6-12m: n=7) and R VL/HIV (ToD: n=21, EoT: n=14, 3m: n=16, 6-12m: n=6) patients. **C.** Quantification of the total expression of HIV mRNA in

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blood from P VL/HIV (ToD: n=12, EoT: n=18, 3m: n=8, 6-12m: n=7) and R VL/HIV (ToD: n=23, EoT: n=14, 3m: n=16, 6-12m: n=21) patients.

Statistical difference between P and R VL/HIV was determined by Mann-Whitney test.

ToD=Time of Diagnosis; EoT=End of Treatment; 3m=3 months post EoT; 6-12m=6-12 months post EoT.

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Table 6: Liver size

	P VL/HIV	R VL/HIV	*p* values
ToD	4.0±0.6	2.0±0.7	0.2191
EoT	0.0±0.2	0.0±0.4	0.7136
3m	0.0±0.3	0.0±0.4	0.9263
6-12m	0.0±0.3	0.0±0.3	0.6133
***p* values**	<0.0001	0.009	

Liver size was measured in cm below the costal margin on P VL/HIV (ToD: n=21, EoT: n=17, 3m: n=14, 6-12m: n=18) and R VL/HIV (ToD: n=28, EoT: n=22, 3m: n=18, 6-12m: n=22) patients.

Statistical differences between P VL/HIV and R VL/HIV patients at each time point were determined using a Mann-Whitney test and statistical differences between the 4 different time points for each cohort of patients were determined by Kruskal-Wallis test.

ToD=Time of Diagnosis; EoT=End of Treatment; 3m=3 months post EoT; 6-12m=6-12 months post EoT. ns=not significant.

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Table 7A: White blood cell counts (WBC, cells/μl of blood)

WBCs	P VL/HIV	R VL/HIV	*p* values
ToD	1,500±165	1,900±374	0.0659
EoT	4,100±325	3,100±440	0.8399
3m	4,350±514	3,485±307	0.0606
6-12m	3,800±613	2,450±338	0.0388
p values	<0.0001	0.0162	

Table 7B: Red blood cell counts (RBC, cells/μl of blood)

RBCs	P VL/HIV	R VL/HIV	*p* values
ToD	2,930,000±121,116	3,455,000±125,648	0.0090
EoT	3,235,000±133,465	3,490,000±172,403	0.1042
3m	4,460,000±191,972	3,760,000±153,258	0.0254
6-12m	4,200,000±243,966	3,750,000±176,013	0.1279
p values	<0.0001	0.7023	

Table 7C: Platelet counts (PLT, cells/μl of blood)

PLT	P VL/HIV	R VL/HIV	*p* values
ToD	69,000±17,551	97,000±11,478	0.1291
EoT	207,500±29,018	172,000±19,964	0.6678
3m	222,000±29,542	118,500±11,618	<0.0001
6-12m	209,000±21,697	135,000±17,100	0.0246
p values	0.0018	0.0523	

A. White blood cell, **B.** red blood cell and **C**. platelet counts were measured in P VL/HIV (ToD: n=21, EoT: n=18, 3m: n=14, 6-12m: n=14) and R VL/HIV (ToD: n=28, EoT: n=21, 3m: n=18, 6-12m: n=14) patients.

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Controls (n=25): WBC=6,700±439 cells/μl of blood; RBC=5,290,000±111,627 cells/μl of blood; PLT=269,000±11,705 cells/μl of blood.

Statistical differences between P VL/HIV and R VL/HIV patients at each time point were determined using a Mann-Whitney test and statistical differences between the 4 different time points for each cohort of patients were determined by Kruskal-Wallis test.

ToD=Time of Diagnosis; EoT=End of Treatment; 3m=3 months post EoT; 6-12m=6-12 months post EoT. ns=not significant.

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Table 8A: Production of IFNγ in response to PHA

	P VL/HIV	R VL/HIV	*p* values
ToD	10.5±12.0	5.7±114.4	0.7534
EoT	24.6±143.7	39.1±197.2	0.4746
3m	55.5±170.9	5.3±126.1	0.1476
6-12m	30.4±36.2	15.8±209.7	0.8002
***p* values**	0.3327	0.5585	

Table 8B: Production of IL-10 in response to PHA

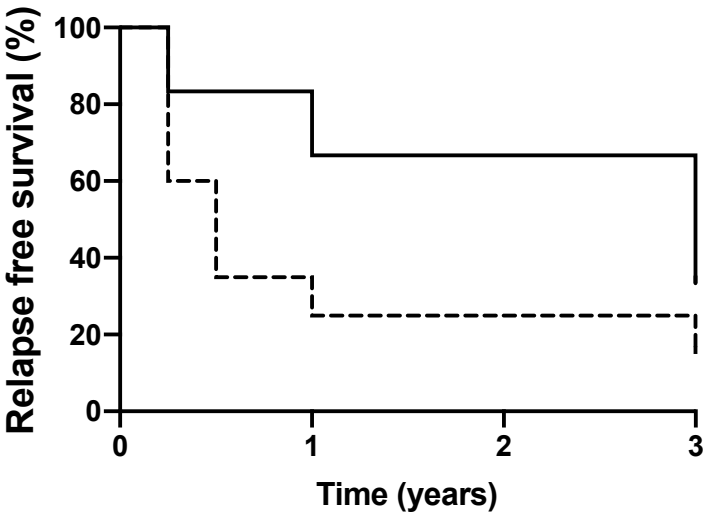
	P VL/HIV	R VL/HIV	*p* values
ToD	17.1±13.1	30.0±28.5	0.6089
EoT	145.8 ±49.3	224.9±54.8	0.5298
3m	360.0±62.7	306.9±56.1	>0.9999
6-12m	486.7±147.0	131.8±43.2	0.0126
***p* values**	<0.0001	0.008	

Whole blood cells from P VL/HIV (ToD: n=16, EoT: n=16, 3m: n=9, 6-12m: n=6) and R VL/HIV (ToD: n=22, EoT: n=23, 3m: n=16, 6-12m: n=17) patients were cultured in the presence of d PHA. **A**. IFNγ and **B.** IL-10 levels in the supernatant were measured by ELISA after 24hrs.

Statistical differences between P and R VL/HIV patients at each time point were determined using a Mann-Whitney test; statistical differences between the 4 different time points for each cohort of patients were determined by Kruskal-Wallis test.

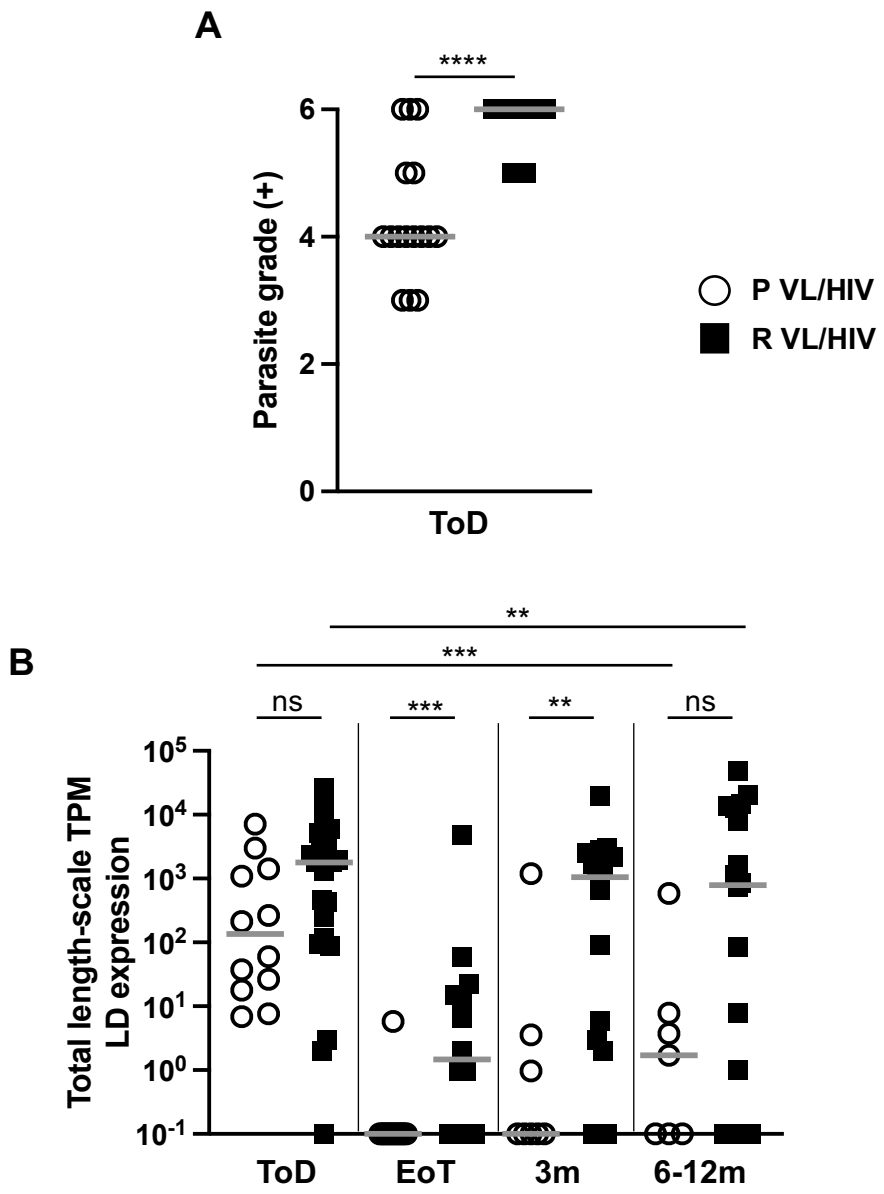
ToD=Time of Diagnosis; EoT=End of Treatment; 3m=3 months post EoT; 6-12m=6-12 months post EoT.

FIGURES



	Events	Relapse rate (%)	HR	95% CI	*p value*
P HIV/VL ———	8	67	0.39	0.15-0.99	0.03
R HIV/VL - - - -	17	85			

Figure 1


A
****
6
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Parasite grade (+)
ToD
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10^1
10^0
10^{-1}
Total length-scale TPM
LD expression
ToD
EoT
3m
6-12m

Figure 2

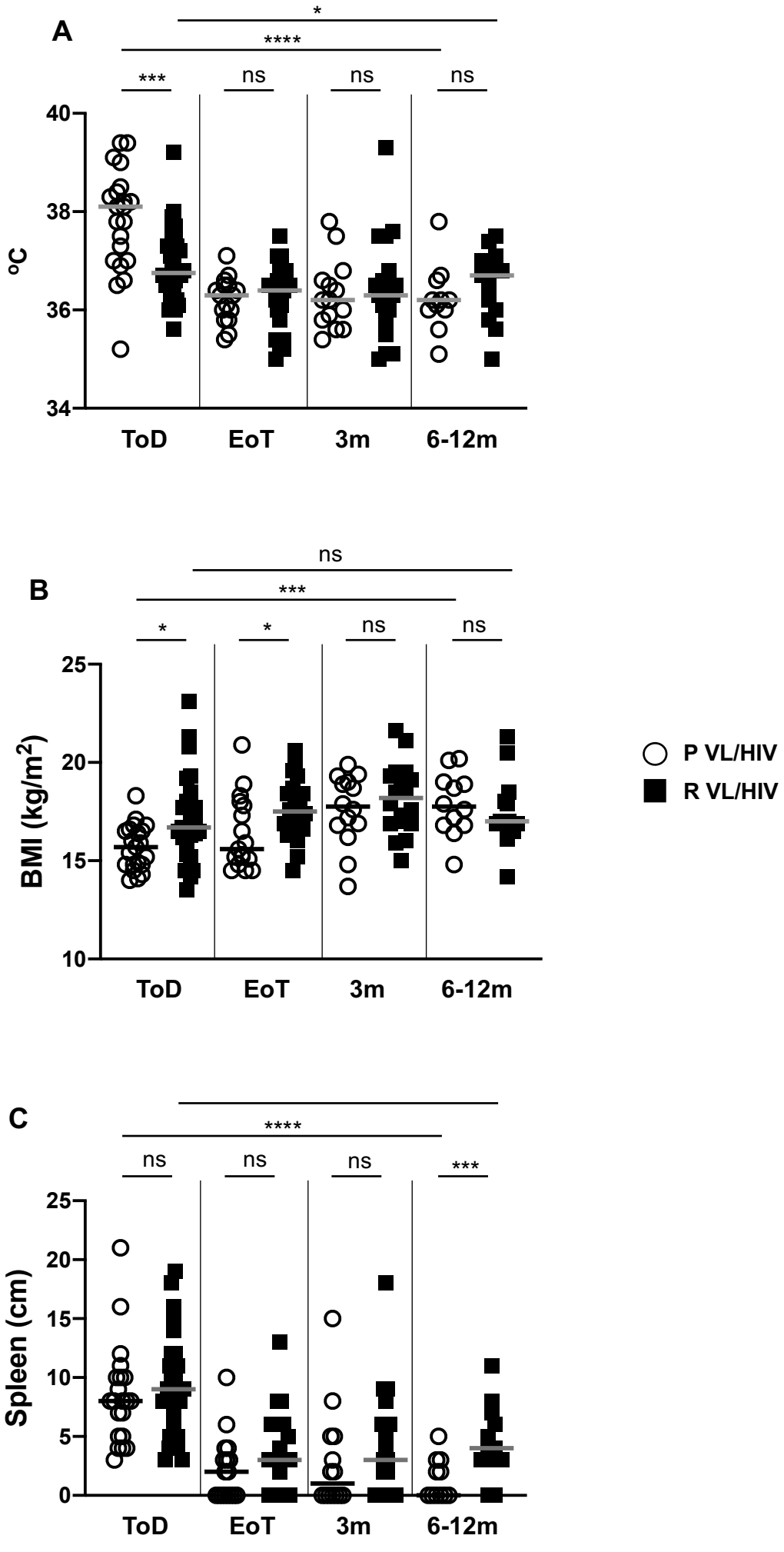
A
*
****
***
ns
ns
ns
40
38
36
34
°C
ToD
EoT
3m
6-12m
B
ns
***
*
*
ns
ns
25
20
15
10
BMI (kg/m2)
ToD
EoT
3m
6-12m
P VL/HIV
R VL/HIV
C
****
ns
ns
ns
***
25
20
15
10
5
0
Spleen (cm)
ToD
EoT
3m
6-12m

Figure 3

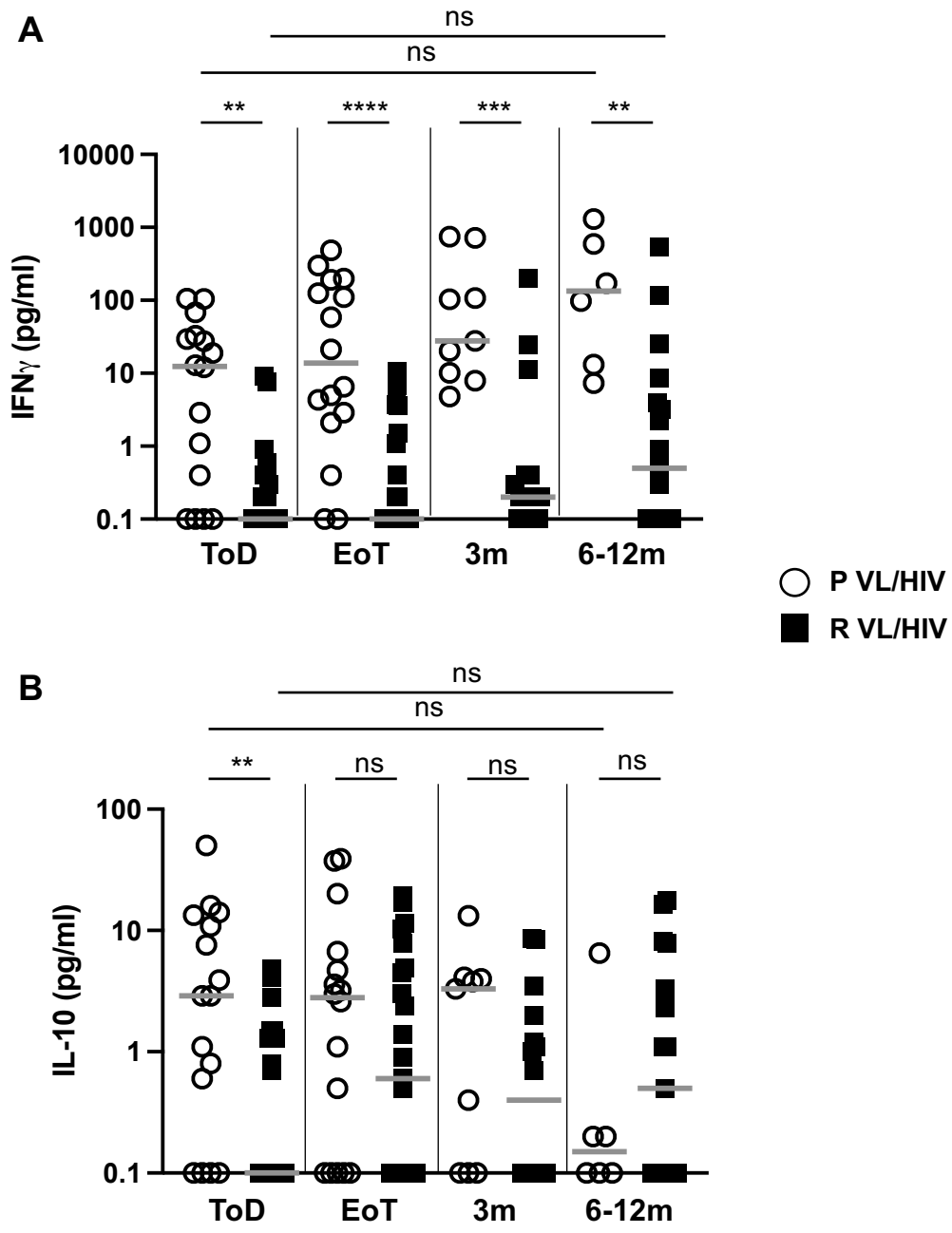
A
ns
ns
**
****
***
**
10000
1000
100
10
1
0.1
IFNγ (pg/ml)
ToD
EoT
3m
6-12m
P VL/HIV
R VL/HIV
B
ns
ns
**
ns
ns
ns
100
10
1
0.1
IL-10 (pg/ml)
ToD
EoT
3m
6-12m

Figure 4

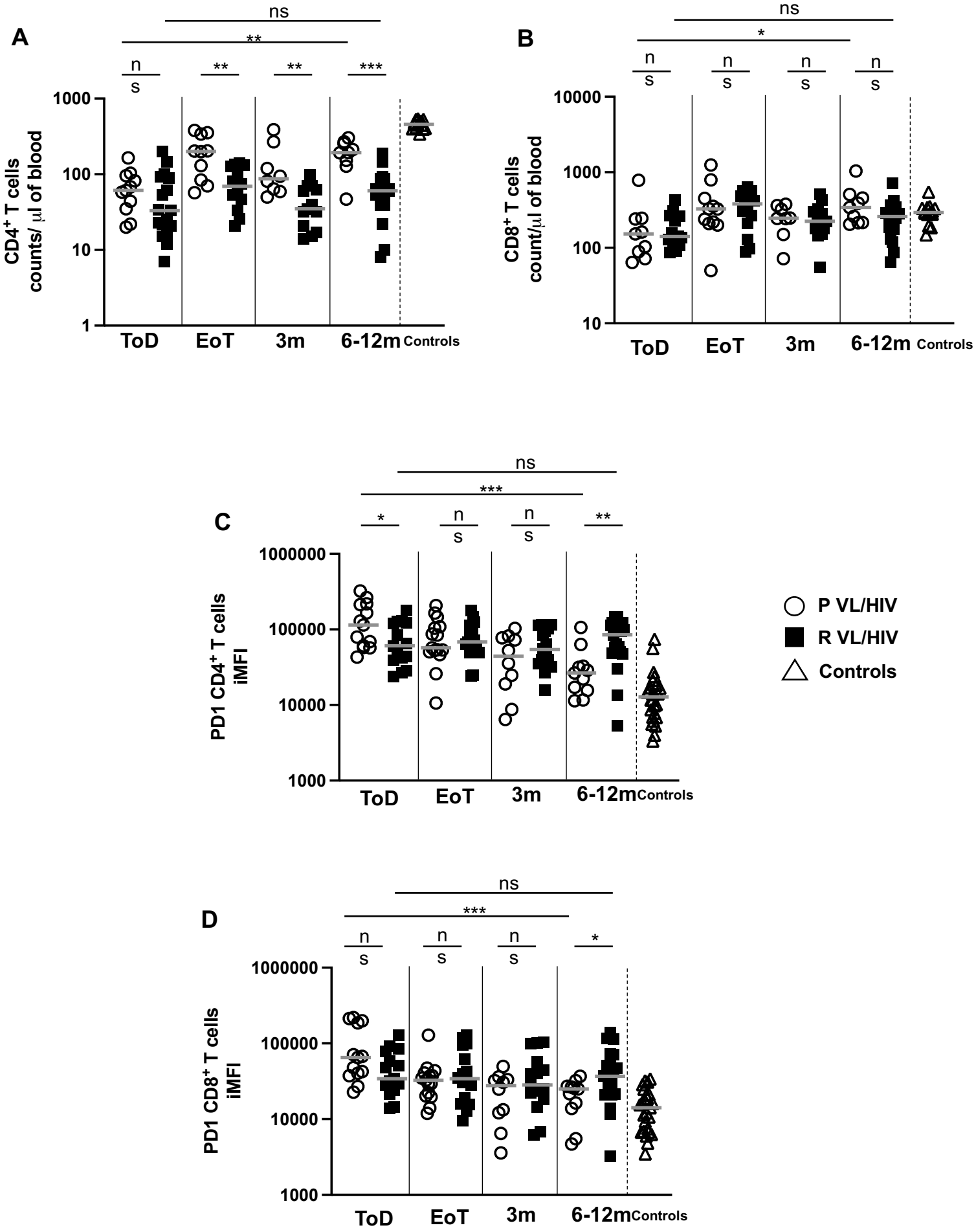
A
ns
**
n s
**
**
***
1000
100
10
1
CD4+ T cells counts/ µl of blood
ToD
EoT
3m
6-12m
Controls
B
ns
*
n s
n s
n s
n s
10000
1000
100
10
CD8+ T cells count/µl of blood
ToD
EoT
3m
6-12m
Controls
C
ns
***
*
n s
n s
**
1000000
100000
10000
1000
PD1 CD4+ T cells iMFI
ToD
EoT
3m
6-12m
Controls
P VL/HIV
R VL/HIV
Controls
D
ns
***
n s
n s
n s
*
1000000
100000
10000
1000
PD1 CD8+ T cells iMFI
ToD
EoT
3m
6-12m
Controls

Figure 5

FIGURE LEGENDS

Figure 1: Relapse free survival: Kaplan-Meier curves of participant VL relapses comparing P VL/HIV to R VL/HIV patients. The hazard ratios (with 95% confidence intervals and *p* values) obtained from the Cox model indicated the change in relapse-free survival following treatment for VL for these groups. HR, hazard ration; CI, confidence interval.

Figure 2: Parasite load: A. Quantification of *Leishmania* amastigotes in smears of splenic aspirates collected from P VL/HIV (n=16) and R VL/HIV (n=26) patients at ToD. **B.** Quantification of the total expression of *L. donovani* mRNA in blood from P (ToD: n=12, EoT: n=16, 3m: n=8, 6-12m: n=7) and R VL/HIVVL/HIV (ToD: n=23, EoT: n=14, 3m: n=16, 6-12m: n=18)

Each symbol represents the value for one individual, the straight lines represent the median. Statistical differences between P and R VL/HIV patients at each time point were determined using a Mann-Whitney test; statistical differences between the 4 different time points for each cohort of patients were determined by Kruskal-Wallis test.

LD mRNA= *L. donovani* mRNA. ToD=Time of Diagnosis; EoT=End of Treatment; 3m=3 months post EoT; 6-12m=6-12 months post EoT.

Figure 3: Clinical parameter: A. Body temperature was measured on P VL/HIV (ToD: n=21, EoT: n=16, 3m: n=14, 6-12m: n=11) and R VL/HIV (ToD: n=28, EoT: n=22, 3m: n=14, 6-12m: n=13) patients. **B.** BMI was calculated for P VL/HIV (ToD: n=21, EoT: n=17, 3m: n=14, 6-12m: n=12) and R VL/HIV (ToD: n=28, EoT: n=22,

3m: n=18, 6-12m: n=13) patients. **C.** Spleen size as measured in cm below the costal margin on VL (ToD: n=21, EoT: n=17, 3m: n=14, 6-12m: n=11), VL/HIV (ToD: n=28, EoT: n=22, 3m: n=18, 6-12m: n=13) patients.

Each symbol represents the value for one individual, the straight lines represent the median. Statistical differences between P VL/HIV and R VL/HIV patients at each time point were determined using a Mann-Whitney test; statistical differences between the 4 different time points for each cohort of patients were determined by Kruskal-Wallis test.

ToD=Time of Diagnosis; EoT=End of Treatment; 3m=3 months post EoT; 6-12m=6-12 months post EoT. ns=not significant.

Figure 4:

Whole blood cells from P VL/HIV (ToD: n=16, EoT: n=16, 3m: n=9, 6-12m: n=6) and R VL/HIV (ToD: n=22, EoT: n=23, 3m: n=16, 6-12m: n=17) patients were cultured in the presence of SLA and PBS. **A**. IFNγ and **B.** IL-10 levels in the supernatant were measured by ELISA after 24hrs and the values obtained with PBS alone was deducted from the value obtained with SLA. Statistical differences between P and R VL/HIV patients at each time point were determined using a Mann-Whitney test; statistical differences between the 4 different time points for each cohort of patients were determined by Kruskal-Wallis test.

ToD=Time of Diagnosis; EoT=End of Treatment; 3m=3 months post EoT; 6-12m=6-12 months post EoT.

Figure 5: $CD4^+$, $CD8^+$ T cell counts and PD1 expression: A. $CD4^+$ T cell counts were measured by flow cytometry in the blood P VL/HIV (ToD: n=11, EoT: n=10 3m:

n=8, 6-12m: n=9) and R VL/HIV (ToD: n=17, EoT: n=14, 3m: n=13, 6-12m: n=16) patients. **B.** $CD8^+$ T cell counts were measured by flow cytometry in the blood of P VL/HIV (ToD: n=13, EoT: n=12, 3m: n=8, 6-12m: n=6) and R VL/HIV (ToD: n=11, EoT: n=12, 3m: n=13, 6-12m: n=10) patients. **C.** CD4 PD1 iMFI was measured by multiplying the % of $CD4^+$ T cells and the median fluorescence intensity of PD1 as measured by flow cytometry in the PBMCs of P VL/HIV (ToD: n=13, EoT: n=15, 3m: n=10, 6-12m: n=11) and R VL/HIV patients (ToD: n=15, EoT: n=17, 3m: n=16, 6-12m: n=21). **D.** CD8 PD1 iMFI was measured by multiplying the % of $CD8^+$ T cells and the median fluorescence intensity of PD1 as measured by flow cytometry in the PBMCs of P VL/HIV (ToD: n=13, EoT: n=15, 3m: n=10, 6-12m: n=11) and R VL/HIV patients (ToD: n=15, EoT: n=17, 3m: n=16, 6-12m: n=21).

Each symbol represents the value for one individual, the straight lines represent the median. Statistical differences between P VL/HIV and R VL/HIV patients at each time point were determined using a Mann-Whitney test and statistical differences between the 4 different time points for each cohort of patients were determined by Kruskal-Wallis test.

ToD=Time of Diagnosis; EoT=End of Treatment; 3m=3 months post EoT; 6-12m=6-12 months post EoT. ns=not significant.