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Research Paper

Thalidomide is Associated With Increased T Cell Activation and Inflammation in Antiretroviral-naive HIV-infected Individuals in a Randomised Clinical Trial of Efficacy and Safety

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ABSTRACT

Trial Design: Open-label, randomised, controlled, pilot proof-of-concept clinical trial.
Methods: Participants: Antiretroviral naïve adult males with CD4 count ≥350 cells/mm^3.
Interventions: Patients were randomised to receive thalidomide 200 mg QD for 3 weeks (Thalidomide group) or not (Control group) and followed for 48 weeks.
Objective: We hypothesized that short-term Thalidomide use would reduce HIV related inflammation and HIV replication among antiretroviral naïve HIV infected individuals.
Outcome: Viral loads, CD4/CD8 counts, ultra-sensitive C-reactive protein (US-CRP), cell activation markers, and plasma lipopolysaccharide (LPS) were analyzed.
Randomisation: Unrestricted randomisation.
Blinding: No blinding was used.
Results: Numbers randomised: Thirty recruited individuals were randomised to Thalidomide (16 patients) or Control (14 patients) groups.
Recruitment: Patients were recruited from April 2011 to January 2013.
Outcome: Viral loads remained stable in both groups. During thalidomide treatment, a decrease in CD4/CD8 ratio ($p = 0.04$), a decrease in CD4 count ($p = 0.04$), an increase in cell activation calculated by the percentage of CD38$^+$/HLA-DR$^+$ CD8 cells ($p < 0.05$) and an increase in US-CRP ($p < 0.01$) were observed in the Thalidomide group, with all parameters returning to baseline levels after thalidomide interruption. We confirmed that thalidomide increased HIV replication *in vitro* of 6 of 7 samples from long-term antiretroviral suppressed individuals.
Harms: No class 3/4 adverse events occurred.
Conclusions: Short-term use of thalidomide led to an intense transient increase in T cell activation and inflammation, with a decrease in the CD4$^+$ cell count without changes to the CD8$^+$ cell count. We confirmed that thalidomide acts *in vitro* as a latency reversal agent and speculate that the *in vivo* results obtained were due to an increase in HIV replication.

1. Introduction

Generalized immune activation and inflammation are hallmarks of the pathogenesis of HIV/AIDS (d'Ettorre et al., 2011). Important factors driving HIV-induced immune activation include ongoing viral replication, microbial translocation from the gastrointestinal (GI) tract and co-infections (Marchetti et al., 2013). Irreversible damage to the integrity of the GI tract's mucosal barrier occurs early during HIV-1 acute infection caused by the irreversible massive depletion of CD4$^+$ T (memory) cells from the GI tract mucosa, resulting in systemic activation of the immune system *via* the translocation of GI tract microbes and lipopolysaccharide (LPS, as a marker of microbial translocation) across the

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damaged barrier (Marchetti et al., 2013). New ultrasensitive techniques can detect very low levels of detectable HIV RNA in rectal samples of HIV-infected individuals with undetectable plasma viremia. Despite being on antiretroviral therapy (ART), this ongoing low-level replication at sites such as the GI tract contributes to the disease burden. During ART, microbial translocation declines but does not return to normal levels, indicating the potential role of microbial translocation in HIV-induced immune activation (Palmer, 2013).

The HIV-induced dysregulation of $CD4^+$ T regulatory (Treg) cells also results in a permanently over-activated immune system due to the lack of immunomodulation (Chevalier and Weiss, 2013). The overburden of viral gene products together with those of foreign pathogens causes the overactivation of both T and B cells. The non-specific production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), IL-6 and several others, causes the bystander activation of T and B lymphocytes and ultimately leads to immunosenescence (Reeves, 2014). HIV-1 replication *per se* also relates to immune activation, and the higher the viral load, the higher the immune activation process (Hunt et al., 2003). It has been demonstrated that even defective HIV may produce HIV proteins, which conceivably maintains a state of inflammation in antiretroviral-suppressed individuals (Imamichi et al., 2016). Interestingly, cell activation is higher among HIV-infected elite controllers than among uninfected individuals or antiretroviral-treated individuals with viral loads below detection limits (Hunt et al., 2006).

This persistent immune activation and inflammation is responsible for enhanced $CD4^+$ T cell death, lymphoid tissue destruction, kidney and liver problems, atherosclerosis, cardiovascular disease and neurocognitive impairment (Reeves, 2014). Therefore, the future treatment of HIV/AIDS requires the consideration of anti-inflammatory strategies with antivirals to overcome chronic inflammation-associated complications.

HIV-related immune activation is associated with a rapid and persistent increase in TNF levels (McMichael et al., 2010), resulting in the induction of adhesion molecules and chemokines from the vessel wall (Signorelli et al., 2007), endothelial cell activation and the increased adhesion of leukocytes (Mu et al., 2007).

One of the drugs known for its immunomodulatory potential is thalidomide.

Thalidomide has a beneficial effect on skin lesions of leprosy, erythema nodosum leprosum (Teo et al., 2002) and multiple myeloma (Chen et al., 2010). Thalidomide is used in the treatment of various autoimmune or inflammatory disorders, such as Behçet disease, cutaneous lupus erythematosus, prurigo nodularis, sarcoidosis, chronic graft-*versus*-host disease, and Kaposi's sarcoma (Chen et al., 2010; Franks et al., 2004).

The pharmacological effects of thalidomide have established it as an anti-inflammatory, immuno-modulatory and anti-angiogenic drug. The mechanisms underlying these features involve the modulation of inflammatory cytokines, such as TNFα, interferon gamma (IFNγ), IL-10, IL-12, cyclooxygenase 2 (COX-2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Franks et al., 2004).

The transcription factor NFκB controls the anti-inflammatory and anti-angiogenic properties of thalidomide. NFκB is located in the cytoplasm in its inactive form bound to the inhibitory protein IκBα. Once stimulated by inducers such as TNF-α or IL-1β, the phosphorylation of IκBα proteins by IκB kinase (IKK) releases NFκB, which translocates to the nucleus and induces the activation of genes responsible for immune and inflammatory responses, cellular proliferation, angiogenesis and the suppression of apoptosis (Wang et al., 1999). Thalidomide exerts its anti-inflammatory effect by enhancing the degradation of TNF-α mRNA, as observed in endotoxin-stimulated monocytes and macrophages (Moreira et al., 1993). In contrast to this anti-TNF activity of thalidomide, IL-2-dependent upregulation of TNF-α by $CD4^+$ and $CD8^+$ T lymphocytes has also been observed *in vitro* (Marriott et al., 2002).

NFκB is an enhancer of HIV-1 transcription, which, upon the activation of an infected cell, migrates to the nucleus and induces the expression of HIV-1 genes (Pomerantz et al., 1990). Because of the convergence of the regulatory pathways for the transcription of HIV and of cytokine genes, it is possible that interventions directed at limiting the cytokine response to HIV-1 and opportunistic infections could have a profound impact on the replicating HIV-1 levels as well as the associated morbidity and mortality.

The link between circulating HIV and TNF-α (Klausner et al., 1996) or TNF-α receptors (Bilello et al., 1996) has long been identified in the plasma of infected individuals, and TNF-α induces viral replication *via* a shared NFκB-dependent transcriptional control mechanism (Pomerantz et al., 1990). Targeting TNF-α may reduce viral replication, resulting in lower inflammation. Thalidomide has shown promising results in inhibiting the production of TNF-α both *in vitro* and *in vivo* (Sampaio et al., 1993). These observations have provided the rationale for exploring the anti-inflammatory potential of thalidomide to inhibit TNF-α, thereby reducing HIV replication and associated inflammation.

The present study was designed to examine whether thalidomide treatment of HIV-1-infected individuals would reduce the levels of HIV surrogate markers of inflammation. We recruited HIV-infected individuals who had not undergone ART, in whom any potential anti-inflammatory properties of thalidomide would be better quantified. We treated these individuals for a period of 3 weeks with thalidomide and followed the patients for 23 weeks. The results were compared to those obtained in a control group consisting of ART-naïve individuals who remained naïve throughout the study period.

Hypothesis. We hypothesized that the use of thalidomide would be safe and able to decrease HIV-related inflammation and bacterial translocation. If this hypothesis is confirmed, new trials should include HIV antiretroviral-treated individuals with viral loads below detection limits to further explore the impact of anti-TNF drugs on HIV-1-related inflammatory markers.

2. Materials and Methods

2.1. Study Design and Participants

This was an open-label, randomised pilot proof-of-concept trial. Thirty individuals were recruited in Rio de Janeiro, Brazil and followed for 23 weeks from April 2011 to January 2013. The subjects were randomly allocated into two groups: the Thalidomide group or Control group (with no thalidomide exposure).

Patients were ART-naïve males from 18 to 60 years of age with a body weight ≥ 50 kg and $CD4^+$ T cell counts > 350 cells/mm^3. The participants were not eligible to begin ART according to local guidelines for the study period. (http://www.aids.gov.br/sites/default/files/consensoAdulto005c_2008montado.pdf)

Informed consent forms were signed by the participants, and the study protocol was approved by the Ethics Committee and Institutional Review Board at the Universidade Federal de São Paulo, Brazil (#17686/09) and Universidade Federal do Rio de Janeiro (#658/10).

2.2. Interventions

Patients were randomised to either receive 100 mg of thalidomide BID for 3 weeks upon study entry (Thalidomide group, 16 patients) or not (Control group, 14 patients); all were followed for 23 weeks. Blood samples were collected for safety tests and HIV monitoring tests, and laboratory work was performed at the Laboratório de Retrovirologia of the Universidade Federal de São Paulo, Brazil. The cell activation markers were double-checked at the Laboratório Interdisciplinar de Pesquisas Médicas, Instituto Oswaldo Cruz/FIOCRUZ, Rio de Janeiro, Brazil.

2.3. Sample Size

As a pilot study, a convenience sample size of 30 patients was determined.

2.4. Randomisation

Unrestricted randomisation. Allocation to distinct groups was determined using blind envelopes.

2.5. Clinical and Laboratory Assessments

Clinical and laboratory assessments were performed at the screening, baseline and at weeks 1, 2, 3, 7, 11, 15, 19, and 23. Patients were evaluated for HIV-1 viral loads, $CD4^+$ and $CD8^+$ T cell counts, ultra-sensitive C-reactive protein (US-CRP), CD38 and HLA-DR on $CD4^+$ and $CD8^+$ T cells, and LPS quantitation in plasma samples. Blood cell counts and biochemical parameters such as aminotransferase enzymes (ALT and AST), creatinine, and glucose were determined at the screening and at each visit. Electrocardiography was performed at the screening and the last visit.

Clinical assessment included clinical signs and symptoms related to HIV infection, adverse events, and adherence to thalidomide intake using a predefined questionnaire.

2.6. Laboratory Methods

2.6.1. Flow Cytometric Evaluation of Cellular Activation

Peripheral blood mononuclear cells (PBMC) were separated and cryopreserved. After thawing, PBMCs were labeled with anti-CD3 APC and anti-CD4 PercP (for the lymphocytic subpopulation) and anti-CD38 FITC and anti-HLA-DR PE (for cellular activation). Fixed cells were acquired on a FACSCalibur apparatus and analyzed with CellQuest software (BD Biosciences, San Diego CA, USA). The results were expressed as the percentage of $CD38^+$ $HLA\text{-}DR^+$ cells among $CD4^+$ and $CD8^+$ T lymphocytes.

2.6.2. Assessment of Plasmatic LPS Levels

Microbial translocation was inferred by plasmatic LPS assessments using QCL-1000 Limulus Amebocyte Lysate (Lonza Walkersville Inc., Walkersville, MD, USA) according to the manufacturer's instructions. The results were expressed as pg/mL, and the minimum limit of detection was 10 pg/mL.

2.6.3. Cytokine Measurement

Cytokine levels were measured in plasma by Cytometric Beads Array kit (CBA, BD Biosciences, San Diego, CA, USA) processed according to the manufacturer's protocol. The following cytokine were quantified: IL-2, IL-4, IL-6, IL-10, IL-17, TNF and IFN-γ. A FACSCalibur were used to acquire the samples and data analysis was performed using the software provided by the manufacturer (FCAP Array, BD Biosciences). The standard curve for each cytokine covers a range of 20 to 5000 pg/mL.

2.6.4. Determination of CXCR4 and CCR5 Co-receptor Genotropism

After RNA extraction, reverse transcription nested PCR to amplify the HIV-1 C2V3 region of gp120 and sequencing were carried out as previously described (Lavigne et al., 2014). V3 loop sequences were interpreted to infer co-receptor tropism by web-based genotyping algorithms in Geno2pheno [coreceptor] (http://www.coreceptor.geno2pheno.org). Because only one PCR product was subjected to sequencing, in accordance with European guidelines, a false positive rate (FPR) of 10% was used to infer tropism (Poveda et al., 2012), and samples were classified as R5 (FPR > 10%) or non-R5 (FPR ≤ 10%) tropic.

2.6.5. In Vitro Determination of Thalidomide's Effect on the HIV-1 Proviral Compartment

$CD8^+$ T-cell depleted PBMCs (EasySep Human CD8 Positive Selection kit, STEMCELL TECHNOLOGIES) isolated from 7 HIV-infected ART-treated patients with undetectable viral loads over a period of two years were used. We measured HIV-1 recovery in *ex vivo* cell cultures first activated by phytohemagglutinin and treated with thalidomide (Sigma Aldrich) for 48 h and then cultured in RPMI medium (Gibco, CA, USA) supplemented with IL-2 20 IU/mL (Roche) and fetal bovine

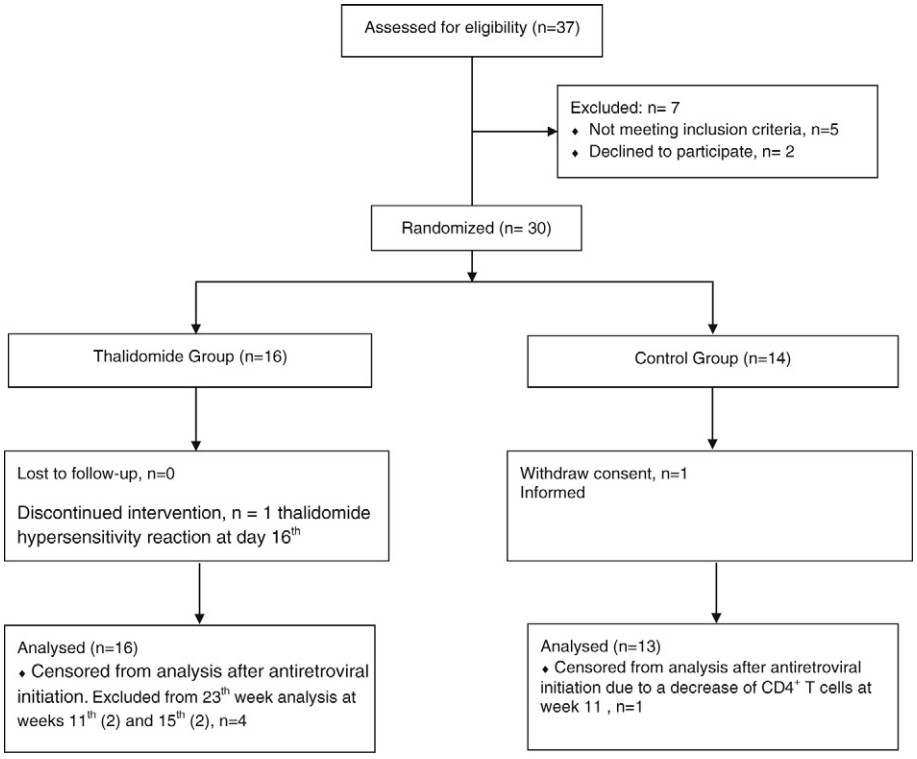

Fig. 1. Participant's flowchart.

serum (Gibco). Fetal bovine serum was used as the control sample. Supernatant samples were taken at days 3, 7, 10, 14, 17, 21, 24, 28 and 31 and monitored by commercial HIV viral load assay (Abbott RealTime HIV-1 assay, Abbott, Chicago IL).

2.7. Statistical Analyses

The Mann-Whitney *U* test, Student's *t*-test, analysis of variance (ANOVA), and the Kruskal-Wallis test with Dunn's *post hoc* test were used for comparisons between groups. Spearman's test was used to perform correlations between different parameters. GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was employed for the analyses.

3. Results

3.1. Patients' Dispositions, Baseline Characteristics and Virologic/Immunologic Response to Therapy

The participant's Flow chart is described in Fig. 1. Thalidomide-treated patients and control patients were comparable in terms of age, T cell counts and HIV viral load at the beginning of the study (Table 1). One patient in the Thalidomide group was co-infected with HCV, and one patient in the Control group was co-infected with HBV.

Adherence was measured weekly using a predefined questionnaire, and the pill count indicated 100% thalidomide use. One patient stopped the medication on day 16 due to a mild cutaneous rash that disappeared upon thalidomide interruption. There were no grade 3 or 4 adverse events during thalidomide use (Table 2).

Table 1
Baseline characteristics of the thalidomide and control groups.

Characteristics	Thalidomide	Control	p-Value
N	16	14	
Age (±SD)	36.88 (±9.28)	31.36 (±5.47)	0.16
Viral load log10			
Mean (±SD)	3.67 (±1.33)	3.82 (±0.57)	0.59
Median	4.11	3.75	
IQR	3.83–4.62	3.46–4.21	
$CD4^+$ T cells/mm^3			
Mean (±SD)	623 (±231)	605 (±255)	0.93
Median	602	625	
IQR	468–660	388–716	
$CD8^+$ T cells/mm^3			
Mean (±SD)	1162 (±396)	1014 (±360)	0.57
Median	1117	1061	
IQR	865–1383	734–1278	
CD4/CD8 ratio			
Mean (±SD)	0.58 (±0.23)	0.67 (±0.36)	0.77
Median	0.55	0.52	
IQR	0.38–0.71	0.41–0.85	
CD4/CD38/HLA-DR			
Mean (±SD)	7.10 (±4.7)	5.98 (±3.42)	0.75
Median	5.64	5.68	
IQR	2.82–12.1	3.18–7.36	
CD8/CD38/HLA-DR			
Mean (±SD)	20.41 (±13.6)	20.07 (±8.8)	0.93
Median	18.32	16.72	
IQR	7.73–32.74	13.90–24.25	
US-PCR mg/dL			
Mean (±SD)	2.70 (±1.6)	2.15 (±1.09)	0.45
Median	2.72	2.03	
IQR	1.34–4.28	1.21–3,07	
LPS level pg/mL			
Mean (±SD)	59.51 (±28,7)	46.59 (±21.7)	0.30
Median	51.05	42.9	
IQR	35.15–79.03	30.70–66.70	
B subtype			
R5	8	11	
Change of tropism	0/8	4/11	

Table 2
Adverse events during the clinical trial period. No grade 3 and 4 adverse events were detected.

Thalidomide group	Week 1	Week 2	Week 3	Week 7	Week 15	Week 21	Week 23
	N	N	N	N	N	N	N
Probably unrelated to thalidomide							
Syphilis		1			1		
HPV					1		
Possibly unrelated to thalidomide							
Diarrhea	1					1	
Common cold symptoms		3	2*	2*	1*		
Infectious gastroenteritis		1	1*				
Sinusitis			1				
Furunculosis						1	
Intestinal constipation			1				
Possibly related to thalidomide							
Somnolence	12	10	9				
Skin rash			1				
Dry mouth			1				
Dry skin		1	1				
Control group							
Gingivitis	1	1*	1*	1*	1*	1*	
Cold			1		1		1*
Dental abscess					1	1*	
Tonsillitis					1		
Syphilis					1		
Gonorrhea					1		

* Patient presented the same symptoms at subsequent visit. N = number of patients.

The mean baseline viral loads were 3.67 (range: <2.6–4.85) and 3.82 (range: 2.93–4.95) log_{10} HIV-1 RNA copies/mL for the Thalidomide and Control groups, respectively (Table 1). A non-significant increase in viral load was observed in Thalidomide group during the three weeks of thalidomide therapy; the mean viral load increased from 3.67 to 3.83 log_{10} HIV-1 RNA copies/mL, returning to baseline values after thalidomide withdrawal (Supplemental Table 1). At week 23, the mean viral loads were 3.65 (range: <50–5.67) and 4.06 (range: 3.38–5.04) log_{10} copies/mL for the Thalidomide and Control groups, respectively (Supplemental Tables 1 and 2). One patient (TV13) in the Thalidomide group was an elite controller, and at week 8, detectable viremia of 67 (1.83 log_{10}) copies/mL was observed, which returned to undetectable levels in subsequent visits (Supplemental Table 1).

The screening mean $CD4^+$ T cell count was 623 cells/mm^3 ± 230 (median: 602 cells/mm^3, IQR: 468–660 cells/mm^3) and 605 cells/mm^3 ± 255 (median: 625 cells/mm^3, IQR: 388–716 cells/mm^3) for the Thalidomide and Control groups, respectively. Declines in the $CD4^+$ T cell count ($p = 0.04$) and the CD4/CD8 ratio ($p = 0.05$) were observed during thalidomide use in the Thalidomide group, returning to baseline levels after drug withdrawal (Fig. 2) (Supplemental Tables 1 and 2). During the study period, the $CD4^+$ T cell count of four patients (TV03, 11, 29 and 31) in the Thalidomide group and one patient (TV12) in the Control group declined to <350 cells/mm^3 (were excluded from the subsequent analysis, as they initiated ART (Fisher's exact test: $p = 0.6$)).

The overall screening mean $CD8^+$ T cell counts were 1162 ± 396 cells/mm^3 (median: 1117 cells/mm^3, IQR: 865–1383 cells/mm^3) and 1014 ± 360 cells/mm^3 (median: 1061 cells/mm^3, IQR: 734–1278 cells/mm^3) for the Thalidomide and Control groups, respectively. No significant change in the $CD8^+$ T cell count was detected in either group (Table 1, Supplemental Tables 1 and 2).

As seen in Fig. 3, the mean Leucocytes count in the Thalidomide group was 6577.33 cells/mL as compared to 5402 cells/mL at week 23 ($p = 0.03$), and mean lymphocytes counts was 2283.79 cells/mL at

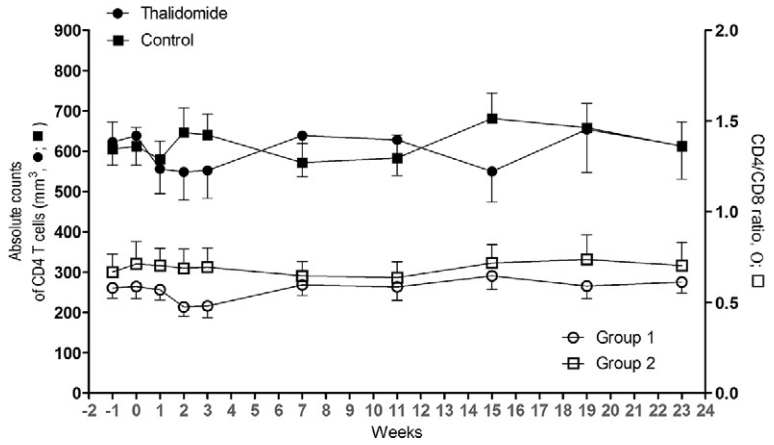

Fig. 2. Ratio of $CD4^+$ T cell counts and $CD4/CD8^+$ T cell counts over time among groups of Thalidomide and Control patients. Peripheral blood from patient of all groups was collected at each visit during 24 weeks for the absolute quantification of $CD4^+$ and $CD8^+$ T lymphocytes by flow cytometry. The closed symbols relate to absolute $CD4^+$ T cell counts, and open symbols relate to the $CD4/CD8^+$ ratio. The results are expressed as the mean ± standard deviation. N = number of participants.

baseline and 1842 cells/mL at week 23 ($p = 0.001$), whereas the mean Leucocytes in the Control group were 5610.77 and 5653.08 cells/mL at baseline and week 23 respectively ($p = 0.9$), and mean lymphocytes counts were 1973.54 and 1009.92 cells/mL at baseline and week 23 respectively ($p = 0.7$).

3.2. Thalidomide was Accompanied by Increased Inflammation and Cell Activation Levels

At study onset, the levels of $CD4^+$- or $CD8^+$-activated T lymphocytes, as inferred by the percentages of cells expressing CD38 and HLA-DR, were similar in both groups, as shown in Table 1.

Counterintuitively, the Thalidomide group presented an increased cell activation status inferred by the percentage of $CD38^+/HLA\text{-}DR^+$ $CD4^+$ cells and the $CD8^+$ T cell counts ($p < 0.05$). This analysis was repeated in the two participating laboratories at UNIFESP and FIOCRUZ, and the results were consistent (Fig. 4). In the elite controller patient, the percentage of $CD38/HLA\text{-}DR^+$ on $CD8^+$ cells increased during the thalidomide period from 3.2% to 11.3% after 3 weeks of thalidomide and then decreasing to 2.2, 2.4 and 1.8% at weeks 8, 12 and 20, respectively. No activation of $CD4^+$ T cells was observed (data on file).

Furthermore, an increase in the unspecific inflammatory marker US-CRP was also detected in the Thalidomide group exclusively during the thalidomide intake period (Fig. 5, $p = 0.004$). All 30 enrolled volunteers showed a US-CRP level above 0.1 mg/dL during the screening visit; this level was above 1 mg/dL in 88% of the Thalidomide group and 86% of the Control group.

In relation to LPS levels, there was no significant baseline difference between the Thalidomide group (mean: 59.5 ± 28.7 pg/mL) and Control group (mean: 46.6 ± 21.7 pg/mL). However, a non-statically

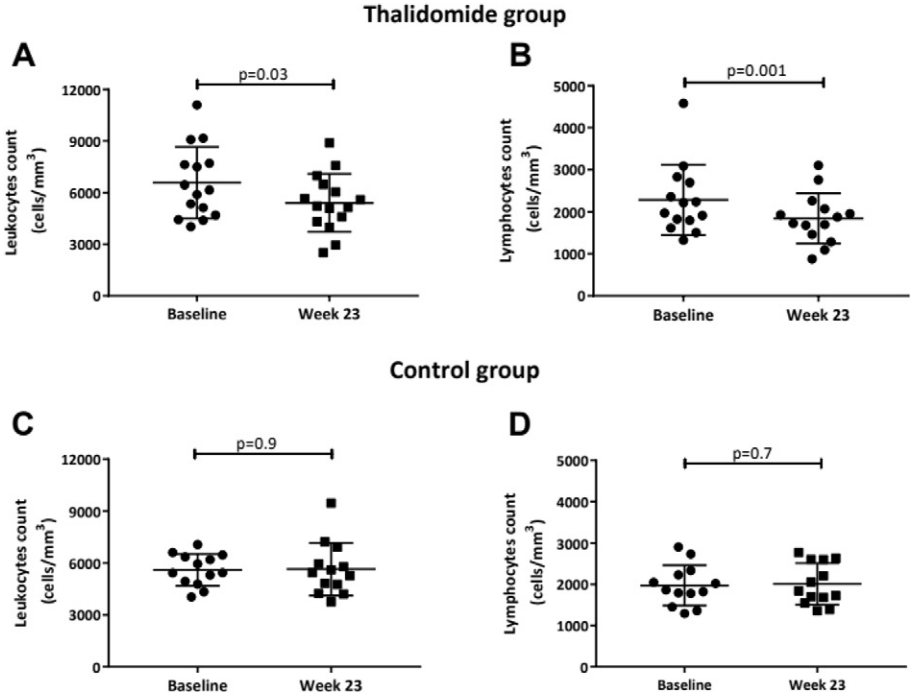

Fig. 3. Leukocytes and Lymphocytes count at baseline and at the end of the study. Results are shown for the Thalidomide group (panels A and B respectively), and the control group (panels C and D respectively), and statistical analysis was performed using Pared parametric *t*-test.

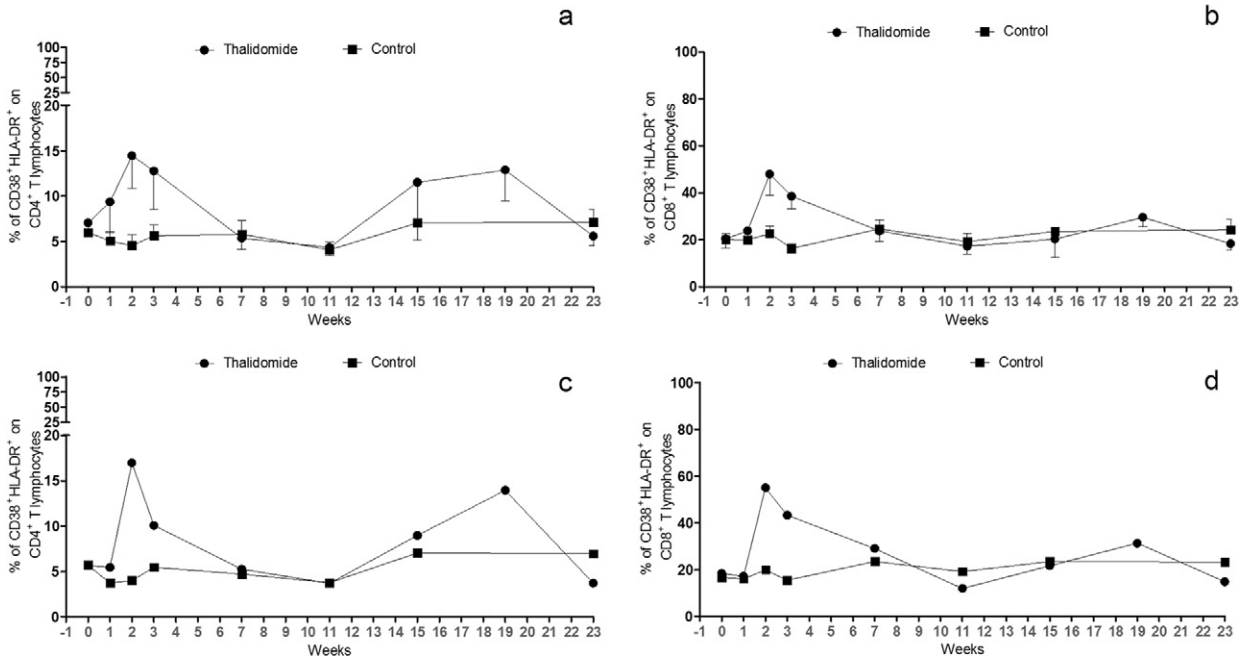

Fig. 4. T cell activation status over time among the Thalidomide and Control groups. Peripheral blood mononuclear cells (PBMC) from patients of all groups were obtained by Ficoll-Hypaque gradient centrifugation. PBMC were thawed and used for immunophenotyping at the pre-established periods over 24 weeks. The percentages of $CD38^+$/HLA-DR^+ $CD4^+$ T cells (2A) and $CD8^+$ T cells (2B) are expressed as the mean ± standard deviation (Panel A) and median (Panel B). The Thalidomide group showed an increased cell activation status compared to the Control group ($p < 0.05$). Thalidomide intake began in week 1 and was completed at the end of week 3. Patients were followed up until the end of week 23.

significant increase in the LPS levels was observed in the Thalidomide group during thalidomide intake with a reduction in the levels observed at subsequent visits.

3.2.1. Additional Correlations

There was also a positive correlation between T cell activation and viral load, demonstrated in both the percentage of CD38/HLA-DR^+ $CD8^+$ cells and the $CD4^+$ T cell counts in both the Thalidomide and Control groups ($p = 0.001$, Supplemental Fig. 1). Interestingly, the increase in LPS level was accompanied by an increase in the US-CPR level in the Thalidomide group ($p = 0.05$, Supplemental Fig. 2). Interestingly, there was an apparent increase in the TNF levels in some individuals on the Thalidomide arm as compared to the control arm when comparing baseline levels to the levels encountered on the last day of Thalidomide intake (Fig. 6). However, no statistical significance in the mean and median TNF levels between different time points in each group. No statistical difference was detected in the other cytokine levels related to the period of Thalidomide intake (data on file).

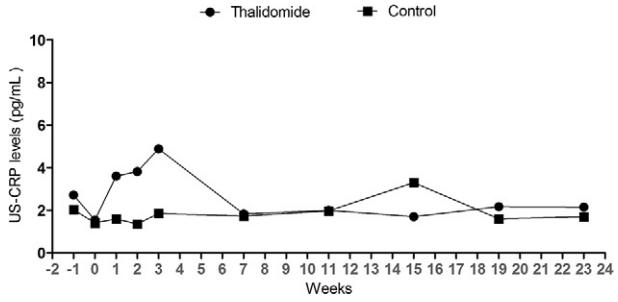

Fig. 5. Inflammatory status over time in the Thalidomide group and Control group inferred by the levels of ultra-sensitive C-reactive protein (US-CRP) were assessed in the patients plasma by colorimetric assay. The levels are expressed as medians.

3.3. HIV Evolution Vis a Vis Co-receptor Tropism

To understand the changes in the $CD4^+$ T cell count during thalidomide therapy and the possible link with the HIV-1 subtype or co-receptor used, co-receptor tropism was assessed from plasma RNA. Initially, co-receptor tropism was assessed at screening and week 24. In case of amplification failure and/or detection of non-R5 viruses, intermediate collection time points were also analyzed. Amino acid alignments of the HIV-1 envelope V3 loops along with their inferred viral tropism and subtypes are shown in Supplemental Figs. 3 and 4. The co-receptor tropism of both groups at successive visits is shown in Table 3. No significant differences were observed in either group in terms of viral load and $CD4^+$ and $CD8^+$ T cell counts on the basis of the tropism profiles (data on file).

As shown in Table 3, a change/switch in co-receptor tropism was not observed in 7 individuals in the Thalidomide group harboring R5 viruses at the final visit of the study period. However, in the Control group, 3 out of 12 patients switched to using CXCR4 during the study period (Fisher exact test, $p = 0.2$). HIV-1 subtypes and the co-receptor tropism profiles of the individuals were not found to influence the changes in $CD4^+$ T cell counts observed in our study. The observed changes in $CD4^+$ T cell counts were therefore an effect of thalidomide therapy (Table 3).

3.4. The In Vitro Effect of Thalidomide on HIV-infected Cells

The altered laboratory values detected in the Thalidomide group returned to baseline levels after thalidomide withdrawal. Although safe, the short-term use of thalidomide among ART-naïve individuals led to a counter-intuitive intense transitory increase in T cell activation and inflammation, with a decrease in $CD4^+$ T cells without a detectable change in $CD8^+$ T cells and viral loads, although it is inherently difficult to detect changes in viral load among naturally viremic individuals. We therefore asked whether these results might be related to the potential purging activity of thalidomide.

Samples from 7 individuals not participating in this study and under ART with viral loads below the detection limits for a mean period of

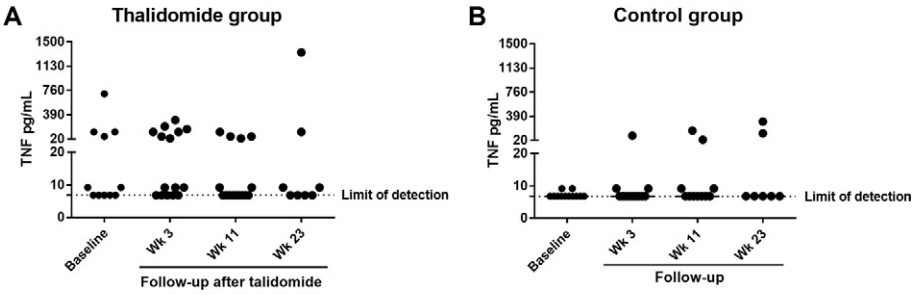

Fig. 6. Levels of TNF in the Thalidomide arm and control arm. Despite the apparent increase in the TNF levels in some individuals on the Thalidomide arm as compared to the control arm when comparing baseline levels to the levels encountered on the last day of Thalidomide intake, no statistical significance in the mean and median TNF levels between different time points in each group was observed.

10.4 years (from 2.1 to 18.2 years) were collected, and $CD8^+$ T cells depleted of other PBMCs were used. Thalidomide treatment induced purging in 6 out of 7 samples (Fig. 7). On the second day after treatment with thalidomide, the culture supernatants were tested for viral load using qPCR, and the results revealed HIV-1 emergence at day 4 with a change in viral load from 2.2 $_{log10}$ to 6.0 $_{log10}$ (median of 5.7 $_{log10}$) HIV-1 RNA copies/mL, whereas viruses were not detected in control samples (Table 4). Relating viral emergence with clinical parameters showed that culture positivity was independent of the $CD4^+$ T cell nadir, the time of viral load below detection limits, the antiretroviral scheme, viral tropism and the subtype of virus (data on file), reinforcing the role of thalidomide in this phenomenon.

4. Discussion

In this study, we explored the effects of thalidomide among ART-naïve HIV-infected individuals with stable $CD4^+$ T cell levels for the short period of three weeks in comparison to an equivalent control group, both to be followed by a period of 23 weeks. As a potent TNF-α inhibitor, our hypothesis was that thalidomide use would be safe and able to mitigate one of the major changes associated with chronic HIV infection: HIV-related inflammation. We also hypothesized that in the absence of antiretroviral medications, the anti-inflammatory thalidomide effect would become more evident. Thus, we carefully monitored HIV-related inflammatory markers, such as T cell activation, unspecific inflammatory markers, such as US-CRP, and bacterial translocation markers, such as plasmatic LPS levels, in addition to viral loads and $CD4^+$ and $CD8^+$ T cell counts.

The basis of our hypothesis in this study was that TNF-α can trigger the cytokine production cascade by inducing cytokine secretion by target cells. TNF-α acts by activating the cellular transcription factor NF-kB, which induces the expression of cytokines and other cellular genes (Breen, 2002). TNF-α can bind to two membrane receptors on target cells, TNF receptor 1 (TNFR1, also known as TNFRSF1A and p55) or TNF receptor 2 (TNFR2, also known as TNFRSF1B and p75), to activate two distinct intracellular signaling pathways for gene transcription (Faustman and Davis, 2010). TNFR1 is present in almost all cells of the body, and its signaling induces the cascade related to apoptosis. TNF binding to TNFR1 activates apoptosis through two pathways involving the adaptor proteins TNFR1-associated death domain (TRADD) and

Table 3
HIV-1 and V3 co-receptor tropism of the Thalidomide group at successive visits inferred through Geno2pheno with an FPR set at 10%. -: HIV-1 env V3 region amplification failed or co-receptor tropism not determined.

	ID	Screening	Baseline	Week 1	Week 2	Week 3	Week 7	Week 11	Week 17	Week 19	Week 23
Thalidomide	TV01	R5	R5	–	–	–	–	–	–	–	–
	TV03	–	X4	–	–	–	–	–	–	–	X4
	TV05	–	–	–	–	–	–	–	–	–	–
	TV07	R5	R5	–	–	–	–	–	–	–	R5
	TV09	–	R5	R5	R5	–	–	R5	R5	R5	R5
	TV11	R5	R5	–	–	–	–	–	–	–	R5
	TV13	–	–	–	–	–	–	–	–	–	–
	TV15	–	–	–	–	–	–	–	–	–	–
	TV21	R5	R5	–	–	–	–	–	–	–	R5
	TV23	R5	R5	–	–	–	–	–	–	–	R5
	TV25	R5	R5	–	–	–	–	–	–	–	R5
	TV27	–	–	–	–	–	–	–	–	X4	X4
	TV29	–	–	–	–	–	–	–	–	–	–
	TV31	R5	R5	–	–	–	–	–	–	R5	–
	TV33	–	X4	X4	X4	X4	X4	X4	X4	–	X4
	TV37	–	–	–	–	–	–	–	–	–	R5
Control	TV02	–	–	R5	R5	R5	R5	R5	–	–	R5
	TV06	–	–	R5	R5	R5	R5	R5	X4	–	X4
	TV08	–	–	X4	X4	X4	X4	X4	R5	X4	X4
	TV12	–	R5	–	–	–	–	R5	–	–	–
	TV14	–	X4	–	X4	X4	X4	X4	–	X4	–
	TV16	–	R5	–	–	–	–	R5	–	R5	–
	TV18	–	R5	–	–	–	–	–	–	–	R5
	TV20	R5	R5	–	–	–	–	–	–	–	R5
	TV22	R5	R5	R5	R5	R5	R5	R5	X4	–	X4
	TV24	R5	R5	–	–	–	–	–	–	–	R5
	TV28	R5	R5	–	–	–	–	–	–	–	R5
	TV32	R5	R5	X4	–	R5	R5	X4	–	X4	X4
	TV34	R5	R5	–	–	–	–	–	–	–	R5
	TV36	R5	R5	–	–	–	–	–	–	–	R5

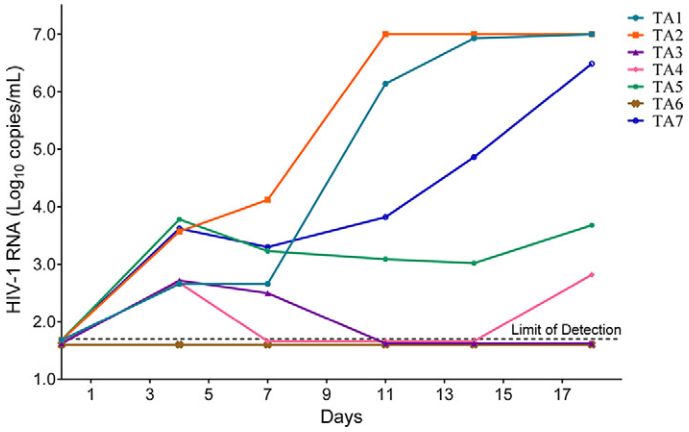

Fig. 7. The *in vitro* effect of Thalidomide on increase in viral load from PBMCs samples from seven patients on undetectable viral load. PBMCs were activated with Thalidomide in the presence of Phytohaemagglutinin and IL-2 for 48 h. Viral RNA was quantified by qPCR. Y-axis shows HIV-1 RNA (log_{10} copies/ml). X-axis shows the days at which supernatants were collected for viral RNA quantification. Symbol TA indicates patient ID.

Fas-associated death domain (FADD). TNFR2 is related to T cell survival, and its signaling involves the mobilization of the transcription factor NF-kB to promote the transcription of pro-survival genes. NF-κB contributes to a variety of components of the immune system through its role in the development and differentiation of immune cells and lymphoid organs. NF-κB's strongest inducers are the cytokines TNF and IL-1, as well as pathogen-derived LPS.

Surprisingly, however, we were able to document that T cell activation markedly increased in both $CD4^+$ and $CD8^+$ T cells during thalidomide exposure, returning to basal levels right after thalidomide withdrawal, whereas no specific trends were detected in the control group. The same result was observed in the Thalidomide group with regard to the US-CPR level. Furthermore, the $CD4^+$ T cell counts decreased during thalidomide use without any changes in the $CD8^+$ T cell counts, leading to a decrease in the CD4/CD8 ratios, although these levels returned to the baseline ranges immediately following thalidomide withdrawal, thus confirming that these changes were exclusively related to drug exposure. Again, no trends were observed in the control group. Interestingly, no changes in the viral loads were observed in both groups, and no changes were observed in the LPS plasma levels. We then speculated that an HIV viral replication process could be triggered by thalidomide, leading to an increase in inflammation and the death of infected $CD4^+$ T cells without any apparent disturbance of $CD8^+$ T cells and without changing the HIV-associated translocation process. As the studied individuals were naturally viremic, we were not able to detect any increase in the viral load levels because untreated individuals are able to produce and eliminate 10 billion HIV virions every day (Ho et al., 1995). However, previous studies using Thalidomide among HIV infected individuals have demonstrated a trend in the increase of HIV viral load after Thalidomide exposure, when this drug was used for oral aphthous ulcers (Jacobson et al., 1999) and HIV wasting syndrome (Kaplan et al., 2000). In another placebo controlled study, 28 days of thalidomide treatment was associated with the modest overshoot in the rebound of viremia, which vanished after stopping the drug (Haslett et al., 1999). Interestingly, one of the individuals recruited in the Thalidomide group was an elite controller, and we detected increased expression of T cell activation markers and an increase in the US-CRP level during thalidomide use followed by a blip of viremia to 67 (1.83 log_{10}) copies/mL. We therefore conducted *in vitro* assays to confirm that thalidomide was able to transactivate HIV, by showing that this drug could act as a latency reversal agent. Surprisingly, our preliminary results were unequivocal, showing a very potent latency-reverting effect of thalidomide in samples from 6 out of 7 tested patients. Thalidomide has been associated with inhibition of NF-kB activity through a mechanism that involves the suppression of Inhibitor of kappa kinase (IKK) activity (Keifer et al., 2001). Since kappa kinase cannot phosphorylate the inhibitor, NFKB stays arrested in the cytoplasm and cell activation is halted at this step, which is the alleged mechanism for the anti-inflammatory properties usually seen with Thalidomide (Keifer et al., 2001). For complete T-cell activation, two signals delivered by antigen presenting cells are required: (*i*) T cell receptor binding to its cognate antigen and (*ii*) a co-stimulation signal in which CD28 molecule expressed by T cell binds with CD80/86 molecules expressed by Antigen presenting Cells. Beside suppression of IKK, Thalidomide is also known to co-stimulate T-cells *via* CD28 receptor (Haslett et al., 1998). This effect of Thalidomide could be associated with T-cell activation and increase in HIV replication, which reconciles with our *in vivo* and *ex-vivo* results. In fact, HIV viral load increase has been previously reported upon thalidomide therapy in trials treating oral aphthous ulcers (Jacobson et al., 1999) and HIV wasting syndrome (Kaplan et al., 2000). In another placebo controlled study, 28 days of thalidomide treatment was also associated with the modest overshoot in the rebound of HIV viremia, which vanished after stopping the drug (Haslett et al., 1999).

In spite of its inflammatory properties, TNF-α production can be a restrictive factor for HIV replication, and it is conceivable that thalidomide use was able to block this process. TNF-α is detected after HIV-1 infection of primary macrophages *in vitro* and is present in plasma and

Table 4
Viral load in the culture supernatants from thalidomide-treated samples. Subjects with viral rebound after drug treatment are highlighted. C = Supernatant collection; VL = Viral load (copies/mL); BDL = Viral load below detection limits.

ID	Day	VL	Day	VL	Day	VL	Day	VL	Day	VL
TA1	0	<50	4	459	7	462	11	1378169	14	8469592
TA2	0	<50	4	3740	7	13,091	11	>10,000,000	14	>10,000,000
TA3	0	<50	4	522	7	318	11	BDL	14	BDL
TA4	0	<50	4	478	7	BDL	11	BDL	14	BDL
TA5	0	<50	4	6009	7	1685	11	1230	14	1047
TA6	0	<50	4	BDL	7	BDL	11	BDL	14	BDL
TA7	0	<50	4	4191	7	1992	11	6610	14	71792

tissues of patients with AIDS (Herbein et al., 1996). *In vitro*, TNF-α is secreted by primary macrophages infected in culture by HIV-1 or treated with envelope glycoprotein gp120 and by HIV-infected monocytes isolated from patients (Merrill et al., 1989). TNF-α is able to enhance host cellular resistance to HIV-1 infection through selective inhibition of HIV-1 entry into primary tissue culture-differentiated macrophages (Herbein et al., 1996). One plausible strategy for the inhibition of HIV entry by TNF-α in macrophages may be through negative modulation of the transcription of CD4 and CCR5, which might explain the inhibition of HIV entry in these permissive cells (Karsten et al., 1996). For this reason, we also monitored the ability of thalidomide to select CXCR4, although no positive results were obtained in the current study. Of note and as mentioned before, we have not been able to find any difference in the cytokine levels between groups or during Thalidomide intake.

Interestingly, we observed in the Control group that T cell activation slightly and linearly increased over time, even with no changes in the basal viral loads. As seen in other studies, there was a positive correlation between the viral loads and CD4 and CD8 T cell activation levels (Hunt et al., 2003).

We recognize that the pilot proof-of-concept open-label nature of this study, specifically related to the small sample size of recruited patients, may preclude conclusions that are more definite. Furthermore, the lack of cytokine profile analysis also prevents us from further understanding the mechanisms related to the inflammatory processes documented here. In addition, a comparison group of HIV-uninfected individuals using thalidomide for the same period of time would enable us to further address the specific effects of thalidomide in association to HIV. Nevertheless, our results clearly demonstrated that thalidomide induces a potent inflammatory profile in the presence of HIV, indicating that this drug may serve as an HIV purging tool in the new era of HIV-sterilizing treatment discoveries.

Trial Registration

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Authors' Contributions

TV: cohort follow-up and manuscript preparation; SS: *in vitro* studies for HIV latency reversal; JS-O: cell activation and LPS assays and statistical analysis; LG: cell activation assays; MSA: HIV genome sequencing and analysis and manuscript preparation; MLS-F: laboratory assistance; LACC: patient inclusion and clinical evaluation; MST: patient inclusion and clinical evaluation; AC: patient inclusion and clinical evaluation; MCS: laboratory supervision; AMC: laboratory supervision, statistical analysis, and manuscript preparation; RSD: study design, data interpretation, and manuscript preparation.

Conflict of Interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2017.08.007.

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