

**Functional biomarker signatures of circulating T-cells and its association with distinct clinical status of leprosy patients and their respective household contacts**

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## Abstract

**Background:** Leprosy is a chronic infectious disease classified into two subgroups for therapeutic purposes: paucibacillary (PB) and multibacillary (MB), closely related to the host immune responses. In this context it is noteworthy looking for immunological biomarkers applicable as complementary diagnostic tools as well as a laboratorial strategy to detect subclinical leprosy in household contacts. **Methods:** The main goal of the present study was to characterize the global cytokine signatures of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells from leprosy patients with distinct clinical forms and their respective household contacts (HHC) upon *in vitro* antigen-specific stimuli. Short-term culture of peripheral blood mononuclear cells was done. After incubation, cells were harvested and prepared for surface and intracytoplasmic cytokine staining **Results:** The cytokine signature analysis demonstrated that leprosy patients presented a polyfunctional profile of T-cells subsets, with increased frequency of IFN- $\gamma$ <sup>+</sup> T-cell subsets along with IL-10<sup>+</sup> and IL-4<sup>+</sup> from CD4<sup>+</sup> T-cells. Moreover, L(PB) displayed a polyfunctional profile characterized by enhanced percentage of IFN- $\gamma$ <sup>+</sup>, IL-10<sup>+</sup> and IL-4<sup>+</sup> produced by most T-cell subsets, as compared to L(MB) that presented a more restricted cytokine functional profile mediated by IL-10<sup>+</sup> and IL-4<sup>+</sup> T-cells with minor contribution of IFN- $\gamma$  produced by CD4<sup>+</sup> T-cells. Noteworthy was that HHC(MB) exhibited enhanced frequency of IFN- $\gamma$ <sup>+</sup> T-cells, contrasting with HHC(PB) that presented a cytokine profile limited to IL-10 and IL-4. **Conclusions:** Together, our findings provide additional immunological features associated with leprosy and household contacts. These data provide evidence that biomarkers of immune response can be useful complementary diagnostic/prognostic tools as well as insights that household contacts may present subclinical infection.

Keywords: leprosy, *Mycobacterium leprae*, cytokines, household contacts

## Background

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*), that represents a serious public health problem with more than 200,000 cases reported worldwide [1]. The disease is characterized by skin and peripheral nerves lesions leading to a broad range of clinical manifestations. Leprosy patients can be classified based on the number of skin lesions as paucibacillary (PB) or multibacillary (MB) [2,3].

The early diagnosis and prompt initiation of treatment are key strategies for leprosy control. Currently the diagnosis of leprosy is achieved essentially by clinical evaluation. Laboratorial methods may also be used to support Leprosy diagnosis. However, in general, the laboratorial test displays low sensitivity or low specificity and some methods are difficult to be used outside reference services [4]. To improve the leprosy diagnosis performance, it is still necessary to develop and integrated sensitive and specific approaches [5,6]. In this sense, the search of complementary laboratorial biomarkers is relevant to identify novel targets for leprosy diagnosis. Besides that, the development of diagnostic tools to detect subclinical leprosy in household contacts is also crucial to interrupt the transmission of *M. leprae*.

There is a general consensus that the host immune response plays a key role in determining the susceptibility to the mycobacteria and the clinical outcome of distinct clinical forms of leprosy [3]. It has been demonstrated that *M. leprae* antigens can discriminate contacts and leprosy patients from healthy controls [2,7]. There are several immunological events that seem to influence the outcome of leprosy, including mechanisms mediated by CD8<sup>+</sup> and CD4<sup>+</sup> T cells [8,9]. Evaluation of the cell-mediated immune response based on dichotomic cytokine profiles of T-cell subsets has been used to discriminate leprosy clinical presentation [9]. Moreover, it has been proposed that the degree of activation of CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes can also allow the discrimination between leprosy patients and their household contacts [6].

Strategies based on biomarkers have been extensively studied in recent years. In the present study we have employed an innovative strategy to characterize the global cytokine signatures of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and correlate these findings with distinct clinical forms of leprosy patients and their respective household contacts. The panoramic cytokine profiles offered additional insights into the immunological events that are relevant for future clinical studies and patient management.

## **Methods**

### **Subjects, Material and Methods**

#### *Ethics statement*

We hereby declare that this study was approved by the Ethics Committee of the Universidade Vale do Rio Doce (UNIVALE), filed under N° PQ 022/09-009. All participants signed a free and informed consent (IC) at the first evaluation. Parents/guardians provided consent on behalf of participants who were minors.

#### *Study Design and Participants*

The present study was carried out in Governador Valadares, eastern of Minas Gerais State, Brazil, that is considered a hyper endemic area for leprosy with approximately 1.9 cases/10,000 inhabitants compared to Minas Gerais (0.5/10,000) and Brazil (1.2/10,000) [1]. The study comprises a cross-sectional investigation that enrolled 49 participants of both genders, age ranging from 11 to 77 years old, including 19 patients (Leprosy, 12 males and 07 females, age ranging from 11 to 77 years old) and 30 healthy controls (Controls, 15 males and 15 females, age ranging from 13 to 58 years old). According the Brazilian Guidelines [10] the patients were further categorized into two subgroups referred as: L(PB) – patients with tuberculoid-tuberculoid/borderline-tuberculoid clinical form (n=09, 05 males and 04 females, age ranging from 11 to 64 years old) and L(MB) – patients presenting borderline-

borderline/borderline-lepromatous/lepromatous-lepromatous clinical forms (n=10, 07 males and 03 females, age ranging from 21 to 77 years old). The group of healthy controls was further classified as household contacts (HHC) of PB or MB patients and respectively referred as: HHC(PB) – (n=11, 04 males and 07 females, age ranging from 13 to 54 years old) and HHC(MB) – (n=10, 05 males and 05 females, age ranging from 13 to 53 years old). Heparinized whole blood sample was collected from all patients before treatment and from healthy controls and used to quantify the intracytoplasmic cytokine profile of circulating lymphocytes upon short-term *in vitro* culture. The study was submitted and approved by the Ethics Committee at the Universidade Vale do Rio Doce – Univale (PQ 022/09–009). All participants have read and signed the informed consent form prior to the inclusion in the study.

#### *Short-term culture in vitro of peripheral blood mononuclear cells*

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood samples by Ficoll-Hypaque gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, NJ, USA) and cultured as described previously by Antas [11]. Briefly, aliquots of  $1.0 \times 10^6$  PBMC/well were incubated in 24-well flat-bottom plates (Costar Corp., Cambridge, MA, USA) with 1mL of culture medium (RPMI1640 supplemented with 20% fetal calf serum, 2mM L-glutamine, 100IU/mL penicillin and 100 $\mu$ g/mL streptomycin) at 37°C, 5% CO<sub>2</sub> in a humidified incubator. In parallel batches,  $1.0 \times 10^6$  PBMC/well were incubated in the presence of sonicated fraction of irradiated *M. leprae* bacilli (10 bacilli/PBMC). *M. leprae*-stimulated and non-stimulated cultures were pre-incubated prior the addition of 3 $\mu$ g/mL of purified anti-human CD28 antibody (Pharmingen Inc., San Diego, CA, USA) for 1h and the addition of 10 $\mu$ g/mL of Brefeldin A (Sigma Immunochemical, St. Louis, MO, USA) 4h before the end of incubation achieved at 16, 20 and 24h. Positive control cultures were carried out using 50ng/mL PMA plus 1 $\mu$ g/mL ionomycin (Sigma Immunochemical, St. Louis, MO, USA) to confirm cell viability. After incubation, cells were harvested and prepared for surface and

intracytoplasmic cytokine staining according to Teixeira-Carvalho [12], modified as follows: cultured PBMC suspensions were adjusted to  $5.0 \times 10^5$  cells/mL and incubated with anti-CD4/PercP and anti-CD8/PercP-Cy5.5 (BD Bioscience, San Diego, CA, USA) for 30 min at 4°C in the dark. Following, cells were washed with phosphate buffered saline supplemented with 0.5% bovine serum albumin (PBS-FACS), fixed in 4% paraformaldehyde (PFA) for 10min at room temperature, washed twice with PBS-FACS and once with PBS supplemented with 0.3% saponin. After fix/perm procedures the cells cultured by 16, 20 and 24h were stained, respectively with anti-IFN- $\gamma$ , anti-IL-4 or anti-IL-10, labeled with PE (Pharmingen Inc., San Diego, CA, USA) for 30 min at room temperature. After two wash steps with saponin and one wash with PBS, stained cells were suspended with 1% PFA and immediately analyzed in a EPICS MCL® flow cytometer (BD Bioscience, San Jose, CA, USA) equipped with the CellQuest® software. A total of 50,000 events gated on lymphocyte regions were collected per sample. Distinct gating strategies were employed to quantify the percentages of cytokine<sup>+</sup> cells within CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. The percentages of cytokine<sup>+</sup> T-cells were estimated as the sum of cytokine-producing CD4<sup>+</sup> and CD8<sup>+</sup> cells. The results were expressed as mean frequency (%)  $\pm$  standard error of cytokine<sup>+</sup> cells amongst gated lymphocyte subsets upon in vitro culture in the absence/presence of *M. leprae* antigen.

#### *Data analysis*

Multiple comparisons amongst groups were carried out by Kruskal-Wallis test followed by Dunn's post-test for sequential pairwise comparisons. Additionally, Student T or Mann-Whitney Test were also employed for pairwise comparative analysis. Comparative analysis of categorical variables was carried out by Chi-square. In all cases, significant differences were considered at  $p < 0.05$ . The Graph Pad Prism software, Version 5.0 (San Diego, CA, USA) was employed to perform all statistical analyses and graphical arts.

Functional biomarker signature analysis was carried out using the global median values for each biomarker index (*M. leprae*-stimulated/non-stimulated culture) as the cut-off edge to identify the proportion of subjects with high biomarker levels, i.e. above the global median cut-off. The descriptive establishment of cut-offs are provided in the Supplementary Figure 1. Those biomarkers with more than 50% of subjects above the cut-off were underscored and considered for comparative analysis amongst groups. Overlaid ascendant biomarker signatures were employed for comparative analysis amongst groups. Venn diagram analysis (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) was employed to identify biomarkers selectively observed in Leprosy patients as compared to Controls as well as in Household Contacts and Leprosy subgroups.

## **Results**

*Analysis of cytokine<sup>+</sup> cells amongst peripheral blood mononuclear cells upon in vitro culture in the presence/absence of M. leprae antigen*

Short-term cultures *in vitro* of peripheral blood mononuclear cells from Leprosy patients and healthy Controls were carried out to quantify the percentages of cytokine<sup>+</sup> cells (IFN- $\gamma$ , IL-4 and IL-10) amongst T-cells, CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells. The results are presented in Table 1.

Table 1. Frequency of cytokine+ cells amongst peripheral blood mononuclear cells from leprosy patients, household contacts and healthy controls upon *in vitro* culture

Parameters*	Groups		Subgroups				
	Controls	Leprosy	Controls		Leprosy		
			HHC(PB)	HHC(MB)	L(PB)	L(MB)	
Non-stimulated Culture	T-cells						
	IFN- $\gamma^+$	4.6 $\pm$ 1.2	7.5 $\pm$ 2.6	2.4 $\pm$ 1.4	<u>10.9<math>\pm</math>2.8<sup>c</sup></u>	7.3 $\pm$ 4.2	7.8 $\pm$ 2.9
	IL-4 <sup>+</sup>	15.7 $\pm$ 3.8	13.9 $\pm$ 5.8	3.2 $\pm$ 1.5	<u>27.6<math>\pm</math>9.0<sup>c</sup></u>	5.1 $\pm$ 2.2	<u>8.0<math>\pm</math>4.7<sup>e</sup></u>
	IL-10 <sup>+</sup>	43.1 $\pm$ 14.8	30.6 $\pm$ 9.3	29.5 $\pm$ 7.4	<u>88.4<math>\pm</math>40.8<sup>c</sup></u>	43.8 $\pm$ 12.0	<u>7.6<math>\pm</math>3.0<sup>e</sup></u>
	CD4 <sup>+</sup> T-cells						
	IFN- $\gamma^+$	1.2 $\pm$ 0.3	2.3 $\pm$ 0.6	0.7 $\pm$ 0.2	<u>2.8<math>\pm</math>0.8<sup>c</sup></u>	2.0 $\pm$ 0.8	2.7 $\pm$ 1.1
	IL-4 <sup>+</sup>	2.2 $\pm$ 0.5	2.6 $\pm$ 0.9	1.5 $\pm$ 0.4	<u>4.7<math>\pm</math>1.6<sup>cf</sup></u>	3.2 $\pm$ 1.9	1.2 $\pm$ 0.6
	IL-10 <sup>+</sup>	2.5 $\pm$ 0.6	3.2 $\pm$ 1.0	1.0 $\pm$ 0.2	<u>4.7<math>\pm</math>1.7<sup>c</sup></u>	<u>3.6<math>\pm</math>1.3<sup>d</sup></u>	2.5 $\pm$ 1.4
	CD8 <sup>+</sup> T-cells						
	IFN- $\gamma^+$	0.4 $\pm$ 0.1	<u>1.2<math>\pm</math>0.5<sup>a</sup></u>	0.4 $\pm$ 0.2	0.8 $\pm$ 0.3	2.0 $\pm$ 1.1	0.6 $\pm$ 0.2
	IL-4 <sup>+</sup>	1.0 $\pm$ 0.4	1.7 $\pm$ 0.7	1.9 $\pm$ 1.5	1.7 $\pm$ 0.8	2.0 $\pm$ 1.2	1.4 $\pm$ 0.6
	IL-10 <sup>+</sup>	1.1 $\pm$ 0.6	1.1 $\pm$ 0.4	0.4 $\pm$ 0.2	1.9 $\pm$ 1.1	1.3 $\pm$ 0.7	0.7 $\pm$ 0.3
<i>M. leprae</i> -stimulated Culture	T-cells						
	IFN- $\gamma^+$	4.6 $\pm$ 1.1	8.8 $\pm$ 3.5	2.3 $\pm$ 1.2	<u>11.2<math>\pm</math>2.5<sup>c</sup></u>	9.0 $\pm$ 5.9	6.6 $\pm$ 2.5
	IL-4 <sup>+</sup>	12.0 $\pm$ 2.4	10.0 $\pm$ 3.5	3.5 $\pm$ 2.0	<u>19.8<math>\pm</math>6.7<sup>c</sup></u>	10.8 $\pm$ 4.5	10.0 $\pm$ 5.3
	IL-10 <sup>+</sup>	51.8 $\pm$ 13.8	49.2 $\pm$ 16.1	56.1 $\pm$ 22.2	<u>130.0<math>\pm</math>45.4<sup>cf</sup></u>	71.2 $\pm$ 21.4	<u>10.7<math>\pm</math>3.1<sup>e</sup></u>
	CD4 <sup>+</sup> T-cells						
	IFN- $\gamma^+$	1.4 $\pm$ 0.4	2.2 $\pm$ 0.7	0.7 $\pm$ 0.1	<u>3.5<math>\pm</math>1.1<sup>c</sup></u>	2.2 $\pm$ 1.0	2.3 $\pm$ 1.4
	IL-4 <sup>+</sup>	1.5 $\pm$ 0.3	1.9 $\pm$ 0.6	1.0 $\pm$ 0.4	<u>2.5<math>\pm</math>0.6<sup>c</sup></u>	1.7 $\pm$ 0.6	2.0 $\pm$ 1.0
	IL-10 <sup>+</sup>	2.5 $\pm$ 0.8	3.3 $\pm$ 1.0	1.6 $\pm$ 0.6	5.1 $\pm$ 2.0	4.0 $\pm$ 1.4	2.1 $\pm$ 1.2
	CD8 <sup>+</sup> T-cells						
	IFN- $\gamma^+$	0.4 $\pm$ 0.1	<u>1.3<math>\pm</math>0.6<sup>a</sup></u>	0.4 $\pm$ 0.2	0.7 $\pm$ 0.4	2.4 $\pm$ 1.3	0.5 $\pm$ 0.2
	IL-4 <sup>+</sup>	0.7 $\pm$ 0.3	1.1 $\pm$ 0.4	2.7 $\pm$ 0.0	1.3 $\pm$ 0.5	1.3 $\pm$ 0.5	1.0 $\pm$ 0.6
	IL-10 <sup>+</sup>	0.9 $\pm$ 0.5	1.2 $\pm$ 0.6	0.3 $\pm$ 0.1	2.0 $\pm$ 1.2	1.7 $\pm$ 1.0	0.6 $\pm$ 0.3

\* Data are expressed as mean frequency (% $\pm$  standard error of cytokine<sup>+</sup> cells (IFN- $\gamma$ , IL-4 and IL-10) amongst gated lymphocyte subsets upon *in vitro* culture in the presence/absence of *M. leprae* antigen, including: T-cells, CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells. L(PB)= HHC(PB)= Paucibacillary Leprosy patients; L(MB)= Multibacillary Leprosy patients; HHC(PB)=Household Contacts of Paucibacillary Leprosy patients; HHC(MB)=Household Contacts of Multibacillary Leprosy patients. Multiple comparisons amongst groups were carried out by Kruskal-Wallis test followed by Dunn's post-test for sequential pairwise comparisons. Additionally, Student T or Man-Whitney Test were also employed for pairwise comparative analysis. Significant differences at p<0.05 are underscored by letters "a", "b", "c", "d", "e" and "f" in comparisons to Controls, Leprosy, HHC(PB), HHC(MB), L(PB) and L(MB), respectively.

Data analysis demonstrated that Leprosy patients presented higher frequency of IFN- $\gamma^+$  CD8<sup>+</sup> T-cells as compared to healthy Controls. Moreover, HHC(MB) exhibited higher frequency of IFN- $\gamma^+$ , IL-4<sup>+</sup> and IL-10<sup>+</sup> events amongst T-cells and CD4<sup>+</sup> T-cells as compared to HHC(PB). Additionally, L(MB) displayed an enhanced percentage of IL-4<sup>+</sup> T-cells along with decreased frequency of IL-10<sup>+</sup> T-cells as compared to L(PB).

Complementary analysis of biomarker indexes (*M. leprae*-stimulated/non-stimulated culture) further demonstrated that L(MB) showed lower levels of IL-10<sup>+</sup> CD8<sup>+</sup> T-cells as compared to L(PB) (Table 2).

Table 2. Cytokine profile amongst peripheral blood mononuclear cells from leprosy patients, household contacts and healthy controls upon *in vitro* culture

Parameters	Groups		Subgroups				
	Controls	Leprosy	Controls		Leprosy		
			HHC(PB)	HHC(MB)	L(PB)	L(MB)	
<i>M. leprae</i> -stimulated /Non-stimulated Culture INDEX*	<b>T-cells</b>						
	IFN- $\gamma$ <sup>+</sup>	1.4±0.2	1.1±0.2	1.3±0.2	1.3±0.4	1.3±0.2	0.8±0.2
	IL-4 <sup>+</sup>	1.3±0.2	1.0±0.2	1.6±0.6	0.8±0.1	1.0±0.2	1.0±0.2
	IL-10 <sup>+</sup>	2.3±0.7	2.3±0.8	2.1±0.7	3.6±2.0	2.2±0.9	2.5±1.6
	<b>CD4<sup>+</sup>T-cells</b>						
	IFN- $\gamma$ <sup>+</sup>	1.3±0.2	1.0±0.2	1.3±0.3	1.5±0.3	1.1±0.2	0.9±0.3
	IL-4 <sup>+</sup>	0.8±0.1	1.2±0.3	0.7±0.1	0.7±0.1	0.9±0.2	1.4±0.5
	IL-10 <sup>+</sup>	1.1±0.2	1.5±0.5	1.1±0.2	1.1±0.3	1.2±0.2	1.9±1.4
	<b>CD8<sup>+</sup>T-cells</b>						
	IFN- $\gamma$ <sup>+</sup>	1.2±0.2	0.9±0.2	1.0±0.2	1.2±0.4	1.3±0.1	0.8±0.2
	IL-4 <sup>+</sup>	0.8±0.1	0.7±0.1	0.8±0.0	1.0±0.2	0.6±0.1	0.8±0.2
	IL-10 <sup>+</sup>	1.1±0.2	1.1±0.1	1.1±0.1	0.9±0.2	1.2±0.1	<b>0.8±0.1<sup>e</sup></b>

\* Data are expressed as mean INDEX (%) ± standard error of cytokine<sup>+</sup> cells (IFN- $\gamma$ , IL-4 and IL-10) amongst gated lymphocyte subsets upon *in vitro* culture in the presence/absence of *M. leprae* antigen, including: T-cells, CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells. L(PB)= HHC(PB)= Paucibacillary Leprosy patients; L(MB)= Multibacillary Leprosy patients; HHC(PB)=Household Contacts of Paucibacillary Leprosy patients; HHC(MB)=Household Contacts of Multibacillary Leprosy patients. Multiple comparisons amongst groups were carried out by Kruskal-Wallis test followed by Dunn's post-test for sequential pairwise comparisons. Additionally, Student T or Man-Whitney Test were also employed for pairwise comparative analysis. Significant differences at p<0.05 underscored by letters "a", "b", "c", "d", "e" and "f" in comparisons to Controls, Leprosy, HHC(PB), HHC(MB), L(PB) and L(MB), respectively.

*Antigen-specific cytokine signature of peripheral blood mononuclear cells upon in vitro culture in the presence/absence of M. leprae antigen*

The antigen-specific cytokine signature was assembled for each parameter, including IFN- $\gamma$ , IL-4 and IL-10-producing T-cells, CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells. Categorical transformation was carried out using the global median values for each biomarker as the cut-off to identify subjects with high biomarker indexes. The results are presented in Table 3.

Table 3. Antigen-specific cytokine profile of peripheral blood mononuclear cells from leprosy patients, household contacts and healthy controls

Parameters	Groups		Subgroups				
	Controls	Leprosy	Controls		Leprosy		
			HHC(PB)	HHC(MB)	L(PB)	L(MB)	
Proportion of subjects with INDEX above the Global Median cut-off <sup>#</sup>	<b>T-cells</b>						
	IFN- $\gamma$ <sup>+</sup>	46 (11/24)	50 (05/10)	44 (04/09)	50 (03/06)	67 (04/06)	25 (01/04)
	IL-4 <sup>+</sup>	50 (12/24)	47 (08/17)	44 (04/09)	33 (02/06)	57 (04/07)	40 (04/10)
	IL-10 <sup>+</sup>	47 (14/30)	55 (06/11)	64 (07/11)	50 (05/10)	57 (04/07)	50 (02/04)
	<b>CD4<sup>+</sup>T-cells</b>						
	IFN- $\gamma$ <sup>+</sup>	42 (10/24)	50 (05/10)	44 (04/09)	67 (04/06)	50 (03/06)	50 (02/04)
	IL-4 <sup>+</sup>	39 (09/23)	<b>69 (11/16)<sup>a</sup></b>	33 (03/09)	20 (01/05)	57 (04/07)	<b>78 (07/09)<sup>e</sup></b>
	IL-10 <sup>+</sup>	47 (14/30)	73 (08/11)	55 (06/11)	40 (04/10)	86 (06/07)	50 (02/04)
	<b>CD8<sup>+</sup>T-cells</b>						
	IFN- $\gamma$ <sup>+</sup>	39 (07/18)	50 (03/06)	33 (01/03)	33 (02/06)	<b>100 (02/02)<sup>f</sup></b>	25 (01/04)
	IL-4 <sup>+</sup>	78 (07/09)	<b>17 (01/06)<sup>a</sup></b>	100 (01/01)	75 (03/04)	<b>0 (00/03)<sup>d</sup></b>	33 (01/03)
	IL-10 <sup>+</sup>	31 (05/16)	30 (03/10)	25 (01/04)	29 (02/07)	50 (03/06)	<b>0 (00/04)<sup>e</sup></b>

# Data are expressed as proportion (%) of subjects with biomarker INDEX (*M. leprae*-stimulated/non-stimulated culture) above the Global Median cut-off, established for each cytokine<sup>+</sup> cells (IFN- $\gamma$ , IL-4 and IL-10) amongst gated lymphocyte subsets upon *in vitro* culture in the presence/absence of *M. leprae* antigen, including: T-cells, CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells. L(PB)= HHC(PB)= Paucibacillary Leprosy patients; L(MB)= Multibacillary Leprosy patients; HHC(PB)=Household Contacts of Paucibacillary Leprosy patients; HHC(MB)=Household Contacts of Multibacillary Leprosy patients. Comparative analysis of was carried out by Chi-square and significant differences at p<0.05 underscored by “a”, “b”, “c”, “d”, “e” and “f” in comparisons to Controls, Leprosy, HHC(PB), HHC(MB), L(PB) and L(MB), respectively.

Data analysis demonstrated that the Leprosy group comprises a higher proportion of subjects with enhanced levels of IL-4<sup>+</sup> CD4<sup>+</sup> T-cells as compared to health Controls. Conversely, the Leprosy group exhibited a decrease proportion of subjects with IL-4<sup>+</sup> CD8<sup>+</sup> T-cells above the global median cut-off as compared to health Controls. No significant differences were observed between subgroups of Household contacts. Comparative analysis between subgroups of Leprosy patients showed that L(PB) presented a higher proportion of IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T-cells and lower proportion of IL-4 CD8<sup>+</sup> T-cells as compared to L(MB). On the other hand, L(MB) group presented higher proportion of IL-4<sup>+</sup> CD4<sup>+</sup> T-cells and lower frequency of IL-10<sup>+</sup> CD8<sup>+</sup> T-cells as compared to L(PB) (Table 3)

*Functional biomarker signatures in Leprosy patients and healthy Controls*

The comparative analysis of ascendant biomarker signatures between Leprosy patients and healthy Controls is presented in Figure 1. Overlaid biomarker signatures were assembled to select biomarkers with proportion of subjects above the global median cut-off higher than the 50% in each group. These attributes were tagged for further analysis (Figure 1A).

Venn diagram report was employed to identify the set of biomarkers selectively increased in Leprosy patients as compared to healthy Controls. The analysis of Venn diagram report allowed the selection of IFN- $\gamma^+$  T-cells, IFN- $\gamma^+$  CD4 $^+$  T-cells, IFN- $\gamma^+$  CD8 $^+$  T-cells, IL-10 $^+$  T-cells, IL-4 $^+$  CD4 $^+$  T-cells and IL-10 $^+$  CD4 $^+$  T-cells as Leprosy-selective biomarkers. Moreover, the attributes IL-4 $^+$  T-cells and IL-4 $^+$  CD8 $^+$  T-cells were identified as healthy Controls-selective biomarkers (Figure 1B).

*Functional biomarker signatures in subgroups of household contacts and Leprosy patients with distinct clinical forms*

Comparative analyses of ascendant biomarker signatures between subgroups of Household contacts and subgroups of Leprosy patients are presented in Figure 2. Overlaid biomarker signatures were assembled to select biomarkers with proportion of subjects above the global median cut-off higher than the 50% in each group. Data analysis demonstrate that the attributes IL-10 $^+$  CD4 $^+$  T-cells, IL-10 $^+$  T-cells and IL-4 $^+$  CD8 $^+$  T-cells were increased in HHC(PB), whereas IFN- $\gamma^+$  T-cells, IL-10 $^+$  T-cells, IFN- $\gamma^+$  CD4 $^+$  T-cells and IL-4 $^+$  CD8 $^+$  T-cells were highlighted in HHC(MB). Furthermore, the attributes IFN- $\gamma^+$  CD4 $^+$  T-cells, IL-10 $^+$  CD8 $^+$  T-cells, IL-4 $^+$  T-cells, IL-4 $^+$  CD4 $^+$  T-cells, IL-10 $^+$  T-cells, IFN- $\gamma^+$  T-cells, IL-10 $^+$  CD4 $^+$  T-cells and IFN- $\gamma^+$  CD8 $^+$  T-cells were tagged in L(PB), while IFN- $\gamma^+$  CD4 $^+$  T-cells, IL-10 $^+$  T-cells, IL-10 $^+$  CD4 $^+$  T-cells and IL-4 $^+$  CD4 $^+$  T-cells were underscored in L(MB) (Figure 2A).

Venn diagram report was employed to identify the set of biomarkers selectively increased in each subgroup of Household contacts and subgroups of Leprosy patients. Using this approach,

the attribute IL-10<sup>+</sup> T-cells was identified as a universal biomarker observed in all subgroups. The attribute IL-4<sup>+</sup> CD8<sup>+</sup> T-cells was a general biomarker to identify both Household contacts, whereas IL-4<sup>+</sup> CD4<sup>+</sup> T-cells was a general biomarker for both subgroups of Leprosy patients. L(PB) selective biomarkers comprise IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T-cells, IL-4<sup>+</sup> T-cells and IL-10<sup>+</sup> CD8<sup>+</sup> T-cells (Figure 2B).

## Discussion

Leprosy still persists as a relevant public health problem in several countries in Asia, Africa, and Latin America [1]. The host immune response plays a critical role in the pathophysiology of Leprosy, that together with genetic factors influence the clinical course of disease [13].

The use of immunological biomarkers as diagnostic/prognostic tools is a relevant complementary strategy to classify leprosy patients into distinct clinical forms and support early clinical interventions, prompt initiation of treatment and effective leprosy control. Moreover, it has been proposed that immunological biomarkers can also be a useful laboratorial strategy to detect subclinical leprosy in household contacts [6].

In the present study, we have characterized the functional cytokine signatures of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells from leprosy patients with distinct clinical forms and their respective household contacts (HHC), using a model of *in vitro* antigen-specific stimuli for peripheral blood mononuclear cells. Our data demonstrated that leprosy patients presented an overall polyfunctional profile of T-cells subsets, with increased frequency of IFN- $\gamma$ <sup>+</sup> T-cell subsets along with IL-10<sup>+</sup> and IL-4<sup>+</sup> from CD4<sup>+</sup> T-cells. According to Queiroz [6] the highest degree of activation of T CD4<sup>+</sup> and T CD8<sup>+</sup> lymphocytes in the index cases, could be, a response to antigenic stimulation by the bacillus, with consequent increased CD86 expression by the monocytes that activate these lymphocytes, generating effector and/or memory T cells. Differential costimulatory molecule expression by T-cells was also verified by Shibuya [14], among lepromatous and tuberculoid patients and contacts. Molecules that either signal for (CD86 and CD28) or reduce (CD152 and PD-1) T-cell activation were evaluated. Probably as a consequence of defective APC function, T-cells from lepromatous patients were driven to an anergic state and exhibited reduced expression of both CD86 and CD28, especially when compared with tuberculoid patients. Additionally, our results demonstrated that L(PB) displayed a polyfunctional profile characterized by enhanced percentage of IFN- $\gamma$ <sup>+</sup>, IL-10<sup>+</sup>

and IL-4<sup>+</sup> produced by most T-cell subsets, as compared to L(MB) that presented a more restricted cytokine functional profile mediated by IL-10<sup>+</sup> and IL-4<sup>+</sup> T-cells with minor contribution of IFN- $\gamma$  produced by CD4<sup>+</sup> T-cells. According to the literature, there is a predominance of a Th2 lymphocytes response in the lepromatous form, which induces the production of cytokines such as IL-4, IL-10, and TGF- $\beta$  that inactivate the microbicidal response of macrophages, thereby facilitating the survival of the bacillus [15]. Considering the minor contribution of IFN- $\gamma$  among L(MB), our results corroborate with literature. Noteworthy was that HHC(MB) exhibited enhanced frequency of IFN- $\gamma$ <sup>+</sup> T-cells, contrasting with HHC(PB) that presented a cytokine profile limited to IL-10 and IL-4. It has been described the correlation between the levels of IFN- $\gamma$  and the degree of exposure to *M. leprae*. While the IFN- $\gamma$  levels were (almost) absent in individuals living in areas with low/medium prevalence rates, in residents of high-prevalence neighborhoods, levels were comparable to those seen in household contacts of leprosy patients. These data indicate that in areas with high prevalence rates, the exposure to *M. leprae* is independent of a previous history of contact with leprosy patients [16]. Furthermore, Geluk [17], showed that ratio IFN- $\gamma$ /IL-10 was higher for asymptomatic individuals compared to either leprosy patient. Interestingly, according to Sampaio [18], *M. leprae*-induced IFN- $\gamma$  release was as great if not greater in MB contacts than in contacts exposed to lower antigenic loads (PB contacts). Moreover, the high percentage of responders in those household and occupational contacts suggest a high index of sensitization among exposed persons [18]. On the other hand, Martins [16], showed the IFN- $\gamma$  levels in response to *M. leprae* or *M. leprae*-specific peptides is progressively reduced when groups of increasing levels of exposure to *M. leprae* are compared (HCMB), and are further diminished in leprosy patients. However, the extent of response to *M. leprae* antigen among the contacts primarily depended on the length of contact and degree of infectiousness for the index case rather than the degree of consanguinity with the index patients [18]. Together, our findings provide additional immunological features associated with leprosy and

household contacts. These data provide evidence that biomarkers of immune response can be useful complementary diagnostic/prognostic tools as well as insights that household contacts may present subclinical infection.

Moreover, according to Geluk [17], IFN- $\gamma$  production induced by *M. leprae*-unique proteins can identify individuals highly exposed to *M. leprae* and therefore more at risk for developing disease and/or transmitting the bacterium.

In our group a parallel study was done using samples of slit skin smears (SSS) from asymptomatic household contacts. We demonstrated that 23.89% of the contacts group showed bacillary DNA by qPCR in samples of SSS and blood. The level of bacillary DNA from household contacts was similar to the DNA levels detected in the group of paucibacillary patients, contributing for the early diagnosis in such high risk groups [19]. Moreover, Gama [20], proposed an integrated analysis of molecular and serological methods using artificial intelligence. The model allows for the diagnosis of leprosy with high sensitivity and specificity and the early identification of new cases among household contacts. Marçal [21], evaluating the utility of immunoglobulin isotypes against LID-1 and NDO-LID, demonstrated that serological tests based on the detection of antigen-specific IgG1 antibodies indicate the enhanced performance of the LID-1 and NDO-LID antigens in the serodiagnosis of leprosy. We conclude that, from the significant level of antigen-specific cytokine secretion by peripheral blood cells from household contacts, especially those HHC(MB), provide reliable evidence of subclinical infection in the household contacts.

## **Conclusions**

Our data provide evidence that biomarkers of immune response can be useful complementary diagnostic/prognostic tools as well as insights that household contacts may present subclinical infection, especially those HHC(MB), that showed significant level of antigen-specific cytokine secretion by peripheral blood cells. Those individuals should be monitored for

disease development in the future.

Early detection of leprosy cases and effective chemotherapy are the best strategies to reduce the incidence of new cases of leprosy and prevent transmission. Considering that HHCs comprise a recognizable group of individuals with a high risk of disease, as they live in close proximity to a source of infection, we suggest that, as a prevention strategy, detection of antigen-specific cytokine secretion should be used to follow-up with leprosy HHCs to confirm or rule out subclinical infection.

### **Additional file**

**Supplementary figure 1.** Establishment of Cut-offs to segregate subjects with low or high Biomarker INDEX (*ppt*)

**Abbreviations** BI: Bacilloscopy index; L(PB): leprosy (paucibacillary), L(MB): leprosy multibacillary, HHC(PB): Household Contacts of paucibacillary; HHC(MB): Household Contacts of multibacillary; CREDENPES: Center for Endemic Diseases and Special Programs; IC: Informed consent; *M. leprae*: *Mycobacterium leprae*; N: Number of patients;

**Acknowledgments:** The authors thank the Programa Multicêntrico de Bioquímica e Biologia Molecular at Universidade Federal de Juiz de Fora Campus Gov. Valadares e Universidade Vale do Rio Doce. We are very thankful to Maria de Fatima Silva, Marlucy Rodrigues Lima, Lilia Cardoso Pires, and Wallace Olimpio for their technical support. We are also thankful to all members of CREDEN-PES, especially Dr. Alexandre Castelo Branco for the diagnostics of the patients and Regina L.B. Cipriano for the administrative support.

**Funding:** This study received financial support from the Conselho de Desenvolvimento Tecnológico e Científico/CNPq/BRAZIL, DECIT 2008, DECIT 2012, FAPEMIG, TC 304/2013/FNS/MS and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). OAMF received PQ

fellowships from CNPq. These fundings sources had no role in the design of the study and collection, analysis, implementation, and interpretation of data and in writing the manuscript.

**Availability of data and materials:** All data generated or analysed during this study are included in this published article and its supplementary information files (Additional file 1).

**Authors' contributions:** PHFM and LAOF developed and standardized the protocol and wrote the manuscript. RSG and LBOP collected the samples. ROP, MOM and ENS contributed reagents/materials/analysis tools. OAMF analyzed the data and helped to write the manuscript. All of the authors read and approved the manuscript.

**Ethics approval and consent to participate:** We hereby declare that this study was approved by the Ethics Committee of the Universidade Vale do Rio Doce—Univale, filed under N° PQ 022/09–009. All participants signed a free and informed consent at the first evaluation.

**Consent for publication:** Not applicable.

**Competing interests:** The authors declare that they have no competing interests.

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## Legend for Figures

*Figure 1. Functional biomarker signatures in Leprosy patients and healthy Controls.* (A) Overlaid biomarker signatures of Leprosy patients (■) and Controls (○) were assembled to select biomarkers with proportion of subjects above the global median cut-off higher than the 50% in each group (black/white background rectangles). (B) Venn diagram report was employed to identify the set of biomarkers selectively increased in Leprosy patients as compared to healthy Controls. These attributes were tagged in bold format.

*Figure 2. Functional biomarker signatures in subgroups of Leprosy patients and Household contacts.* (A) Overlaid biomarker signatures of subgroups of Household contacts (HHC(PB)= ■ and HHC(MB)= ●) and subgroups of Leprosy patients (L(PB)= ■ and L(MB)= ■) were assembled to select biomarkers above the global median cut-off with proportion higher than the 50% in each group (black/white background rectangles). These attributes were tagged in bold format. (B) Venn diagram report was employed to identify the set of biomarkers

selectively increased in each subgroup of Household contacts and subgroups of Leprosy patients. These attributes were highlighted in bold underline format.

Supplementary Figure 1. Establishment of cut-offs to segregate subjects with “low” or “high” biomarkers index. The whole data universe (Leprosy+Controls) of each biomarker index (*M. leprae*-stimulated/non-stimulated culture) was plotted to calculate the global median values and establish specific cut-off values for each cytokine<sup>+</sup> cells (IFN- $\gamma$ , IL-4 and IL-10) amongst T-cells, CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells. These cut-offs are provided in the figure and were further employed to categorize the subjects as they present “low” (○) or “high” (●) biomarker index.