T-Cell Regulation in Lepromatous Leprosy

Kidist Bobosha1,2*, Louis Wilson1, Krista E. van Meijgaard1, Yonas Bekele2, Martha Zewdie2, Jolien J. van der Ploeg- van Schip1, Markos Abebe2, Jemal Hussein2, Saraswoti Khadge3, Kapil D. Neupane3, Deanna A. Hagge3, Ekaterina S. Jordanova4, Abraham Aseffa2, Tom H. M. Ottenhoff1, Annemieke Geluk1

1 The Dept. of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands, 2 Armauer Hansen Research Institute and ALERT hospital, Addis Ababa, Ethiopia, 3 Mycobacterial Research Laboratory, Anandaban Hospital, Kathmandu, Nepal, 4 The Dept. of Obstetrics and Gynaecology, Free University Amsterdam, Center for Gynaecologic Oncology, Amsterdam, The Netherlands

Abstract

Regulatory T (Treg) cells are known for their role in maintaining self-tolerance and balancing immune reactions in autoimmune diseases and chronic infections. However, regulatory mechanisms can also lead to prolonged survival of pathogens in chronic infections like leprosy and tuberculosis (TB). Despite high humoral responses against Mycobacterium leprae (M. leprae), lepromatous leprosy (LL) patients have the characteristic inability to generate T helper 1 (Th1) responses against the bacterium. In this study, we investigated the unresponsiveness to M. leprae in peripheral blood mononuclear cells (PBMC) of LL patients by analysis of IFN-γ responses to M. leprae before and after depletion of CD25+ cells, by cell subsets analysis of PBMC and by immunohistochemistry of patients’ skin lesions. Depletion of CD25+ cells from total PBMC identified two groups of LL patients: 7/18 (38.8%) gained in vitro responsiveness towards M. leprae after depletion of CD25+ cells, which was reversed to M. leprae-specific T-cell unresponsiveness by addition of autologous CD25+ cells. In contrast, 11/18 (61.1%) remained anergic in the absence of CD25+ T-cells. For both groups mitogen-induced IFN-γ was, however, not affected by depletion of CD25+ cells. In M. leprae responding healthy controls, treated lepromatous leprosy (LL) and borderline tuberculoid leprosy (BT) patients, depletion of CD25+ cells only slightly increased the IFN-γ response. Furthermore, cell subset analysis showed significantly higher (p = 0.02) numbers of FoxP3+CD8+CD25+ T-cells in LL compared to BT patients, whereas confocal microscopy of skin biopsies revealed increased numbers of CD68+CD163+ as well as FoxP3+ cells in lesions of LL compared to tuberculoid and borderline tuberculoid leprosy (TT/BT) lesions. Thus, these data show that CD25+ Treg cells play a role in M. leprae-Th1 unresponsiveness in LL.

Introduction

The human immune system strives to maintain the delicate balance between preventing host susceptibility to various pathogens and limiting immunopathology due to an exacerbated immune response to infections. Sub-populations of T-cells previously identified as suppressor T-cells and later as Treg cells are the major players in the regulatory network of the immune system [1,2]. Although the idea of suppressor T-cells was a key topic of research already in the 70’s and 80’s it was not successfully established because of poor cellular characterization, and it took until mid-1990’s before Treg cells were recognized as a different lineage [1]. More recently, studies clearly demonstrated the suppressive ability of this sub-population contributing to the re-acceptance of suppressor T-cell as a different T-cell lineage [3,4].

Characterization of this T-cell sub-population has continued and currently the thymus-derived Treg cells (iTreg cells) and peripherally derived Treg cells (pTreg cells) [5] are the two widely accepted categories of Treg cells [1,6,7]. Both T-cell subtypes play a role in limiting immune reactions in autoimmune diseases and chronic infections [8–11]. In addition, CD39+ Treg cells have also been reported as a subset of the CD4+ CD25highFoxP3+ Treg cells in association with chronic infections like tuberculosis (TB) [12], hepatitis B (HBV) and in graft rejections [13,14] and the ability of CD8+ CD39+ Treg cells to suppress antigen specific CD4+ proliferation clearly demonstrated the importance of this sub-population [15].

Leprosy is a chronic infectious disease leading to more than 200,000 new cases every year [16]. The remarkable inter-individual variability in clinical manifestations of leprosy closely parallels the hosts’ abilities to mount effective immune responses to M. leprae. This is clear from the well-known immunological and clinical spectrum in those who progress to disease ranging from polar T helper 1 (Th1) to Th2 responses. TT and BT show more dominant Th1 responses which limit M. leprae growth resulting in clinical paucibacillary (PB) leprosy whereas, BL/LL patients demonstrate dominant Th2 responses as well as more permissive growth of M. leprae resulting in clinical multibacillary (MB) leprosy. TT/BT patients in general show high cellular responses and low antibody titers to M. leprae antigens, and develop localized...
**Author Summary**

Leprosy is a curable infectious disease caused by *Mycobacterium leprae* (*M. lepra*) that affects the skin and peripheral nerves. It is manifested in different forms ranging from self-healing, tuberculoid leprosy (TT) with low bacillary load and high cellular immunity against *M. lepra*, to lepromatous leprosy (LL) with high bacillary load and high antibody titers to *M. lepra* antigens. However, LL patients have poor cell mediated response against *M. lepra* leading to delayed clearance of the bacilli. A possible explanation for this bacterial persistence could lie in the presence of more regulatory cells at infection sites and in peripheral blood. This study shows the recovery of the cell mediated response by depletion of CD25 T cells in a subset of LL patients, while another patient subset was not affected similarly. Moreover, an increased frequency of FoxP3+ T cells together with anti-inflammatory macrophages was observed in LL patients' skin biopsies. Thus, these data show that CD25 Treg cells play a role in the *M. lepra*-unresponsiveness in leprosy patients.

granuloma with often no detectable bacilli in their lesions. The LL/BL patients at the opposite pole are incapable to generate *M. lepra* specific Th1 cell responses, show high antibody titers to *M. lepra* antigens, and poor granuloma formation with numerous bacilli in their lesions. The borderline states of leprosy are immunologically unstable. The different outcomes of infection in leprosy are most likely caused by host defense mechanisms [17]. However, the mechanism underlying the *M. lepra*-specific T cell anergy in LL patients is still not completely understood.

In chronic bacterial or viral infections, evidence exists that Treg cells suppress effector T cells (Teff cells) in order to limit damage to the host caused by the immune responses against pathogens [18]. In this situation, the regulatory activity of Treg cells may lead to prolonged survival of pathogens in the host [9,19]. As evidenced in a previous study, higher levels of CD4+CD25+FoxP3+ Treg cells were observed in active TB patients in the periphery compared to latently infected individuals and healthy controls [20,21]. Also, an increased number of Treg cells expressing FoxP3, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced tumour-necrosis-factor-receptor-related protein (GITR) were reported in lymphnodes from children with tuberculosis lymphadenitis [22]. Similarly, in leprosy, higher numbers of Treg cells in PBMC from BL and LL patients stimulated with *M. lepra* cell wall antigen (MLCWA) were observed compared to TT/BL forms, indicating the possibility that Treg cells may have a role in persistence of *M. lepra* bacteria as well as unresponsiveness of Th1 cells in BL/LL patients [23]. Recently, the mechanism of action of FoxP3 in CD4+CD25+ T cells derived from BL/LL leprosy patients was shown to result from increased molecular interactions of FoxP3 with Histone deacetylases (HDAC7/9) in the nucleus of CD4+CD25+ T cells derived from BL/LL patients [24].

In the presence of pathogens, Treg cells can also be induced by certain macrophages as evidenced by the anti-inflammatory, CD163+ macrophages, known as type 2 macrophages (m2p2), that exert a suppressive effect on Th1 responses [25,26]. On the other hand, IL-10 induced phagocytosis of *M. lepra* by m2p2 without induction of microbialicidal activity in LL patients has been described [27] indicating the role of IL-10 producing Treg cells in the persistence of the pathogen within the host. Similarly, the presence of higher IL-10 expression correlated with increased CD163 and indoleamine 2,3-dioxygenase (IDO) proteins in tissues and sera of LL patients further evidenced their potential [28].

In this study, we have investigated the functional role of CD25 Treg cells in *M. lepra* unresponsiveness of LL patients as well as the frequency of CD25+ and FoxP3+ cells in the PBMC of leprosy patients. Additionally, lesions of LL and TT/BL patients were assessed for the presence of FoxP3+ cells and CD163+ macrophages (m2p2).

**Materials and Methods**

**Ethical statement**

Ethical approval of the study protocol was obtained from the National Health Research Ethical Review committee, Ethiopia (NERC # RDHE/127-83/08) and the Nepal Health Research Council (NHRC #751). 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 for their treatment. Written and Informed consent was obtained from study participants before enrollment.

**Study participants**

The following HIV-negative individuals were recruited on a voluntary basis: newly diagnosed, non reactional leprosy patients from Ethiopia (ALERT hospital, Addis Ababa, Ethiopia) classified as LL (n = 40) and TT/BL (n = 16) and healthy endemic controls from health centers in Addis Ababa (EC; n = 5); Treated, non reactional LL (n = 6) and TT/BL (n = 9) patients and EC (n = 10) from Anandaban Hospital, Kathmandu, Nepal; and non-endemic Dutch healthy controls (NEC; n = 13). Leprosy was diagnosed based on clinical, bacteriological and histological observations and classified by a skin biopsy evaluated according to the Ridley and Jopling classification [17] by qualified microbiologists and pathologists. All patients were enrolled before treatment was initiated. EC were assessed for the absence of clinical signs and symptoms of tuberculosis and leprosy. Individuals working in health facilities were excluded as EC.

**PBMC isolation, freezing and thawing**

PBMC were isolated by Ficoll-Hypaque density gradient method, 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 °C) for 30 to 40 seconds until thawed half way and resuspended in 10% FCS in AIM-V (37 °C) containing 1/10,000 benzene until completely thawed, washed 2 times (5–7 minutes each) and counted. The percentage viability obtained was >75% and cells were incubated with anti-CD25 magnetic beads or used for FACS analysis.

**CD25+ cell separation**

Frozen PBMC were thawed, washed and incubated with 20 μl of the CD25 micro beads II, human (Miteny Biotec, Bergisch Gladbach, Germany) in 80 μl MACS buffer (Phosphate-buffered saline (PBS) with 0.5% Bovine serum albumin (BSA) and 2 mM EDTA) for 20 minutes at 4 °C. Cells were washed and added to MS column attached to Magnetic Cell Sorter (MACS) (Milteny Biotec) where CD25+ cells were collected as flow through and the CD25+ population was collected by detaching the column from the magnetic cell sorter. Cells were washed with MACS buffer and
resuspended in AIM-V medium. The purity of the CD25− and CD25+ cell populations was >80% (supplementary figure S2A and S2B).

Lymphocyte stimulation tests (LST)

Total PBMC (150,000 cells/well), CD25− cells (150,000 cells/well) or CD25+ cells with proportionally added CD25+ cells (10,000 and/or 25,000) 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% CO2 and 70% humidity. After 6 days, supernatants were collected and kept frozen until used in ELISA.

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 at [http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx]).

IFN-γ ELISA

IFN-γ levels were determined by ELISA (U-CyTech, Utrecht, The Netherlands) [29]. The cut-off value to define positive responses was set beforehand at 100 pg/ml. The assay sensitivity level was 40 pg/ml. Values for unstimulated cell cultures were typically <40 pg/ml.

Flow cytometry

After depletion, the total PBMC, CD25− or CD25+ populations (25,000 to 200,000 cells) were stained for CD3 (clone SK7, PerCP; Becton, Dickinson and Company, New Jersey, USA), CD4 (clone SK3, FITC; BD) and CD25 (PE; MACS) to check the purity.

Frozen PBMC of patients and healthy controls (2 × 106 cells/ml) were thawed, washed and treated with benzonase (10 U/ml, Novagen, MerckBioSciences, Merck KGaA, Darmstadt, Germany) for 2 hours prior to in vitro stimulation with PMA (20 ng/ml)/ionomycine (500 ng/ml) in the presence of 1 μg/ml anti CD28 (Sanquin, the Netherlands) and 1 μg/ml anti CD49d (BD Biosciences, Eberbodegem, Belgium) for 5 hours. Brefeldin A (Sigma Aldrich) was added at 3 μg/ml and cells were left for an additional 6 hours in the incubator at 37°C with 5% CO2 and 70% humidity. After live/dead staining with Vivid (Invirogen, Life technologies, Merelbeke, Belgium), surface staining was performed for 30 minutes at 4°C with the labeled antibodies directed against: CD14- and CD19-Pacific Blue, CD3-PE- TexasRed (all Invitrogen, Life technologies), CD8-Horizon V500, CD4-Pe-Cy7, CD25-APC-H7 (all BD Biosciences), and CD39-PE (Biolegend, ITK Diagnostics, Uithoorn, The Netherlands). Samples were washed, fixed and intracellular staining was performed using the intrastain kit (Dako Diagnostics, Glostrup, Denmark) with IFN-γ -Alexa700 (BD Biosciences), IL-10 APC (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and FoxP3 PE-Cy5 (eBioscience, Hatfield, UK) labeled antibodies. Cells were acquired on a FACS LSR Fortessa with Diva software (BD Biosciences, The Netherlands) and analyzed with FlowJo version 9.4.1 (Tree Star, Ashland, OR, USA). The full gating strategy for live CD4+ CD3+ cells or CD8+ CD3+ cells (supplementary Figure S1A and S1B) was performed in compliance with the most recent MIATA [30] guidelines according to the following procedure: events were first gated using a forward scatter area (FSC-A) versus height (FSC-H) plot to remove doublets. Subsequently, the events were subjected to a lymphocyte gate using a side scatter (SSC) followed by a live/dead gating. Then, live CD3+ cells were gated and CD14+ and CD19+ events were excluded from analysis using a dump channel. Finally, CD3 live cells were separated in to CD4+ and CD8+. After the gates for each function were created, we used the Boolean gate platform to identify all functions within each cell preparation using the full array of possible combinations.

Immunohistochemistry and confocal microscopy

Skin biopsies taken from leprosy lesions of LL (n = 10) and TT/BT (n = 4) patients were fixed in formalin and embedded in paraffin. Tissue sections with 4 μm thickness were prepared using a microtome (LEICA RM2165). The prepared tissue sections were stained for hematoxylin and eosin (H & E; images are shown in supplementary figure S3) and also used as previously described [31] for immunofluorescence staining. Tissue sections were deparaffinised and rehydrated using graded concentrations of ethanol to distilled water. Antigen retrieval was performed in boiling Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0) for 12 minutes. After two hours of cooling at room temperature in antigen retrieval buffer, slides were washed twice in distilled water and twice in PBS, blocked for 15 min with 5% goat serum in PBS, washed again with PBS and stained with primary antibodies for FoxP3 (1:100, mouse anti-human IgG1 Abcam; Cambridge, UK), CD10 (1:100 mouse anti-human IgG2b, Abcam), CD68 (mouse anti-human IgG2a AbD serotec/Bio-Rad; Venneendaal, The Netherlands), CD163 (1:400, mouse anti-human IgG1, Leica; Rijswijk, The Netherlands) and CD39 (1:100, mouse anti-human IgG2a, Abcam). Two antibodies were used per tissue section: FoxP3 with CD68, CD163, CD39 or CD8; CD68 with CD163 and CD39 with CD163. After overnight incubation at room temperature in the dark, sections were washed and incubated for 1 hour in the dark with secondary antibodies; goat-anti-mouse IgG1 coupled with Alexa 488 (1:200) (Invirogen,Bleiswijk The Netherlands), goat-anti-mouse IgG2a or goat-anti-mouse IgG2b with Alexa 546 (1:200) (Invirogen). Tissue sections were then washed three times with PBS and mounted with Vectashield (DAPI, 4',6-diamidino-2-phenylindole; Vector Laboratories, Brussels, Belgium). Immunofluorescence of skin sections was examined and images were taken from 5 different fields per section using a Leica-TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany). Nucleated cells that positively stained for the specific marker were counted from five different fields per section by two laboratory persons independently. Average counts for each marker per section were compared for all samples.

Statistical analysis

Differences in cytokine concentrations were analyzed with the two-tailed Mann-Whitney U test or Wilcoxon signed rank test for non-parametric distribution using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA; www.graphpad.com) P-values were corrected for multiple comparisons. The statistical significance level used was p<0.05.

Results

Depletion of CD25+ cells enhanced pro-inflammatory response in LL patients

To analyse the role of CD25+ cells in the production of IFN-γ, PBMC from Ethiopian LL patients (n = 17) and Dutch healthy
controls (n = 12) were depleted of CD25+ cells and cell subsets with and without re-added CD25+ cells were stimulated with M. leprae WCS in 6 days culture.

PBMC from treated Nepali LL (n = 6), BT (n = 9) and EC (n = 10) were depleted for CD25+ cells but only the total PBMC and CD25+ cell subset were stimulated with M. leprae WCS. When compared according to clinical classification, there was a trend of higher IFN-γ production in PB compared to MB samples. IFN-γ production of total PBMC (undepleted fraction) from LL patients in response to M. leprae (WCS) was significantly lower (p = 0.001) compared to responses by PBMC from TT/BT patients, whereas IFN-γ responses to PHA were high in both groups (Fig. 1). These data further confirm the M. leprae-specific lack of cell mediated immunity (CMI) in LL patients.

Analysis of IFN-γ production in response to M. leprae (WCS) by CD25− cells alone or CD25− cells (150,000 cells/well) supplemented with the CD25+ fraction (10,000 or 25,000 cells/well) discriminated two groups of LL patients: those that produced IFN-γ in response to M. leprae after CD25+ cell depletion and those that did not (Fig. 2A, 2B and 2E). Among the 18 LL Ethiopian patients, 7 (38%) responded to M. leprae WCS after depletion of CD25+ cells whereas they lacked any response in total PBMC. IFN-γ production in response to PHA in both groups was not affected by the depletion of or enrichment with CD25+ cells.

In the LL patient group, in which recovery of IFN-γ responses was observed to M. leprae WCS after depletion of CD25+ cells, this could be reversed proportionally by the addition of CD25+ cells (Fig. 2A). In the patient group in which CD25+ cell depletion did not reverse anergy to M. leprae, there was no effect observed by addition of CD25+ cells to the depleted fraction (Fig. 2B).

In similar analysis of treated leprosy patients (LL and BT) and endemic controls from a Nepali population, PBMC responded to M. leprae WCS in the presence of CD25+ cells and a slight increase in IFN-γ levels after CD25+ cell depletion was also observed (Fig. 2C). Similarly, healthy Dutch controls (n = 8) responding to M. leprae WCS before depletion of CD25+ cells showed a slight increase after depletion (Fig. 2D left panel) as well, while other NEC (n = 5) remained unresponsive after CD25+ cell depletion (Fig. 2D right panel).

FoxP3 expressing CD8+ CD25+ T-cell are more abundant in PBMC of LL

For cell subset analysis, PBMC from Ethiopian LL (n = 13), TT/BT (n = 5) and EC (n = 7) and Dutch healthy controls (NEC; n = 4) were stained for surface and intra-cellular markers. The frequency of FoxP3+ CD8+CD25+ cells was significantly higher in PBMC of LL patients compared to TT/BT patients (p = 0.02) (Fig. 3). Although not statistically significant (p = 0.05), we also observed a higher frequencies of FoxP3+ CD4+ CD25+ T-cell in the LL group compared to the TT/BT patients (Fig. 3). In contrast, analysis of the frequency of IL-10 producing CD4+ CD25+ or CD8+CD25+ T-cell showed no significant differences between patients and healthy controls. The frequency of IL-10 production in CD4+ CD25+ or CD8+CD25+ T-cell in general was very low in all groups.

Mgp2 (CD68+ CD163+) and FoxP3+ cells are more frequent in skin lesions of LL patients

Confocal analysis of two-colour immunofluorescence was used to localize specific cell markers in skin biopsies of Ethiopian LL (n = 10) and TT/BT (n = 4) leprosy patients. Higher number of CD68+ cells in LL lesions (p = 0.02) (Fig. 4A, 5A and B) indicated the presence of more infiltrating macrophages compared to TT/BT (Fig. 5C and D). In addition, CD68+ CD163+ cells (mgp2) and FoxP3+ cells were present to a larger extent in LL patients' lesions (p = 0.02) compared to TT/BT (Fig. 4B, 4C; 5C and 5D). With respect to the numbers of CD68+ CD163+ cells (mgp2) and FoxP3+ cells, differences were observed among the LL patients which could be explained by variations in the time elapsed since skin lesions were noticeable or by influence of other host factors. Although we found significantly higher frequency of CD8+FoxP3+ in PBMC, we could not clearly detect CD8+FoxP3+ in the skin lesions indicating CD4+FoxP3+ cells could play a regulatory role in these tissues. In addition, skin lesions were stained with CD39 combined with FoxP3 to localize CD39+FoxP3+ regulatory T-cells. However, in most skin tissues, CD39+ cells were not detected except for two LL skin tissues in which CD39 and FoxP3 positivity were observed simultaneously in macrophage-like shaped cells (Fig. 4E). Thus, these results indicate the induction of more FoxP3+ but not CD39+ Treg cells in LL patients’ skin lesions probably by the presence of type 2 macrophages.

Discussion

Decreased M. leprae-specific T-cell mediated immunity is the hallmark of lepromatous multibacillary leprosy and can be assessed by in vitro unresponsiveness to M. leprae (antigens) or clonal anergy [2,23,32]. In this study, we confirm the M. leprae-specific unresponsiveness by the absence of IFN-γ responses to M. leprae WCS.

Several studies have investigated the possible causes leading to hyporesponsiveness in LL patients such as formation of foamy macrophages in presence of IL-10 [27], cholesterol dependent

---

Figure 1. IFN-γ responses to PHA and M. leprae whole cell sonicate (WCS) by PBMC of TT/BT (n = 7), BB/BL (n = 9) and LL (n = 16) patients. Median values for each group are indicated by horizontal lines. doi:10.1371/journal.pntd.0002773.g001
Figure 2. IFN-γ responses of total PBMC, CD25⁺ cells and CD25⁺ cells supplemented with CD25⁺ cells from LL patients. (A) representatives for the group responding to *M. leprae* after depletion of CD25⁺ cells (*n* = 7); (B) representatives for the group not responding to *M. leprae* after depletion of CD25⁺ cells (*n* = 11); (C) LL005 and LL010 representatives for Nepali treated LL patients (*n* = 10), BT004 and BT006 representatives for Nepali treated BT patients (*n* = 7) and EC020 and EC023 representatives for Nepali EC (*n* = 10) before and after depletion of CD25⁺ cells; (D) NEC001 and NEC002 representatives for healthy Dutch controls (*n* = 10) after depletion of CD25⁺ cells with and without response to *M. leprae* WCS; (E) Dot plot graph showing IFN-γ responses of both groups of Ethiopian LL patients in dot-plot graph. Medium indicates AIM-V medium used in the assays as negative control. In 2A and 2B: for LL001, CD25-25000 and for LL052 and LL053, CD25-10000 were not done.

doi:10.1371/journal.pntd.0002773.g002

Figure 3. T-cell subset analysis of PBMC from LL, TT/BT and the control group consisting of EC and NEC showing the frequencies of FoxP3 expressing T-cells and IL-10 producing FoxP3⁺ T-cells.

doi:10.1371/journal.pntd.0002773.g003
However, we did not stain the CD25+ cells from total PBMC of LL patients showed higher expression of IFN-γ in response to M. leprae antigens stimulated PBMC [23,35]. Recently, Saini et al. showed elevated frequencies of circulating Treg cells (CD4+CD25highFoxP3+) in TT patients [35] whereas Palermo et al. showed that PBMC stimulated with M. leprae antigen for 6 days in culture had significantly higher number of Treg cells (CD4+CD25“FoxP3”) in LL patients [23]. Recently, Saini et al., further confirmed the importance of Treg in LL non-responsiveness by measuring TGF-β producing CD4+CD25“FoxP3” cells in stimulated PBMC culture [45]. In this study, we analysed the frequency of Treg cells in PBMC briefly activated with PMA/ionomycin. The frequency of CD4+CD25“FoxP3” cells was higher in LL compared to BT but not statistically significant (Fig. 3). However, with the visible difference observed between LL and BT and with the evidences from previous studies, their presence and role in BL/LL patients cannot be denied. For example, the recent molecular analysis of FoxP3 in CD4+CD25“ T cells nuclei has revealed that the FoxP3 interaction with histone deacetylases drives the immune suppression by CD4+CD25“ Treg cells in BL/LL, unlike in other forms of leprosy [24].

On the other hand, the frequency of CD8+CD25“FoxP3” cells found in this study was significantly higher in LL (Fig. 3). This suggests that FoxP3+CD8+CD25“Treg cells may also play a role in unresponsiveness in LL although not specifically analyzed for their functional role in our depletion experiments. Although lower in frequency compared to the CD4+CD25“FoxP3”, Saini et al., also reported higher numbers of CD8+CD25“FoxP3” in LL compared to BT but without induction of TGF-β [45]. Most studies focused on CD4+CD25“FoxP3” in leprosy [23,35]. In contrast one study on LL lesions showed the presence of increased numbers of CD8+ T cells with suppressive type in LL indicating the importance of CD8+ Treg cells in leprosy [46]. In addition few other studies identified CD8+ Treg as a potential suppressive sub-population [47,48]. Recent evidence from an in vitro study also revealed CD8+ Treg cells (CD8+LAG-3+FoxP3+CTLA-4+) induced by mature plasmacytoid dendritic cells (pDC) with suppression activity on allo-reactive T memory cells [49]. In our opinion, the CD8+ Treg population is not sufficiently studied in leprosy and we believe further analysis of this population in all forms of leprosy in periphery and lesionsy tissues will be vital.
Figure 5. Immunohistochemical analysis (Original magnification, 400×; image size 359 μm × 359 μm) of skin lesions. Sequential skin sections from LL (n = 10) and BT (n = 4) patients were stained with mAb specific for CD68 (red) and FoxP3 (green) [A, B, E, F], for CD68 (red) and CD163 (green) [C, D], and CD39 (red) [G]. Representatives LL [A, B, C, D, and G] and BT [E, F] patients are shown. Insets represent 1500× magnification of FoxP3+ cells [A, B]; 800× magnification of CD68+CD163+ [C, D]; 1000× magnification of CD39+ cells [G].

doi:10.1371/journal.pntd.0002773.g005
The low IL-10 frequency measured by FACS analysis in all groups did not allow detection of significant differences among groups as expected in view of the crucial role of IL-10 as an anti-inflammatory cytokine in the unresponsiveness in LL patients [27,36]. This could be due to the short PMA/ionomycin stimulation inherent to the procedure for ex vivo determination of the frequency of CD25+ cells. However, 6 days stimulation of PBMC from BL patients with M. leprae induced high levels of IL-10 [30].

Although, it will not be easy to generalize or conclude on frequencies and numbers of CD4+ CD25+FoxP3+ Treg cells in different forms of leprosy since the experimental procedures used in each study vary, most of the studies including ours, point to the presence of increased numbers of Treg cells in LL patients either in periphery as well as lesions. Detailed characterization of Treg cell subsets in large cohorts of leprosy patients as well as the ratio to effector T cells may provide additional insights in this area.

The dominant presence of CD163+ macrophages in LL lesions [27,28] and the significantly higher expression of IL-10 and CTLA4 in LL tissues have been reported previously [25]. The role of Treg cells (FoxP3+ GITR+ CD25+) and their induction by CD163+ anti-inflammatory human macrophages was demonstrated in vitro since CD4+ T-cells gained a potent regulatory/suppressor phenotype and functions after activation by mq2 [25]. In the current study, we show the presence of significantly higher number of CD68+ CD163+ cells (mq2) in the vicinity of FoxP3+ cells in LL lesions compared to TT/NT lesions. These findings support the involvement of both cell types in the induction and/or maintenance of M. leprae directed Treg cells in LL lesions.

Since a suppressive effect of CD4+CD25+FoxP3+ Treg cells was described in TB patients [12], we also analysed the frequency of CD39+FoxP3+ cells in PBMC but observed no differences between LL and TT/NT patients except for few LL skin lesions, in which macrophage-shaped CD39+ cells were observed. A recent study has shown that CD39 expression on macrophages has an important role in self-regulation mechanism during inflammation [51]. These cells may also play a similar role in LL patients but this has to be further analysed.

In summary, this study clearly show that CD25+ Treg cells play a role in unresponsiveness in LL, and that there are two subtypes of M. leprae unresponsive LL patients. Furthermore, the co-existence of Treg cells with mq2 in LL lesions further supports the potential role of these regulatory cell subsets at the site of infection.

Supporting Information

Figure S1 A. Gating strategy for live CD4+CD3+ or CD8+CD3+ cells in PBMC. Ungated events were first gated using a forward scatter area (FSC-A) versus height (FSC-H) plot to remove doublets. Subsequently, the events were subjected to a lymphocyte gate by gated through a side scatter (SSC).

Figure S2 A. Dot plot analysis of bulk (total) PBMC, CD25 depleted and CD25 positive population of a representative LL patient (LL055). After separating the CD25 negative and CD25 positive cell population using Magnetic cell sorter, fractions of each cell population including the bulk (total) PBMC were analysed for their expression of CD3, CD4 and CD25. Here the data are presented in dot plots. B. Zebra plots of bulk (total) PBMC, CD25 depleted and CD25 positive population of a representative LL patient (LL053). After separating the CD25 negative and CD25 positive cell population using Magnetic cell sorter, fractions of each cell population including the bulk (total) PBMC were analysed for their expression of CD3, CD4 and CD25. Here the data are presented in zebra plots.

Figure S3 Hematoxylin and Eosin staining of four representative LL patients (original magnification ×100). Tissue sections from paraffin embedded biopsy samples of leprosy patients were stained for H&E. Here images of H&E staining of four representative LL patients are presented.

Acknowledgments

The authors gratefully acknowledge Dr. Nigel Savage (Dept. of Infectious Diseases, LUMC) for expert-advice on tissue staining. We thank Yonas Fantahun, S/Gen Amare, Seflu Girma, Dr. Mihret WoldeTinsae and Dr. Saba M. Lambert from AHRI/ALER, Ethiopia and Murdo Macdonald, Bishwa Sapkota and Chaman Ranjit from MLR, Anandaban Hospital, Nepal for the support in the recruitment and sample collection process. AHRI, LUMC and MLR are members of the IDEAL (Initiative for Diagnostic and Epidemiological Assays for Leprosy) Consortium.

Author Contributions

Conceived and designed the experiments: AG KB KEvM. Performed the experiments: KB KEvM LW JM JL SK. Analyzed the data: AG KB LW KEvM MJP SS ESJ. Contributed reagents/materials/analysis tools: AA AG THMO KDN DAH. Wrote the paper: AG KB THMO. Revised the manuscript: AG THMO AA ESJ DAH.

References

1. Sakaguchi S, Wing K, Miyara M (2007) Regulatory T cells - a brief history and perspective. Eur J Immunol 37 Suppl 1: S116–S125. 10.1002/eji.200737599 [doi].
2. Ottenhoff TH, Elferink DG, Klatser PR, de Vries RR (1986) Cloned suppressor T cells from a lepromatous leprosy patient suppress Mycobacterium leprae reactive helper T cells. Nature 322: 462–464. 10.1038/322462a0 [doi].
3. Moltin RL, Mehta V, Wong L, Fujiyima Y, Chang WC, et al. (1986) Suppressor T lymphocytes from lepromatous leprosy skin lesions. J Immunol 137: 2831–2834.
4. Mutis T, Cornelisse YE, Datema G, van den Elen PJ, Ottenhoff TH, et al. (1994) Definition of a human suppressor T-cell epitope. Proc Natl Acad Sci USA 91: 9456–9460.
5. Abbas AK, Brentjens C, Bhestone JA, Campbell DJ, Ghosh S, et al. (2013) Regulatory T cells: recommendations to simplify the nomenclature. Nat Immunol 14: 307–308. n.2554 [pii].10.1038/ni.2554 [doi].
6. Milli KH (2004) Regulatory T cells: friend or foe in immunity to infection? Nat Rev Immunol 4: 841–853. nri403 [pii].10.1038/nri403 [doi].
7. Belkaid Y (2007) Regulatory T cells and infection: a dangerous necessity. Nat Rev Immunol 7: 875–888. nri2189 [pii].10.1038/nri2189 [doi].
8. Nouri-Aria KT, Durham SR (2008) Regulatory T cells and allergic disease. Inflamm Allergy Drug Targets 7: 237–252.
9. Sanchez AM, Yang Y (2011) The role of natural regulatory T cells in infection. Immunol Inflamm Allergy Drug Targets 7: 875–888. nri2189 [pii].10.1038/nri2189 [doi].
10. Donn EM, Raimondi G, Cooper DK, Thomson AW (2012) Induced regulatory T cells: mechanisms of conversion and suppressive potential. Hum Immunol 73: 329–334. S0198-8859(11)00602-1 [pii];10.1016/j.humimm.2011.12.011 [doi].
11. Belkaid Y, Tarbell K (2009) Regulatory T cells in the control of host-microorganism interactions. Annu Rev Immunol 27: 551–589. 10.1146/annurev.immunol.021908.132723 [doi].
Tang Y, Jiang L, Zheng Y, Ni R, Wu Y (2012) Expression of CD39 on FoxP3+ T regulatory cells correlates with progression of HBV infection. Mol Immunol 13: 2147–2153. 10.1016/j.molimm.2012.03.015 [doi].

Boer MC, van Meijgaarden KE, Bastia J, Ottenhoff TH, Joosten SA (2013) CD39 is involved in mediating suppression by Mycobacterium bovis BCG-activated human CD4+ regulatory T cells. Eur J Immunol 43: 1927–1937. 10.1002/eji.201243286 [doi].

2013 Global Leptospirosis update: on the 2012 situation. Wiley Epidemiol Rev 88: 365–379.

Ridley DS, Jopling WH (1966) Classification of leprosy according to immunity. J Hyg 66: 3–11. 10.1017/S0022171766002651 [pii].

Ribeiro-Rodrigues R, Resende CT, Rojas R, Toossi Z, Dietze R, et al. (2006) A role for CD4+CD25+ regulatory T cells in the early control of Mycobacterium leprae infection. J Infect Dis 193: 1220–1229. 10.1086/499635 [pii].

2012 Expression of CD4+CD25+ regulatory T cells in the granulomatous lesions. Am J Pathol 174: 234–242. 10.1111/j.1524-1558.2008.00915.x [pii].

Gravano DM, Vignali DA (2012) The battle against immunopathology: CD8+ T cell assays and MIATA: the essential minimum for maximum impact. J Immunol 185: 134–143. jimmunol.0803474 [pii]; 10.4049/jimmunol.0803474 [doi].

Kumar S, Naqvi RA, Ali R, Rao DN (2011) Disruption of HLA-DR raft, GITR+CD25POS and FOXP3NEG regulatory T cells in the skin lesions of patients with leprosy. J Am Acad Dermatol 8: 182–189. jaad.090901 [pii]; 10.1016/j.jaad.2010.05.015 [doi].

Kumar S, Naqvi RA, Ali R, Rao DN, Kharana N, et al. (2013) CD4+CD25+ T cells with elevated levels of FoxP3+ regulatory T cells in the granulomatous lesions. Am J Pathol 174: 234–242. 10.1111/j.1524-1558.2008.00915.x [pii].

Joosten SA, Ottenhoff TH (2008) Human CD4+ and CD8+ regulatory T cells in the skin lesions of patients with leprosy. J Clin Immunol 28: 338–346. 10.1007/s10875-008-9193-4 [pii].

Savage ND, de BT, Wulfung KB, Joosten SA, van MK, et al. (2008) Human anti-inflammatory macrophages induce Foxp3+ GFRalpha+ CD25+ regulatory T cells that suppress via membrane-bound TGF-beta. J Immunol 181: 2221–2226. 181/3/2220 [pii].

Verreck FA, de BT, Langenberg DM, van der Zanden L, Ottenhoff TH (2006) Human plasmacytoid dendritic cells induce CD8+ regulatory T cells that suppress allo-reactive memory T cells. Eur J Immunol 42: 1094–1106. eji.1830190421 [pii].

Attia EA, Abdallah M, Saad AA, Afifi A, El TA, et al. (2010) Circulating CD4+ regulatory T cells correlates with progression of HBV infection. BMC Immunol 13: 37. ccr-07-4554 [doi].

Morrya DF, de Mattos KA, Amadeu TP, Andrade PR, Sales JS, et al. (2012) CD163 favors Mycobacterium leprae survival and persistence by promoting anti-inflammatory pathways in leprosy macrophages. Eur J Immunol 42: 2935–2946. 10.1002/eji.201241198 [pii].

Geluk A, van Meijgaarden KE, Wilson L, Bobosha K, van der Ploeg-van Schip HD, et al. (2008) Rational vaccine design for leprosy through the chemokine CC chemokine ligand 4. Proc Natl Acad Sci U S A 105: 20829–20834. 10.1073/pnas.0802571105 [pii].

Lochmiller VL, Hofman FM, Taylor CR, Rea TH (1983) Lymphocyte subsets in the skin lesions of patients with leprosy. J Am Acad Dermatol 8: 182–189.

Joosten SA, van Meijgaarden KE, Savage ND, de BT, Triebel F, et al. (2007) Identification of a human CD8+ regulatory T cell subset that mediates suppression through the chemokine CC chemokine ligand 4. Proc Natl Acad Sci U S A 104: 8029–8034. 0702257104 [pii].

Joosten SA, Ottenhoff TH (2008) Human CD4+ and CD8+ regulatory T cells in infectious diseases and vaccinology. PLoS Pathog 4: e1000192. 10.1371/journal.ppat.1000192 [pii]; P1000192 [pii].

Corthay A (2009) How do regulatory T cells work? Scand J Immunol 70: 326–334. sj1093276 [pii]; 10.1111/j.1365-3083.2009.02496.x [doi].

Geluk A, van der PJ, Teles RO, Franken KL, Prins C, et al. (2008) Rational vaccine design for leprosy through the chemokine CC chemokine ligand 4. Proc Natl Acad Sci U S A 105: 20829–20834. 10.1073/pnas.0802571105 [pii].

Jordanova ES, Gorter A, Ayachi O, Prins F, Durrant LG, et al. (2008) Human regulatory memory phenotype. Am J Transplant 10: 2410–2420. 10.1111/j.1600-6143.2010.03291.x [doi].

2011 T regulatory cells and plasmacytoid dendritic cells in hansen disease: a new insight into pathogenesis? Am J Dermatopathol 33: 365–379.

Boer MC, van Meijgaarden KE, Bastia J, Ottenhoff TH, Joosten SA (2013) CD39 is involved in mediating suppression by Mycobacterium bovis BCG-activated human CD4+ regulatory T cells. Eur J Immunol 43: 1927–1937. 10.1002/eji.201243286 [doi].

Rahman S, Gudetta B, Fink J, Granath A, Ashenafi S, et al. (2009) MMP-2 and IL-10 expression in lepromatous leprosy. Am J Trop Med Hyg 86: 878–883. 86/5/878.