In search of biomarkers for leprosy diagnosis : in silico identification, screening & field application

Aboma, K.B.

Chapter 7

Immune responses in Ethiopian leprosy patients coinfected with HIV or helminthes

Kidist Bobosha*,¶, Yonas Bekele¶, Saba M. Lambert#,▼, Martha Zewdie¶, Digafe Tsegaye ▼, Jolien J van der Ploeg van Schip*, Louis Wilson*, Diana Lockwood #, Abraham Aseffa¶ and Annemieke Geluk*

*From the *Dept. of Infectious Diseases Leiden University Medical Center, The Netherlands; ¶Armauer Hansen Research Institute, Addis Ababa, Ethiopia; ▼All Africa Leprosy Rehabilitation and Training Center, Addis Ababa, Ethiopia; #London School of hygiene and Tropical medicine*

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Abstract

The host immune response against *Mycobacterium leprae* (*M. leprae*) determines the type and severity of the disease. Early detection of leprosy and asymptomatic *M. leprae* infection is key to reducing transmission. We have developed diagnostic tools based on cellular immune responses to *M. leprae* antigens. However, coinfections with HIV or helminths may reduce the host immune response to *M. leprae* thereby possibly hampering diagnosis of infected cases in tests based on anti-mycobacterial cellular immunity. This study characterized the immune profiles of HIV- or helminth coinfected leprosy patients in order to estimate the effect of coinfection in immunodiagnostic tools. Production of selected host biomarkers from peripheral blood mononuclear cells (PBMC) stimulated with *M. leprae* whole cell sonicate (WCS) and mRNA expression level of 76 genes was measured.
Similar T helper 1 (Th1) and Th2 responses were measured in WCS stimulated PBMC of leprosy patients with HIV coinfection and matched leprosy patients without HIV infection. In non-reaction BL/LL patients with and without helminth infection, the IFN-γ production was similar in both groups but the reductive effect of helminth coinfection in patients with T1R suggests the interference of helminth driven Th2 responses.
mRNA expressions of IL15 (p=0.0001), CTLA4 (p=0.003) and TLR10 (CD290) (p=0.0001) were significantly higher in HIV coinfected patients than in non-HIV patients, whilst ZNF532 expression was significantly lower (p =0.002). In BL/LL patients with and without helminth coinfection, similar mRNA expressions were observed for all 76 genes tested.
In summary, the similarity in immune responses in leprosy patients with and without HIV and helminth coinfections allows the use of identified immune- and transcriptomics biomarkers in diagnostic tests irrespective of patients' coinfection status. However, in patients with reactions, helminth infections may mask the severity of the reaction in Th1-based diagnostic tests, which warrants further investigations in larger/ longitudinal cohorts.

Author Summary

Early detection of leprosy is the main strategy to reduce transmission. Recently, we have developed diagnostic tools based on cellular immune responses of the host to *M. leprae* antigens. HIV or helminth coinfections in leprosy patients may interfere and hamper the diagnostic potential of these new tools. Therefore, in this study, the immune profiles of coinfected leprosy patients were characterized and compared with non coinfected leprosy patients to estimate the effect of coinfection in immunodiagnostic tools.

Similar host immune responses were measured in both HIV co-infected and non-coinfected leprosy patients. The IFN-γ production in helminth co-infected patients without T1R (type 1 reaction) was similar with that of non coinfected patients. However, a reduced IFN-γ production was measured in helminth coinfected patients with T1R.

mRNA expressions for majority of the genes were similar except for a few genes namely IL15 CTLA4 and TLR10 (CD290) which were found significantly higher in HIV coinfected patients and ZNF532 expression was significantly lower. In BL/LL patients with and without helminth coinfection, similar mRNA expressions were observed for all genes tested.

In summary, the similar immune responses shown in this study for both groups allows the use of identified host biomarkers in diagnostic tests irrespective of patients' coinfection status. However, further investigations in larger/ longitudinal cohorts are warranted in helminth coinfected patients with reaction.

Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*) and about 200,000 new cases of leprosy are reported by the WHO every year [1]. The disease manifestation has a wide spectrum which ranges from the tuberculoid form with high cell mediated immunity (CMI) and low number of bacilli to the lepromatous form with poor CMI and high bacillary load [2]. Host immunity determines the clinical manifestation after infection with *M. leprae*. However, coinfections may interfere with host immunity, thereby determining clinical manifestations either by up regulating or down regulating the different arms of immunity [3;4].

Over the last three decades, HIV has killed millions through increasing susceptibility of infected people to many opportunistic infections. Mycobacterial infections are among these opportunistic threats, and tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) is the leading cause of death among HIV infected individuals [5;6]. After initiation of anti retroviral therapy (ART), diseases like TB may manifest as a result of immune reconstitution inflammatory syndrome (IRIS) which is an exaggerated immune reaction against re-current or unrecognized sub-clinical infections [7].

Similarly, increased manifestation of leprosy, especially of the lepromatous form, was predicted in HIV infected patients taking ART [8;9]. In contrast to the expectations, the impact of HIV has not worsened the leprosy situation, although some patients on ART are being diagnosed as new leprosy cases [10]. Few studies have analysed some clinical, immunological and pathological parameters [11-14]. Most of these features in *M. leprae*-HIV coinfected patients were reported to be similar compared to patients with one infection, indicating that each disease progresses independently [15].

The occurrence of type 1 leprosy reactions (T1R) in association with anti-retroviral therapy (ART) initiation was reported and is often considered as immune reconstitution inflammatory syndrome (IRIS) in *M. leprae*-HIV coinfected patients on ART [11;14;16-20]. In a non-HIV leprosy patient with T1R, our group has previously shown increased expression of cytotoxicity-associated genes granzyme A (GZMA), granzyme B (GZMB) and perforin 1 (PRF1) [21]. In *M. leprae*-HIV coinfected patients, increased frequency of $CD8^+$ T cells was also reported as a potential triggering factor for the occurrence of T1R along with increased production of PRF and GZMB compared to non-HIV leprosy patients [22]. Therefore, in this study, we compared the mRNA expressions of these and other related genes in both groups of patients.

Coinfection with intestinal parasites, mainly helminthic infections are known to elicit immune modulation characterized by up-regulating Th2 responses in the infected host [23-28]. In mycobacterial infections like TB, studies have shown the effect of helminth infestation through weakening Th1 immunity [29]. Moreover, poor immunogenicity induced by BCG vaccination was also observed in helminth infested groups compared to de-wormed groups in an Ethiopian cohort [30;31]. There are also evidences for Omega-1 (with glycosylation and ribonuclease activity) secreted by *Schistosoma mansoni* eggs in conditioning dendritic cells in priming Th2 responses [32;33]. In addition, helminthic infections are common in HIV infected people [34]. The presence of intestinal helminths in leprosy patients may potentially facilitate the progression of *M. leprae* infection to more severe forms of leprosy [35]. Significant association of lepromatous leprosy (LL) with

helminths and higher production of Th2 type cytokines such as IL-4 and IL-10, were reported in coinfected patients [35;36].

Our group has selected specific *M. leprae* proteins and identified host biomarkers such as IFN-γ, IP-10, MIP-1β, MCP-1, IL-1β, IL-6 for detection of *M. leprae* infection and/ or exposure in different groups including leprosy patients, household contacts and endemic controls [37-40]. These biomarkers are currently applied in development of field friendly rapid diagnostic tests. Assessing the effect of coinfections on these biomarkers is essential for the interpretation of these tests in leprosy patients with coinfections.

In this study, we analysed immune responses to *M. leprae* antigens in HIV or helminth coinfected Ethiopian leprosy patients to assess the effect of these coinfections on host immune biomarkers.

Materials and Methods

Ethical statement. This study was performed according to the Helsinki Declaration (2008 revision). Ethical approval of the study protocol was obtained from the National Health Research Ethical Review committee, Ethiopia (NERC # RDHE/127-83/08). Participants were informed about the study objectives, the required amount and kind of samples and their right to refuse to take part or withdraw from the study at anytime without consequences to their treatment. Written and informed consent was obtained from study participants before enrollment. Pre- and post counseling for HIV testing was performed by the recruiting nurse and patients identified as HIV-positive were referred to ALERT ART clinic.
Study participants. During 2009 and 2012 *M. leprae*-HIV coinfected patients (n=21) and leprosy patients without HIV (n=256) were enrolled in this study. Leprosy was diagnosed based on clinical, bacteriological and histological observations and classified by a skin biopsy evaluated according to the Ridley and Jopling classification [2] by qualified microbiologists and pathologists. All patients were enrolled before initiation of MDT antibiotic treatment for leprosy infection or steroid treatment for leprosy associated inflammation or reactions. or steroid. The HIV test was done using KHB (Shanghai kehua Bioengineering CO-Ltd, Shanghai, China) and if positive a second test was performed using STAT-PAK™ (Chembio HIV1/2, Medford, New York, USA). Stool samples were prepared using the direct stool smear (wet smear) protocol and examined under microscope within 30 min for the presence of ova, cysts and parasites. Patients with positive microscopic result were further categorized into patients with helminth and protozoan infection.
PBMC isolation, freezing and thawing. PBMC were isolated by density gradient method using Ficoll-paque, cells were washed and suspended in 20% fetal calf serum (FCS) in AIM-V (Invitrogen, Carlsbad, CA) and kept cool on ice, counted and frozen using a cold freshly prepared freezing medium composed of 20% FCS, 20% dimethyl sulphoxide (DMSO) in AIM-V. Cells were kept at -80 °C for 2-3 days and transferred to liquid nitrogen until use. During thawing, cells were transported in liquid nitrogen to a water bath (37^{o}C) incubated for 30 to 40 seconds until thawed half way and resuspended in 10% FCS in AIM-V (37^{o}C) containing 1/10,000 benzonase until completely thawed, washed twice (5-7 minutes each) and counted.
M. leprae Whole Cell Sonicate (WCS). Irradiated armadillo-derived *M. leprae* whole cells were probe sonicated with a Sanyo sonicator to >95% breakage. This material was kindly provided by Dr. J.S. Spencer through the NIH/NIAID "Leprosy Research Support" Contract N01 AI-25469 from Colorado State University (now available through the Biodefense and

Emerging Infections Research Resources Repository listed at http://www.beiresources.org/TBVTRMResearch Materials/tabid/1431/Default.aspx).

Lymphocyte stimulation tests (LST). PBMC (200,000 cells/well) were added in triplicate into 96 well U bottom tissue culture plates and cultured with *M. leprae* whole cell sonicate (WCS; 10 μg/ml), phytohaemagglutinin (PHA; 1 μg/ml) or AIM-V medium at 37°C with 5% CO_2 and 70% humidity. After 6 days, supernatants were collected and kept frozen until used in ELISA.

IFN-γ ELISA. IFN-γ levels were determined by ELISA (U-CyTech, Utrecht, The Netherlands) [41]. The cut-off value to define positive responses was set beforehand at100 pg/ml. The assay sensitivity level was 40 pg/ml. Values for unstimulated cell cultures were typically < 40 pg/ml.

Multiple cytokine and chemokine assays. The concentrations of 12 analytes (IL-1β, IL-10, IL-12p70, IL-17, IFN-γ, IP-10 (CXCL10), G-CSF, GM-CSF, MCP-1 (CCL2), MIP-1β (CCL4), VEGF and TNF) in supernatants from 6 days LST were measured using the Bio-Plex suspension array system powered by Luminex xMap multiplex technology (Bio-Rad Laboratories, Veenendaal, The Netherlands) and analyzed using the Bio-Plex Manager™ software 6.0 (Bio-Rad laboratories, Veenendaal, The Netherlands). The assay protocol described in Bobosha, K et al., 2012 was followed [42].

PGL-I ELISA. IgM antibodies against *M. leprae* PGL-I were detected with natural disaccharide of PGL-I linked to BSA (ND-O-BSA (0.01 ng/ well) provided through the NIH/NIAID Leprosy Contract N01-AI-25469) as previously described [43]. Serum dilutions (100 μl/ well; 1:300) were incubated at 37°C for 90 min in flat-bottomed microtiter plates (Nunc) coated with NDO-BSA. After washing diluted enzyme linked secondary antibody solution (100 μl/ well) was added to all wells and incubated at 37°C for 30 min. After washing diluted TMB solution (100 μl/ well) was added to all wells and incubated in the dark for 15 min at RT. The reaction was stopped by adding 100 μl/ well 0.5 N H_2SO_4. Absorbance was determined at wavelength of 450 nm. Samples with a net optical density at 450 nm (OD) above 0.199 were considered positive. The ELISA performance was monitored using a positive and negative control serum sample on each plate.

PAXgene whole blood RNA isolation. PAXgene tubes were stored at -80°C and shipped to LUMC. Total RNA from venepuncture PAXgene blood collection tubes was extracted and purified using the PAXgene Blood RNA kit (BD Biosciences) including on-column DNase digestion according to the manufacturers' protocol. The RNA yield from 2.5 ml of whole blood was determined by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and ranged from 4.2 to 8.5 μg of total RNA (average 6.02 ± 1.5 μg) with an average $OD_{260/280}$ ratio of 2.0 ± 0.04.

Dual color Reverse Transcription Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA) assays. dcRT-MLPA assay was performed as described previously [44]. Briefly, for each target-specific sequence, a specific RT primer was designed that is complementary to the RNA sequence and located immediately downstream of the probe target sequence. Half-probes consisted of chemically synthesized oligonucleotides and right hand half-probes were 5' phosphorylated to facilitate ligation. As a positive control, chemically synthesized oligonucleotides were used that were complementary to the RNA sequence and encompassed the combined target-specific sequences of the left and right hand half-probes. Primers and probes were purchased from Sigma-Aldrig Chemie (Zwijndrecht, The Netherlands) and MLPA reagents from MRC Holland (Amsterdam, The Netherlands). To avoid detection of contaminating DNA fragments, all target sequences have an exon boundary near the probe ligation site. Also, splice variants and SNPs present in the mRNA were taken into account. Trace data were analyzed using GeneMapper software package (Applied Biosystems, Warrington, UK). The areas of each assigned peak (in arbitrary units)

were exported for further analysis in Microsoft Excel spreadsheet software. Signals below the threshold value for noise cut-off in GeneMapper (log2 transformed peak area ≤ 7.64) were assigned the threshold value for noise cut-off. Results from target genes were calculated relative to the average signal of the reference gene, *GAPDH*, present within the gene sets. Following normalization of the data, signals below the threshold value for noise cut-off (peak area ≤ 7.64) were again assigned the threshold value for noise cut-off. To monitor assay performance, a negative control (without RNA), a positive control (using synthetic template oligonucleotides as hybridization templates) and a commercial Human Universal Reference RNA (Clontech, Palo Alto, CA, USA) were included on each 96-well plate. dcRT-MLPA experiments for RNA samples of all time points were performed simultaneously.

Statistical analysis. Data were analyzed with the two-tailed Mann-Whitney U test for non-parametric distribution using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA; www.graphpad.com). The statistical significance level used was $p<0.05$.

Results

T1R in *M. leprae*-HIV coinfected patients

to assess the effect of HIV coinfection, we recruited, 10 male and 10 female HIV coinfected leprosy patients with age range of 18 to 50 including 16 BB/BL/LL patients and 4 BT. The BI ranged from 0 to +4 (Table 1).

Table 1. *M. leprae*-HIV coinfected patients' demographic and clinical status

	Lep-HIV Coinfected	Sex	Age	Rxn	Status	BI	PGL-I (OD_{450})	ART status*	CD4 count
1	Lep HIV 002	M	39	T1R	BT	0	0.02	ART	242
2	Lep HIV 011	F	23	T1R	BT	0	0.018	ART	90
3	Lep HIV 008	F	18	T1R	BT	0	0.103	Not received	581
4	Lep HIV 001	M	40	T1R	BB	0	0.726	ART	NA
5	Lep HIV 006	F	35	T1R	BB	0	0.2	ART	21
6	Lep HIV 018	F	50	T1R	BB	0	0.027	Not received	247
7	Lep HIV 004	M	25	T1R	BL	0	0.096	ART/IRIS	206
8	Lep HIV 009	M	36	T1R	BL	0	0.068	ART	86
9	Lep HIV 012	F	30	T1R	BL	0	0.599	ART	332
10	Lep HIV 014	M	38	T1R	BL	0	0.981	Not received	359
11	Lep HIV 016	M	28	T1R	BL	0	0.08	Not received	216
1	Lep HIV 017	F	39	T1R	BL	0	0.042	Not received	218
13	Lep HIV 019	F	26	T1R	BL	2	2.183	ART	216
14	Lep HIV 020	M	30	T1R	BL	0	0	ART	144
15	Lep HIV 007	F	30	T2R	BL	0	0.612	ART	109
16	Lep HIV 003	M	30	T2R	LL	3	0.091	Not received	238
17	Lep HIV 025	M	38	T2R	LL	2	1.375	Not received	375
18	Lep HIV 015	M	34	No rxn	BT	0	0.026	Not received	565
19	Lep HIV 021	F	40	No rxn	LL	4	ND	Not received	59
20	Lep HIV 005	F	24	No rxn	LL	3.3	1.375	ART	425

Rxn: leprosy reaction; T1R: type 1 reaction; T2R: type 2 reaction

*: at diagnosis of leprosy

The patients who were referred from the ART clinic at ALERT were all on ART (11/20), whereas the other patients who were first diagnosed for leprosy had not received ART (9/20). T1R was diagnosed in 14 out of 20 (70 %) and T2R in 3 out of 20 (15 %) (LL= 2 and BL= 1). Only 3 patients did not have reactions (LL=2 and BT=1) at the time of leprosy diagnosis. Among the HIV coinfected patients on ART; 9/11 (69.2%), and among those naive to ART 5/9 (30.7%) had a clinical T1R. The CD4 count ranges from 21 to 425 cells/µl in patients on ART and ranges from 59-581 cells/ul in patients naïve to ART.

Similar cytokine responses in non-HIV and HIV coinfected leprosy patients

To assess the influence of HIV infection on recently identified potential biomarkers for early diagnosis, cytokine/chemokine responses against *M. leprae* WCS in both HIV uninfected and HIV coinfected leprosy patients were analysed. As shown in Figure 1, the IFN-γ responses to *M. leprae* WCS measured by ELISA in both groups were found similar.

The IFN-γ responses to PHA in both groups were higher in general compared to responses to *M. leprae* WCS. Also, the IFN-γ response in non-HIV leprosy patients to PHA was significantly, though slightly, higher (p =0.045) compared to that in those infected with HIV (Figure 1).

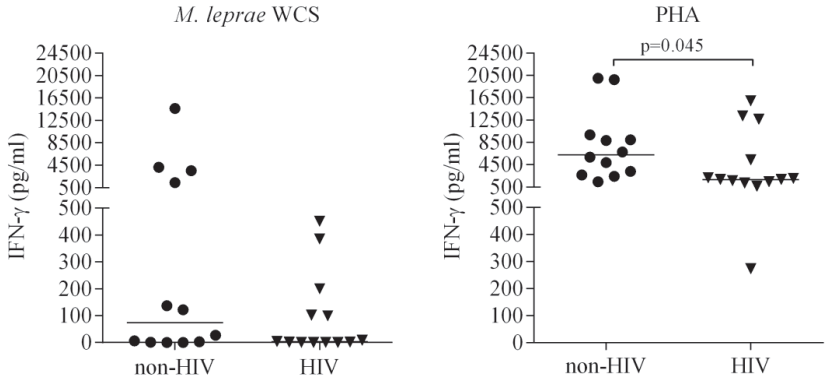


Fig 1: IFN-γ responses of PBMC from HIV coinfected (n=13) and non-coinfected leprosy patients (n=13) stimulated with *M. leprae* Whole Cell Sonicate (WCS) and PHA positive control in 6 days culture.

Similarly, the 11 analytes (IL-1β, TNF, IL-12p70, IL-17, IL-10, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α, G-CSF, GM-CSF and VEGF) measured in supernatants of 6 days PBMC culture were also found similar in both groups (Figure 2) affirming the possibility to use the previously identified host biomarkers as diagnostic markers in non-HIV as well as HIV coinfected individuals.

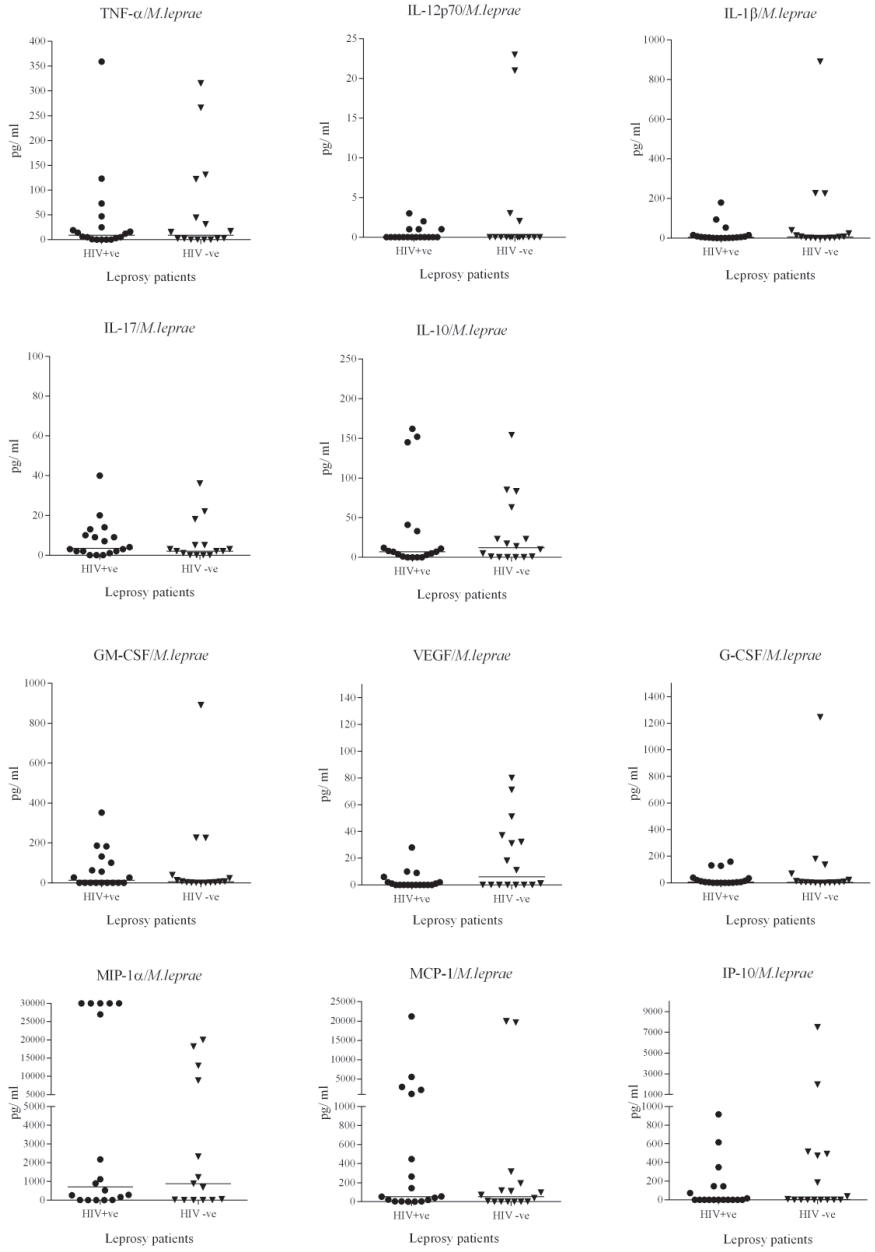


Fig 2: Multiple cytokine and chemokine responses of PBMC of HIV coinfected (n=18) and non-coinfected leprosy patients (n=15) stimulated with *M. leprae* Whole Cell Sonicate (WCS).

Increased mRNA expression of IL15 and CTLA4 in HIV coinfected leprosy patients

mRNA expression of 76 target genes involved in innate and adaptive immunity or associated with leprosy [21] were analysed in both HIV-coinfected (n=20) and HIV-uninfected leprosy patients (n=20). Only a few genes showed significant differences between both groups of

leprosy patients. Significantly higher CD4 (p <0.0001) and CD8 (p <0.002) mRNA expression levels were measured in non-HIV and HIV coinfected respectively (Figure 3). The expressions of IL15 (p =0.0001), CTLA4 (p =0.003) and TLR10 (CD290) (p =0.0001) were found significantly higher in HIV coinfected leprosy patients (Figure 3). In contrast, ZNF532 mRNA expression was found significantly higher (p =0.002) in non-HIV patients. The expression of all other tested genes including IL1β, CCL4, CCR7 and VDR did not show significant differences between the two groups.

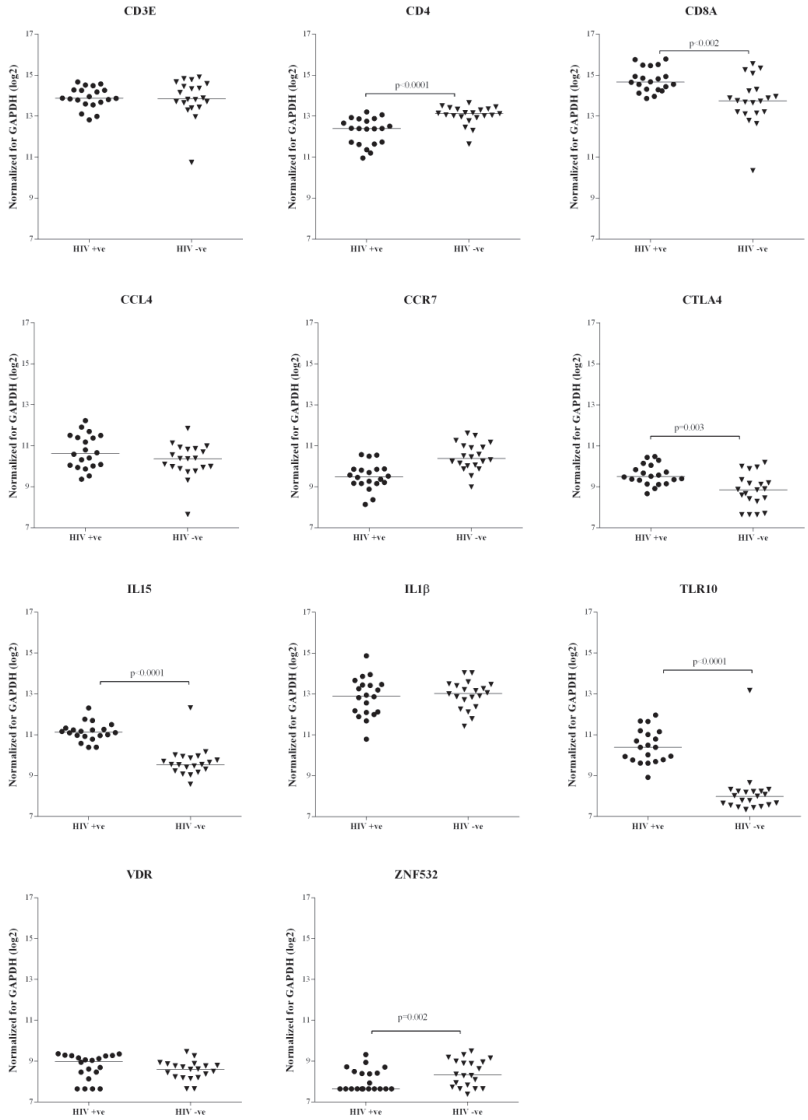


Fig 3: mRNA expression of 11 target genes in peripheral blood of HIV coinfected (n=20) and non-coinfected leprosy patients (n=20).

Comparable frequency of helminth coinfection in MB and PB leprosy patients

Leprosy patients were recruited consecutively at ALERT hospital and tested for helminth infestation where 218 BL/LL and 38 BT patients were included from 2009 – 2013. Among these patients, 54 out of 218 BL/LL patients (24.7%) and 6 out of 38 (15.7%) BT patients were found coinfected with parasites; helminths (51.6%) and protozoans (48.3%) (Table 2).

Table 2. Type and distribution of parasites in leprosy patients

Stool result	# of patients	%	Sex ratio (M/F)	Age range (yrs)	Type of leprosy	Reaction (No/Yes)
Cyst of *Giardia lamblia*	8	3.13	5/3	18-55	BL/LL	4/4
Cyst of *Giardia lamblia* and *Entamoeba histolytica*	1	0.39	1/0	33	BL	1/0
Cyst of *Entamoeba histolytica*	9	3.50	6/3	24-46	BL/LL	5/4
Trophozoites of *Giardia lamblia*	7	2.72	6/1	20-55	BL/LL (5) BT (2)	4/3
Trophozoites of *Giardia lamblia* and *Entamoeba histolytica*	1	0.39	1/0	18	LL	1/0
Trophozoites of *Entamoeba histolytica*	3	1.17	2/1	29-40	BL/LL	1/2
Larvae of *Strongyloides stercolaris*	4	1.56	4/0	22-28	BL/LL	1/3
Ova of *Ascaris lumbricoides*	10	3.89	7/3	18-47	BL/LL	7/3
Ova of *Enterobius vermicularis*	1	0.39	1/0	31	BL	1/0
Ova of hookworm	10	3.89	8/2	18-46	BL/LL (6) BT (4)	6/4
Ova of *Hymenolepis nana*	1	0.39	0/1	21	BL	1/0
Ova of *Taenia Spp*	2	0.78	1/1	25-29	BL/LL	0/2
Ova of *Trichuris trichiura*	1	0.39	0/1	18	LL	1/0
Ova of *Trichuris trichiura* and *Ascaris lumbricoides*	2	0.78	2/0	25	LL	2/0
No parasites	196	76.26	133/63	18-65	BL/LL (164) BT (32)	97/89
Total	256					

Protozoans	29	48.3
Helminths	31	51.6

Parasites	60	23.35
No parasites	196	76.26
	256	100

M/F: Male –Female ratio; BT: Borderline tuberculoid; BL: Borderline lepromatous

The most frequent protozoans were *Giardia lamblia* and *Entamoeba histolytica* and the most frequent helminths were *Ascaris lumbricoides* and hookworm. Helminth infected BL/LL patients were 27 out of 218 (12.4%) and BT were 4 out of 38 (10.5%).

Helminth coinfection suppresses the Th1 response in leprosy patients with T1R

As shown in Figure 4, the IFN-γ (hallmark cytokine for Th1 immunity) production in non-reactional BL/LL patients with and without helminths in response to *M. leprae* WCS were low (less than 100 pg/ml) and no difference was observed between the BL/LL patients with and without helminths. However, when PBMC of BL/LL patients (n=4) with T1R not treated with steroids were stimulated with *M. leprae* WCS, patients free of helminth infestation showed significantly higher (p=0.028) IFN-γ responses than those coinfected with helminths (n=4).

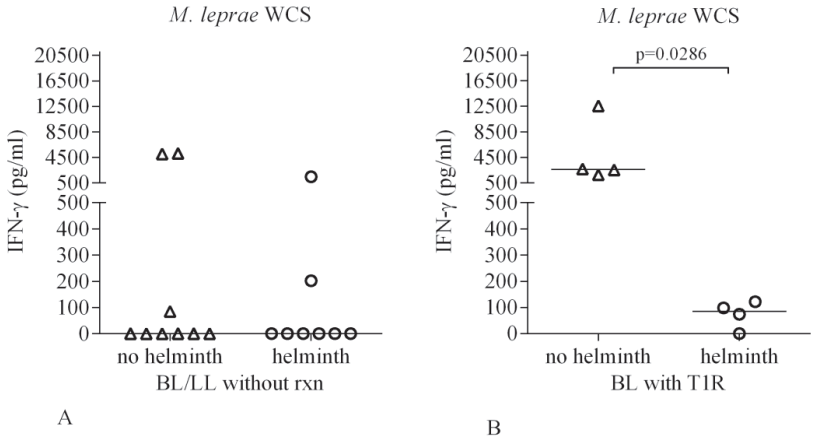
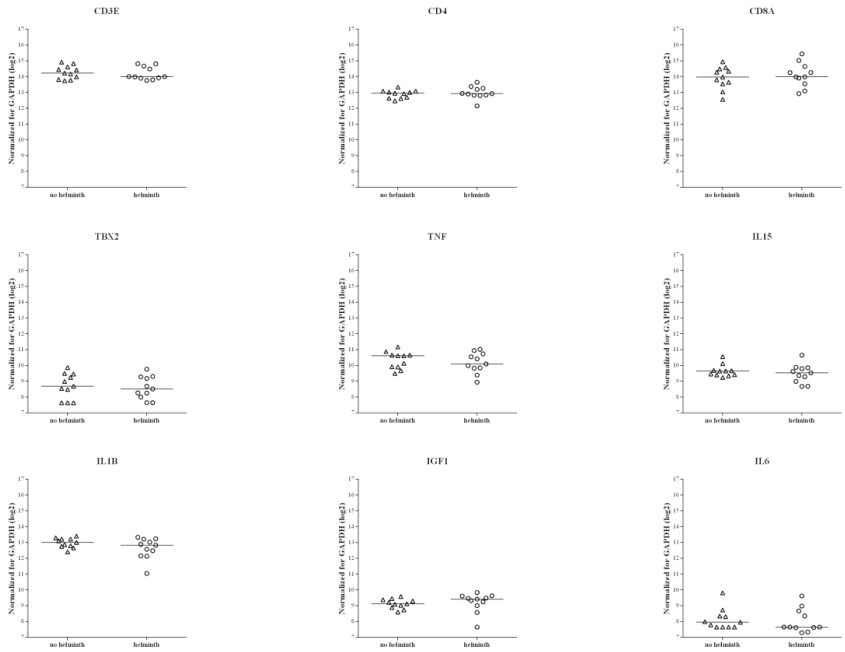


Fig 4: IFN-γ responses of PBMC of helminth coinfected and non-coinfected leprosy. Patients stimulated with *M. leprae* Whole Cell Sonicate (WCS) in 6 days culture A. patients without reaction: no helminth n=9 and helminth infested n=8 B. patients with T1R: no helminth n=4 and helminth infested n=4

Similar mRNA expressions of leprosy associated genes in patients with and without helminth

The mRNA expression of 76 genes associated with innate and adaptive immunity were assessed using dcRT-MLPA [41] in whole venous blood of BL/LL patients without helminth (n=11) and coinfected with helminth (n=11). The mRNA expressions in both groups were found similar. In figure 5, the mRNA expression levels for a selection of 27 genes are shown.



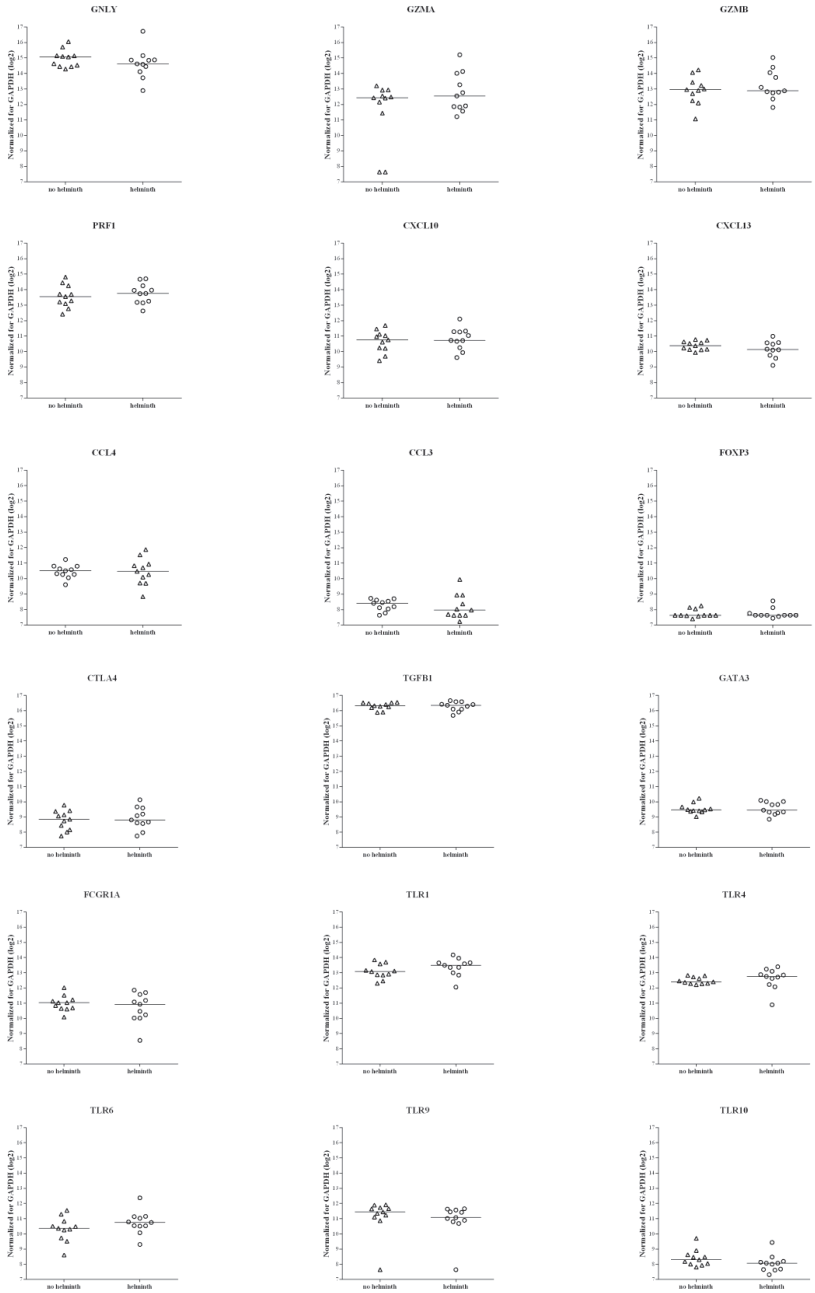


Fig 5: mRNA expression of 27 target genes in peripheral blood of BL/LL patients without helminths (n=11) and with helminths (n=11).

Discussion

Coinfections in leprosy have not been studied intensively although some reports have shown coinfections as risk factors for leprosy reactions (both type 1 and 2) [45;46]. Reports on the occurrence of TB in HIV patients who are naïve to ART as a result of diminished $CD4^+$ T cells [47;48] and in those who are on ART as IRIS [7] prompted the leprosy research community to evaluate HIV-infection in leprosy patients [13;17;18;49-51]. HIV coinfection was reported previously to occur in any form of leprosy [11] and a strong association of ART with the occurrence of T1R was shown in several studies [11;12;17;22] including this study. ART is known to restore $CD4^+$ T cell numbers but also drives an excessive and tissue destructive inflammation in some individuals [52]. This inflammatory environment may facilitate the occurrence of T1R in *M. leprae*-HIV coinfected patients. However, taking into consideration that T1R represents a common phenomenon that occurs in 30-50% of leprosy patients at any time before, during or after MDT, the interference or influence of ART needs to be investigated. In addition, studies on the interaction of various factors including ART, MDT, steroids, reactions, leprosy forms and various immune cells including T cells, dendritic cells and macrophages will certainly generate valuable information which could be used in management of coinfected patients in general and specifically those with T1R.

The clinical and histopathologcial features of leprosy in HIV coinfected patients did not differ from non-HIV leprosy patients in our study, as also evidenced by previous studies [15;49] except the above discussed ART and T1R association. No differences were observed in immune responses against *M. leprae* WCS as measured by different cytokines/chemokines suggesting the feasibility of the use of the newly identified diagnostic biomarkers in coinfected patients as well. Still, helminths-induced Th2 response may suppress stronger Th1, which could become evident upon treatment with anti-helminth prophylaxis. Therefore, further longitudinal studies are required to monitor the effect on anti-*M.leprae* responses effected by treatment.

Lower CD4:CD8 ratio [14] and an increased $CD8^+$ memory T cells in HIV coinfected patients with T1R [22] were reported previously. In our study, mRNA expression levels in whole blood of CD4 was significantly lower ($p<0.0001$), whereas CD8 expression was significantly higher ($p<0.002$) in HIV coinfected leprosy patients. In addition, IL15 and TLR10 were higher expressed in coinfected and ZNF532 was higher expressed in leprosy patients without HIV. IL-15 is known as a pleiotropic cytokine dominant in lesions of TT/BT patients [53;54]. Increased IL15 mRNA expression in coinfected patients may be associated with the Th1 dominated immune response as a result of ART and/or T1R. Toll like receptors (TLR) are important in triggering inflammatory and adaptive immune responses in a host invaded by pathogens. A recent study revealed that TLR10 shares microbial derived agonists of TLR1 and also requires TLR2 for innate immune recognition [55]. Although not particularly demonstrated for TLR10, there is evidence that HIV infected individuals in general show pro-inflammatory responsiveness to TLR agonists [56]. Therefore, the significantly increased TLR10 mRNA expression ($p =0.0001$) in the leprosy HIV coinfected patients demonstrated in this study, indicates its potential as a biomarker for HIV infection in leprosy patients. Zinc fingers in general are interaction modules that bind to different compounds including nucleic acids, proteins and small molecules [57]. There is insufficient information particularly on Zinc Finger 532 (ZNF532) function and the significantly higher mRNA expression ($p =0.002$) shown in non-HIV patients in this study calls for further investigation in this domain. There are few recent reports on Zinc finger nucleases, which are generated by fusing Zinc finger DNA-binding domain to a DNA cleavage as therapy for HIV.

Therefore, our finding on ZNF532 may have relevance in future diagnostic or therapeutic research in HIV.

On the other hand, in non-helminth and helminth coinfected leprosy patients, the comparable mRNA expressions may implicate the minimal interference of helminth but assessing the protein expression levels is warranted. Decreased Th1 responses in presence of helminths in leprosy patients was reported previously [35]. In this study, the majority of helminth coinfected and non-helminth leprosy patients were BL/LL patients and showed comparable Th1 responses because the cellular immune responses in both groups were low, in line with the general phenomenon in BL/LL patients. Importantly, however, we were able to show the effect of helminths in leprosy patients with T1R as their IFN-γ responses were significantly lower compared to non-helminthic leprosy patients with T1R. This suggested interference of helminth infestation and domination of helminth driven Th2 type response in patients with reaction as T1R generally induces a dominant Th1 type responses. Furthermore, since delay in clearance of bacilli is a major issue in BL/LL patients, the risk of helminth infection in further delaying the bacterial clearance and whether de-worming puts patients at risk of developing T1R have to be investigated in longitudinal studies to develop proper management algorithms of coinfected patients and potentially adapt diagnostic tests.

In general, characterization of host immune profiles in coinfected patients is relevant for the validation of newly developed diagnostic tools. In this study, the previously identified biomarkers for early diagnosis such as IP-10, CCL4 and CCL2 [58;59] were evaluated and comparable responses were observed in mono- and coinfected groups indicating the potential use of these biomarkers in both groups. Therefore, it is essential for future biomarker screenings or diagnostic tool development for leprosy to consistently include patients who are infected with other microbes besides *M. leprae* and thereby account for the interference of coinfections.

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