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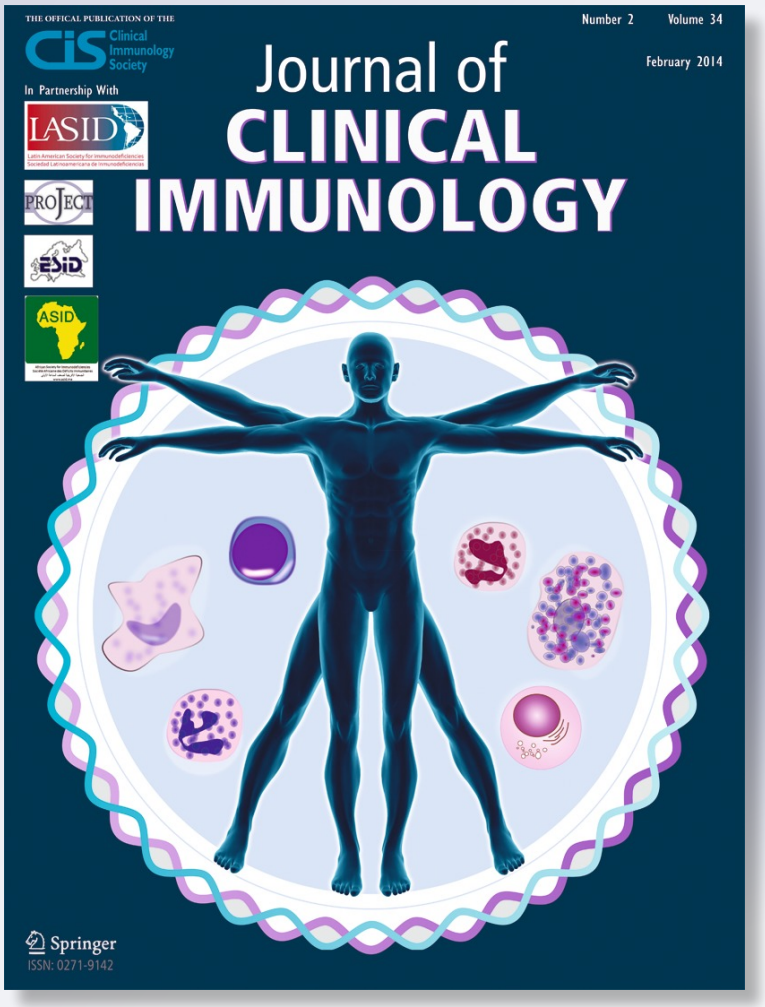

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ORIGINAL RESEARCH

Longitudinal Immune Responses and Gene Expression Profiles in Type 1 Leprosy Reactions

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Abstract
Purpose Leprosy, a chronic disease initiated by *Mycobacterium leprae*, is often complicated by acute inflammatory reactions. Although such episodes occur in at least 50 % of all leprosy patients and may cause irreversible nerve damage, no laboratory tests are available for early diagnosis or prediction of reactions. Since immune- and genetic host factors are critical in leprosy reactions, we hypothesize that identification of host-derived biomarkers correlated to leprosy reactions can provide the basis for new tests to facilitate timely diagnosis and treatment thereby helping to prevent tissue damage.
Methods The longitudinal host response of a leprosy patient, who was affected by a type 1 reaction (T1R) after MDT-treatment, was studied in unprecedented detail, measuring cellular and humoral immunity and gene expression profiles to identify biomarkers specific for T1R.
Results Cytokine analysis in response to *M. leprae* revealed increased production of IFN-γ, IP-10, CXCL9, IL-17A and VEGF at diagnosis of T1R compared to before T1R, whereas a simultaneous decrease in IL-10 and G-CSF was observed at T1R. Cytokines shifts coincided with a reduction in known regulatory $CD39^{+}CCL4^{+}$ and $CD25^{high}$ T-cell subsets. Moreover, RNA expression profiles revealed that IFN-induced genes, (V)EGF, and genes associated with cytotoxic T-cell responses (GNLY, GZMA/B, PRF1) were upregulated during T1R, whereas expression of T-cell regulation-associated genes were decreased.
Conclusions These data show that increased inflammation, vasculoneogenesis and cytotoxicity, perturbed T-cell regulation as well as IFN-induced genes play an important role in T1R and provide potential T1R-specific host biomarkers.

Keywords Biomarker · early diagnosis · gene profiles · leprosy reactions · T1R

Introduction

Leprosy is one of the six diseases considered by the WHO as a major threat in developing countries [1]. It is a complex dermato-neurological disease that presents with different clinico-pathological forms often resulting in severe, life-long disabilities and deformities [2]. Nearly 250,000 new cases of leprosy are diagnosed each year and many more go undetected/untreated [1]. Moreover, with increasing migration, new cases of leprosy are more regularly diagnosed in developed countries as well [3].

Fifty percent of the patients develop leprosy reactions, representing the major cause of neurological disability and consequent anatomical deformities in leprosy [4, 5]. Reactions can present during treated or untreated leprosy and after completion of MDT. Two types of nerve-damaging reactions are recognized: type 1 reactions (T1R) and type 2 reactions (T2R), erythema nodosum leprosum (ENL). T1R affect about

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30 % of the leprosy patients [6], and occured more frequently than ENL in Asian and African populations [7].

Substantial evidence describes increased numbers of $CD4^+$ T-cells in skin lesions, high levels of IL-2 receptors, TNF-α, IL-6, IP-10 and IL17F in sera and increased gene expression of pro-inflammatory cytokines during reactions [8–11]. Also, TNF-α, iNOS and TGF-β detected by immunohistochemistry in skin lesions is associated with T1R [5].

Since leprosy reactions have such detrimental effects on leprosy-associated morbidity, timely treatment is fundamental to reduction of patients' sequellae. Nevertheless, there are no biomarkers specific for or predicting reactions that can be applied in diagnostic tests. Identification of such compounds requires longitudinal analysis [12]. Most studies, however, measured one time point in cross sectional designs and the few longitudinal, serological analyses showed limited significance in detecting reactional patients [13, 14], although IP-10 in sera, even if not predictive, may be useful to help diagnose T1R [10].

In this study, we had the unique opportunity to longitudinally analyse blood as well as skin samples of a leprosy patient during development and treatment of T1R, assessing not only cellular and humoral immunity parameters but also genetic and histological markers in order to identify biomarkers associated with T1R.

Materials and Methods

Patient Description

PatientD05 (male from Angola) was treated at the LUMC and Erasmus Medical Center. On 04-29-2005, patientD05 (17 years) was diagnosed with multibacillary leprosy (BL) downgrading with nerve-T1R, based on bilateral ulnar and median nerve palsy, glove and stocking complete anaesthesia of four extremities and vaguely defined coppery macules on face, shoulders and back. Diagnosis was confirmed by histopathology and bacillary index (BI) was +5. MDT for multibacillary leprosy (rifampicin, clofazimine and dapsone) was given from June 2005 through June 2006 with prednisolone (40 mg od; reduced with 5 mg every 4 weeks to 20 mg and then maintained for 4 months). One month after finalizing MDT, multiple ENL episodes occurred which were first treated with a short course prednisolone (60 mg, tapered down in the following weeks) and in subsequent months with up to 120 mg prednisolone. In November 2006 MDT was reinstated, replacing clofazimine with minocycline. Low dose steroids were provided for pain relief. In June 2007 high dose prednisolone pulse therapy was reinstated for ENL (skin, joints and nerves in addition to drop foot). Clofazimine was reinstated together with dapsone since steroids alone could not control the ENL. In 2009 and most of 2010 the patient was not treated for active leprosy reactions but preventive with clofazimine and methotrexate (7.5 mg once weekly). In the end of December 2010 new hypopigmented (indeterminate leprosy-like) patches developed diagnosed as skin-T1R and treated with dapsone, rifampicin and ofloxacin and after 01-18-2011 with prednisolone pulse for 2 weeks and topical clobetasol propionate. Histopathology did not show any acid-fast bacteria. At the time point without signs of reactions (06-01-2009) the patient was treated with low dose clofazimine; during T1R (01-18-2011) the patient was 1 month on MDT; On 03-27-2012 the patient had not been treated with steroids for 14 months and had no clinical signs of reactions. Ethical approval of the study (MEC-2012-589) and informed consent (before venepuncture) were obtained.

Antigens

M. leprae recombinant proteins were produced as described [15]. *M. leprae* whole cell sonicate (WCS) 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).

Analysis of Cytokine and Chemokine Production

PBMC were cultured in 6 days lymphocyte stimulation tests (LST) with *M. leprae* WCS or -proteins, PPD (all 10 μg/ml) or PHA (1 μg/ml) as described elsewhere [15]. The concentrations of IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFN-γ, IP-10(CXCL10), G-CSF, GM-CSF, CCL2(MCP-1), MIG(CXCL9), CCL4(MIP-1β), VEGF and TNF-α in supernatants of LST or in sera were measured as described [15] using the Bio-Plex-suspension-array-system (Bio-Rad, Veenendaal, NL).

PAXgene Whole Blood RNA Isolation

Total RNA from venepuncture PAXgene blood collection tubes (stored at− 80 °C) 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)

dcRT-MLPA was performed as described [16]. 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. 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.

Polychromatic Flow Cytometry

PBMC were stimulated in vitro with *M. leprae* WCS (10 μg/ml) for 24 h and treated as described [17] using TexasRed-conjugated anti-CD3-PE (Invitrogen), dot605-conjugated anti-CD8 (Invitrogen), Pacific Blue-conjugated anti-CD14 (eBioscience), Pacific Blue-conjugated anti-CD19 (eBioscience), Pe-Cy7-conjugated anti-CD4 (BD Biosciences, Eerbodegem, Belgium), APC-H7-conjugated anti-CD25 (BD Biosciences), LAG3-Atto647 (Enzo Life Sciences, Antwerpen, Belgium) and CD39-PE (Biolegend, ITK Diagnostics, Uithoorn, The Netherlands). For intracellulair staining the intrastain kit (Dako Diagnostics, Glostrup, Denmark) was used with Alexa700-conjugated anti- IFN-γ (BD Biosciences), PE-Cy5-conjugated antiFoxP3 (eBioscience, Hatfield, UK), FITC-conjugated anti-CCL4 (R&D Systems, Abingdon, UK), Alexa700-conjugated anti-granzymeB, PE-conjugated anti-perforin (all BD Biosciences). Anti-granulysin mAb was kindly provided by Dr. A.M. Krensky (Stanford University, CA, USA) and combined with FITC-labeled goat anti-rabbit (BD Biosciences, The Netherlands). Cells were acquired on a FACS LSR Fortessa with Diva software (BD-Biosciences) and analyzed with FlowJo version 9.4.1 (Tree Star, Ashland, OR).

Immunohistochemistry

Skin biopsies were taken on 04-29-2005 (at diagnosis leprosy, in the absence of skin T1R) and 01-18-2011 (T1R) and used in 4 μm paraffin sections as described [18] using antibodies specific for FoxP3 (1:100; mouse antihuman IgG1; Abcam, Cambridge, UK), CD163 (1:400; mouse antihuman IgG1; Leica, Rijswijk, The Netherlands) and CD68 (1:100; mouse-anti-human IgG2a; Abd serotec/Biorad, Veenendaal, The Netherlands). Subsequently sections were incubated with 1:200 dilutions of isotype-specific fluorochrome Alexa488 (green)-labeled goat-anti-mouse IgG1, Alexa546 (red)-labeled goat-anti-mouse IgG1 or -IgG2a (all Invitrogen). As a control for background staining, tissues were incubated with 1:500 dilutions of normal mouse serum (Dako, Heverlee, Belgium) followed by secondary Ig antibodies. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories, Brussels, Belgium). Immunofluorescence of skin sections was examined using a Leica-TCS-SP5 confocal-laser-scanning microscope (Leica Microsystems, Mannheim, Germany).

Statistical Analysis

Differences in cytokine concentrations, gene expression and MFI values were analysed with two-tailed Mann–Whitney-*U* test, one-way ANOVA or paired *t*-test respectively for non-parametric distribution using GraphPadPrism version 5.01 (GraphPad Software, San Diego,CA). The statistical significance level used was $p<0.05$.

Results

M. leprae-Specific Cytokine Production

Blood samples of patientD05 were obtained longitudinally and PBMC isolated in the absence of clinical signs of T1R (06-01-2009), at diagnosis of T1R (01-18-2011) and 14 months after termination of steroid-treatment (03-27-2012), were cultured with *M. leprae*-unique proteins, *M. leprae*-WCS, PPD or PHA. After 6-days, culture supernatants were analyzed for the presence of 16 cytokines (Fig. 1). *M. leprae*-unique proteins and WCS induced increased levels of IFN-γ, IP-10, IL17A, CXCL9 and VEGF at diagnosis of T1R compared to before T1R. PHA- and PPD-induced cytokines remained stable for all time points, indicating that these cytokine profiles were *M. leprae*-specific, consistent with the patient's BL/LL phenotype. Fourteen months after the end of steroid treatment, cytokines induced by *M. leprae*-proteins decreased again (IP-10, IL-17A, CXCL9, VEGF) or remained stable (IFN-γ).

In contrast, IL-10 levels in response to *M. leprae* WCS or -proteins were high before T1R but practically absent during T1R, indicating that regulation of T(h1) cell responses by IL-10 may be perturbed during T1R. After T1R treatment, however, IL-10 increased again to levels measured before T1R. PHA-induced IL-10 levels, however, remained stable at T1R. Patterns similar to those observed for IL-10 were detected for G-CSF with a significant decrease during T1R which was restored to similar levels. A different pattern was observed for TNF-α, remaining similar at diagnosis of T1R, but reached significantly higher levels after treatment for T1R (Supplementary Figure 1).

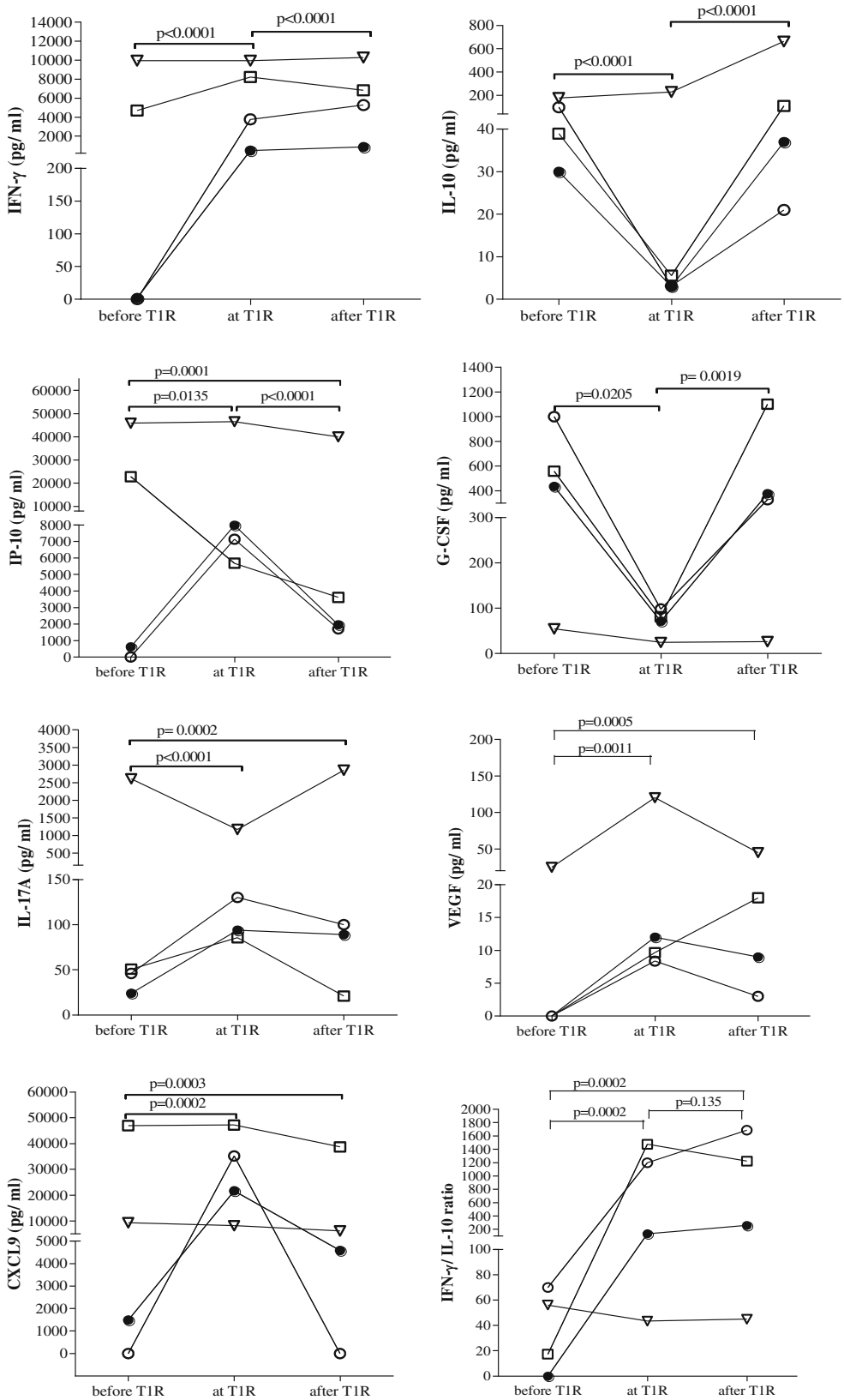

Fig. 1 Cytokine production (corrected for background values) by PBMC of MB patient D05 before (06-01-2009), during (01-18-2011) and after T1R (03-27-2012) in response to 6-day stimulation with *M. leprae* WCS (*white circle*), PHA (*inverted triangle*), PPD (*white square*) and 13 *M. leprae*-unique recombinant proteins, the average of which is indicated (*black circle*). At the time point without signs of reactions (06-01-2009) the patient was treated with clofazimine; during T1R (01-18-2011) the patient was 1 month on MDT; On 03-27-2012 the patient had not been treated with steroids for 14 months and had no clinical signs of reactions

The IFN-γ/IL-10 ratio has been described to significantly correlate with tuberculosis (TB) severity [19]. IFN-γ/IL-10 ratios for PBMC of patient D05 stimulated with *M. leprae*-unique proteins or WCS were low before T1R and high at diagnosis as well as after treatment of T1R, corroborating the usefulness of this ratio as an indicator for pathogenic responses to mycobacteria.

Serological responses of patientD05 at the same time points were determined longitudinally (2005-2012) by determining the antibodies directed against *M. leprae* PGL-I. This analysis showed a gradual decrease without any abrupt peaks or dips in seropositivity (Supplementary Figure 2A) which renders the serological marker not suitable to indicate T1R. Also, similar to the culture supernatants (Fig. 1), the sera were analyzed for the presence of 16 cytokines and chemokines. Besides IP-10, CCL2, CCL4, TNF and VEGF, no cytokines were detectable. Consistent with a study by Scollard et al. [10], IP-10 in serum was increased during T1R (Supplementary Figure 2B), similar to our findings for stimulated PBMC (Fig. 1). However, although the longitudinal pattern of CCL2, CCL4, TNF and VEGF showed an increase during T1R compared to the previous timepoint, the pattern did not allow prediction of T1R.

Longitudinal mRNA Expression Profiles

The availability of human transcriptome-arrays offers cutting-edge tools to identify and assess gene expression profiles for leprosy. Thus, total RNA was isolated from whole blood of patientD05 in the absence of clinical signs of T1R (06-01-2009), at diagnosis of T1R (01-18-2011) and 14 months after termination of steroid-treatment (03-27-2012), and used to identify differential gene expression by dcRT-MLPA using 144 target genes (Table I).

Table I Target genes tested in dcRT-MLPA

AIRE	CSF2	IFI16	IL12B	NLRP4	TBX21
ASAP1	CSF3	IFI35	IL13	NLRP6	TGFB
BLR1	CTLA4	IFI44	IL15	NLRP7	TLR1
BMP6	CX3CL1	IFI44L	IL17A	NLRP10	TLR2
CAMTA1	CXCL10	IFI6	IL18	NLRP11	TLR3
CCL2	CXCL13	IFIH1	IL21	NLRP12	TLR4
CCL3	CXCL9	IFIT2	IL22RA1	NLRP13	TLR5
CCL4	DSE	IFIT3	IL23A	NOD2	TLR6
CCL11	EGF	IFIT5	IL32	OAS1	TLR7
CCL22	ERBB2	IFITM3	INDO	OAS2	TLR8
CCR6	FCGR1A	IFNG	IRF7	OAS3	TLR9
CCR7	FCGR1B	IGF1	KIF1B	PACRGv1	TLR10
CD14	FLCN1	IL1A	LAG3	PARK2v1,2	TNF
CD163	FOXP3	IL1B	LIPE	PHEX	TNFRSF18
CD19	GATA3	IL10	LRRK2	PLP1	TNIP1
CD209	GBP1	IL2	LTA4H	PRF1	TWIST1
CD274	GBP2	IL2RA	LYN	PTPRCv1	VDR
CD36	GBP5	IL4	MARCO	PTPRCv2	VEGFA
CD3E	GNLY	IL5	MBP	RORC	ZNF532
CD4	GZMA	IL6	MMP2	SLAMF7	
CD8A	GZMB	IL7	MSR1	SOCS1	
CD46	HCK	IL7R	NEDD4L	STAT1	
CD68	HDAC1	IL8	NLRP1	STAT2	
CFB	HDAC2	IL9	NLRP2	TAP1	
CFH	HPRT	IL12A	NLRP3	TAP2	

Gene expression profiling was performed on 144 target genes using three distinct dcRT-MLPA gene sets

Substantial increases in expression were observed for several genes (Fig. 2), in particular those associated with T-cell cytotoxicity such as GNLY (granulysin), PRF1 (perforin), GZMA/B (granzyme A and -B). Also, an increase in leukocytes during T1R was indicated by genes encoding T-cell (co-) receptors CD4, CD8, CD3 as well as CCR7 (C-C chemokine receptor type 7), CXCR7 (C-X-C chemokine receptor type 7), PTPRCV1 (protein tyrosine phosphatase receptor type C or CD45) and CD14. In line with the inflammatory nature of T1R, higher than 2 log2 upregulation was observed for genes encoding pro-inflammatory cytokines IP10, IL15, IL23 and TNF as well as FCGR1, VEGF and EGF. Notably, we observed that, analogous to active TB [20], IFN-inducible transcripts of OAS1/2, GBP1/5, IFI44, IFI44L, IFIT5, IFIH1 and STAT1/2 were higher expressed during T1R. This shows that the upregulation of these genes is not specific for leprosy T1R but for a state of infection accompanied by severe inflammatory responses.

Most genes remained expressed at higher levels after treatment, but VEGF, IL15, IL23, IP10 and TNF noticeably decreased again. Although expression of the Th2-cell-specific transcription factor GATA-3 increased during T1R, expression of IL4 remained low and IL13 expression decreased (Fig. 2b), excluding involvement of Th2 cytokines during T1R.

On the other hand, decreased expression was observed for IGF (insulin growth factor), KIF1B (kinesin-like protein B) and LRRK2 (leucine-rich repeat kinase 2) during T1R. Importantly, expression of genes encoding for FoxP3 (forkhead box P3), a regulatory T-cell transcription factor, as well as CD163, a marker for type 2 macrophages (mφ2) that can induce regulatory T-cells (Tregs) [21, 22], was decreased extensively during T1R (1.8 and 2.3 log2 respectively). These profiles indicate diminished immune-regulation during T1R and corroborates the lack of IL-10 production at that time point.

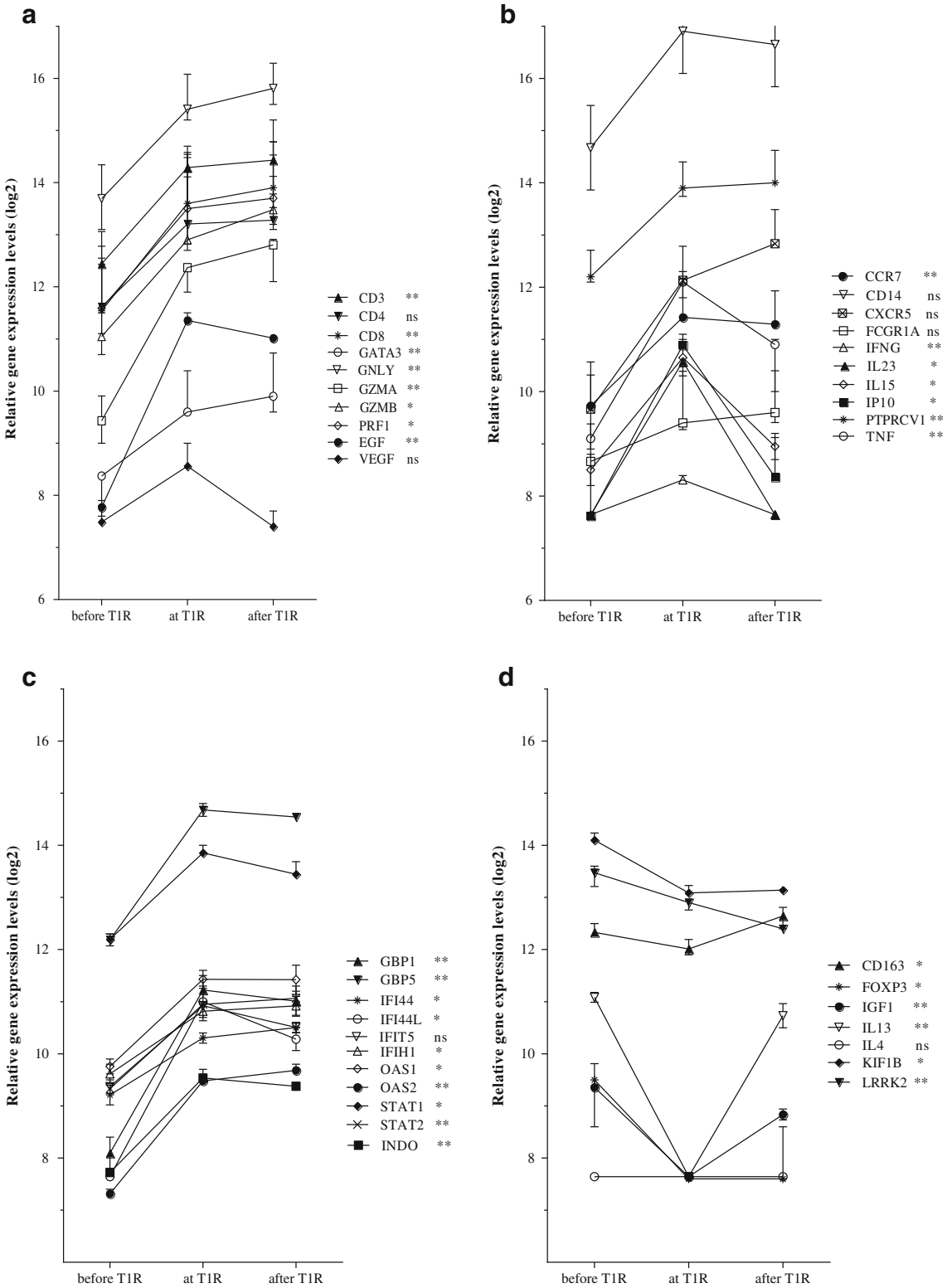

Fig. 2 Gene expression as assessed by dcRT-MLPA using RNA isolated from unstimulated whole blood of MB patient D05 before (06-01-2009), during (01-18-2011) and after T1R (03-27-2012) (see also Fig. 1). Data show upregulation (**a**, **b**, **c**) or downregulation (**d**) of target genes. Log2-transformations of peak areas (normalized for GAPDH expression) are shown on the *y*-axis as median values and standard errors of three independent experiments. Significant differences between gene expression before T1R and at T1R are indicated next to the gene name. *ns* not significant; *: $p<0.05$; **: $p<0.005$

T-cell Subset Analysis

To determine T-cell subsets involved in T1R, we performed intracellular staining after 24 h *M. leprae*-stimulation of PBMC in the absence of clinical signs of T1R (06-01-2009) and at diagnosis of T1R (01-18-2011) (Fig. 3a). IFN-γ was produced only during T1R, while the number of IL-10-producing T-cells was less than 5,000 (data not shown). Analysis of cell surface markers $CD39^+$ or $CD25^+$, indicative for regulatory T-cells, showed two distinct populations both producing low levels of IFN-γ, whereas CCL4 production was specific for $CD39^+$ $CD4^+$ T-cells. Of note is that during T1R the percentage of $CD39^+CCL4^+$ T-cells decreased from 13 to 10 % (Fig. 3a), simultaneously with a shift in the mean fluorescence intensity (MFI) in the $CCL4^+$ population from 611 to 450 (Fig. 3b), indicating that besides the amount of $CCL4^+$ T-cells, the quantity of CCL4 per cell, was also reduced significantly. Since CCL4 can inhibit T-cell activation by interfering with TCR-signaling [23], this indicates that reduction in CCL4-producing $CD39^+$ $CD4^+$ T-cells may affect T-cell regulation during T1R. Although $CD25^+$ T-cells produced no significant amounts of IFN-γ or CCL4, the observed lower MFI for CD25 during T1R indicated a decrease in natural Tregs ($CD25^{high}$ T-cells).

Given that gene expression profiles showed an increase in granulysin, perforin and granzymeB, the presence of these proteins was analyzed in PBMC (03-27-2012) stimulated with *M. leprae* WCS, revealing significant numbers of $perforin^+granulysin^+CD8^+$ T-cells (23.8 %) as well as a $perforin^+granzymeB^+CD8^+$ population (Fig. 3c), although these proteins were not detected in the $CD4^+$ T-cell population (data not shown). Similar analysis of BT leprosy patients after MDT treatment and healthy controls showed higher percentages of $perforin^+granulysin^+$ $CD8^+$ T-cells along with $perforin^+granzymeB^+CD8^+$ T-cells in patients than in healthy controls (Supplementary Figure 3).

Immunohistochemical Profiles of Lesional Skin Biopsies

Confocal analysis of two-colour immunofluorescence of skin biopsies of patientD05 without T1R in skin (04-29-2005) and during T1R in skin (01-18-2011) showed that FoxP3, a transcription factor specific for regulatory T-cells [24], was more abundantly present in T-cells before onset of T1R than during T1R (Fig. 4a vs. b). $FoxP3^+$ cells were distinct from the more prominently present $CD68^+$ population. An additionally important finding was that in the biopsy taken in the absence T1R in the skin, the number of cells expressing $CD163^+$ was higher than during T1R (Fig. 4c vs. d). Since CD163 is a marker for anti-inflammatory type 2 macrophages (mϕ2) that have the ability to induce Tregs, this is consistent with the lack of Tregs during T1R. Furthermore, IL-10 was more prominently found in the skin lesion before T1R than during T1R (Supplementary Figure 4), whereas human β-defensin 3 (hBD-3), an endogenous antimicrobial peptide secreted by keratinocytes that was shown to be upregulated in patients with T1R [25], demonstrateted specific staining of keratinocytes in the epidermis (Supplementary Figure 4). Although the number of hBD-3 producing cells in the skin before T1R was slightly less than during T1R, hBD-3 was present abundantly at both time points.

Discussion

Using longitudinal analysis of blood- and skin samples of a leprosy patient who developed T1R, a candidate blood-derived biomarker profile for T1R was identified composed of immune- and genetic host factors associated with T-cell cytotoxicity, -regulation, vasculoneogenesis and IFN-signalling: during T1R we identified upregulation of the expression of several genes associated with T-cell cytotoxity, inflammation and IFN-induced genes. In contrast, genes involved in T-cell regulation were downregulated. These T1R-specific gene expression patterns correlated well with the stimulated blood cytokine profiles, the presence of specific Treg subsets as well as the histochemistry of skin lesions.

Although it stands beyond any doubt that leprosy control requires skilled health professionals, dependable diagnostic tests for early detection of leprosy can be of great added value, particularly for leprosy reactions. Such tests require identification of new biomarkers that are specific for reactional states and are detectable before tissue damage occurs. In this respect, newly discovered innate immune mechanisms in leprosy [26, 27] along with results from 'unbiased' genome-wide [28] and functional genetic approaches [20] that are deciphering critical host pathways in human infectious diseases may help to identify biomarkers for leprosy reactions.

Our results showed that *M. leprae*-induced production by PBMC of IL-10 and G-CSF acutely decreased during T1R, whereas IFN-γ, IP-10, CXCL9, IL-17A and VEGF increased. Although IFN-γ and IL-10 have been studied in T1R, this is the first report on T1R-related shifts in secretion of VEGF, CXCL9, IL-17A and G-CSF. Since the samples analysed in this study were obtained during treatment an effect thereof on the immune parameters measured cannot be completely excluded. Therefore, we compared the immune responses at time points without or with clinical signs of T1R in order to identify profiles associated with T1R pathology. In this respect it is of note that no effect on PPD- or PHA-induced responses were observed at the time point before T1R when the patient received a low dose of clofazime.

Since overexpression of VEGF can cause vascular disease and as VEGF can discriminate between active and latent TB [29], the increased VEGF levels during T1R likely contribute to its pathology. Similarly, the increased IL-17A levels during

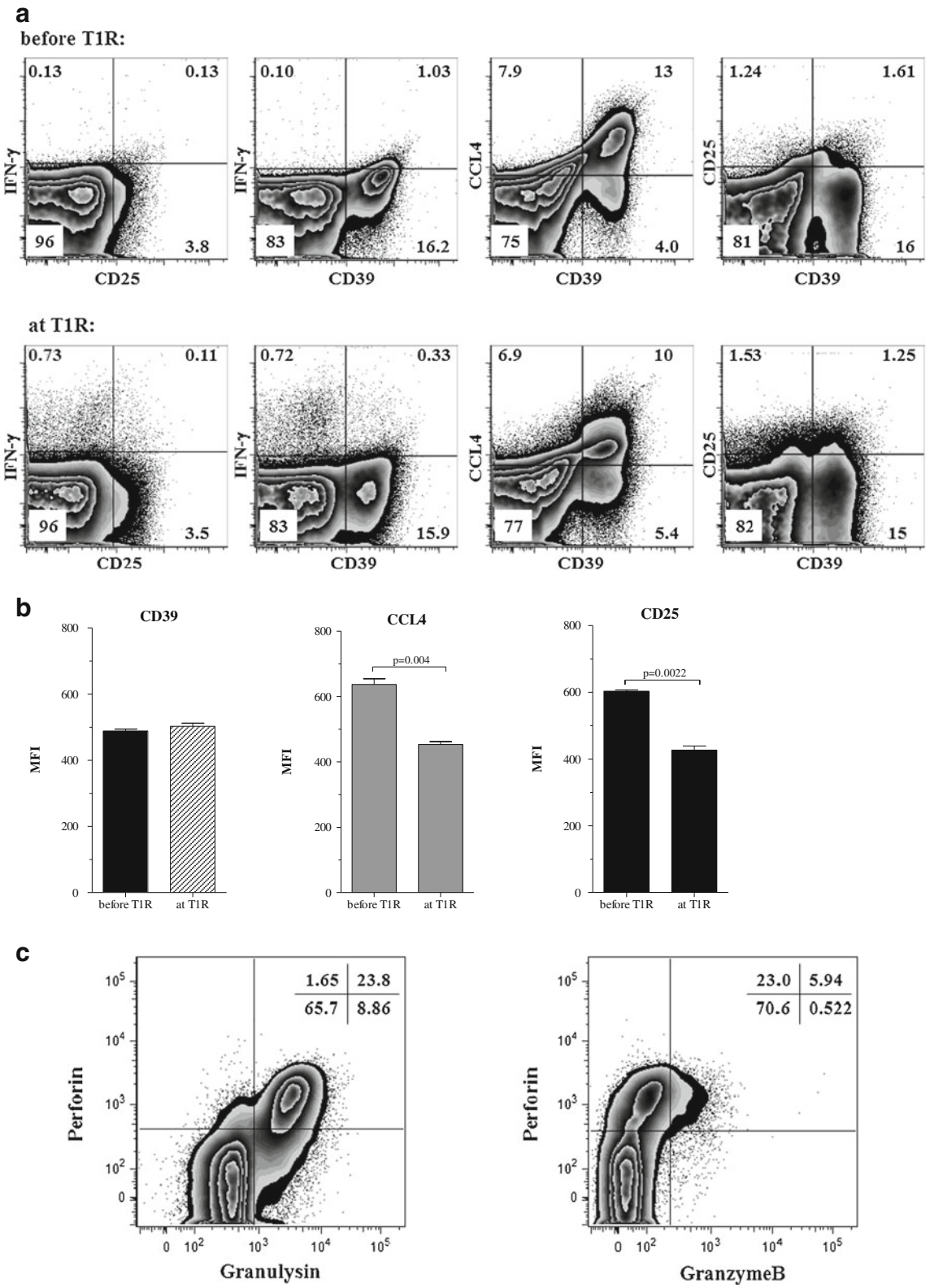

Fig. 3 **a** Bivariate zebra plots (hybrid of contour- and density plots) of PBMC of MB patient D05 before (06-01-2009) and during T1R (01-18-2011) showing $CD25^+$ $CD4^+$ or $CD39^+$ $CD4^+$ T-cells producing IFN-γ or CCL4. **b** Mean fluorescence intensities (MFI) of $CD39^+$ $CD4^+$, $CCL4^+CD4^+$ or $CD25^+$ $CD4^+$ cells are shown as the median and standard errors of three replicate measurements. **c** Presence of granulysin, perforin or granzymeB in $CD8^+$ T-cells 14 months after end of steroid treatment (03-27-2012)

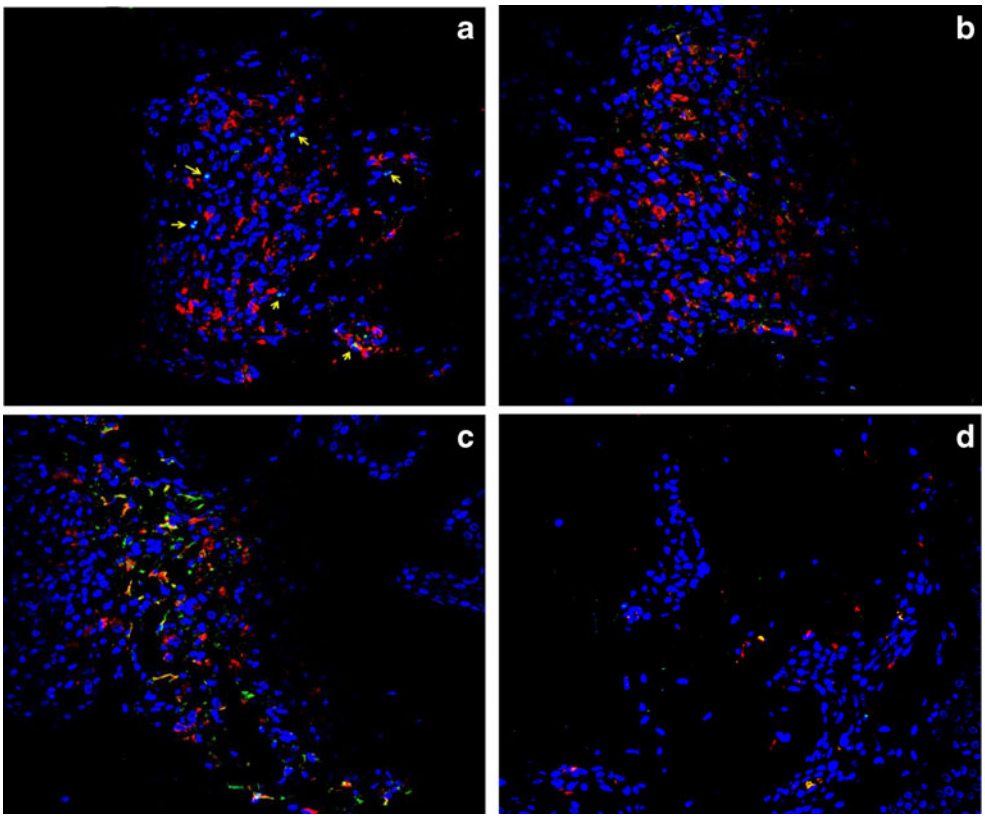

Fig. 4 Immunohistochemical analysis (magnification: ×400; image size 359×359 μm) of lesional skin biopsies of MB patientD05 in the absence of skin T1R (04-29-2005) (**a**, **c**) and at diagnosis of skin T1R (01-18-2011) **b**, **d** Sequential skin sections were stained with mAb for CD68 (*red*) and FoxP3 (*green*) **a**, **b**, *arrows* indicate FoxP3$^+$ cells; CD68 (*red*) and CD163 (*green*) **c**, **d**

T1R, coincided with upregulation of IL23 gene expression levels. Since mϕ1 can produce high quantities of IL-23 [21] that support Th17 expansion in leprosy lesions the damaging inflammatory response of IL-17 concur with leprosy reactional states.

Depletion of CD39$^+$ T-cells has been shown to lead to exacerbation of colitis in mouse models for inflammatory bowel disease [30] indicating the regulatory effect of this cell subtype. Also, BCG was recently shown to induce human CD4$^+$CD39$^+$ as well as CD8$^+$CD39$^+$ regulatory T-cells [31, 32]. Concomitant with T1R we observed a lack of CD163$^+$ and FoxP3$^+$ cells in blood-derived gene expression levels and skin lesions as well as a decrease in the CD39$^+$CCL4$^+$ T-cell subset [31, 32], collectively indicating reduced T-cell regulation during this reaction.

Thus, the combined *M. leprae*-specific decrease in IL-10 secretion by PBMC as well as the breakdown of T-cell regulation profiles in favour of inflammation and IFN-induced transcripts during T1R almost certainly underlies the aetiology of reactional tissue damage, whereas balanced immune responses are protective against T1R [33]. Since steroids such as progestron and sitostanol induce Tregs [34, 35], treatment of T1R with steroids may in fact represent a mechanism to restore the disturbed Th1, Th17/Treg balance during T1R.

The contribution of cytotoxic T-cells to the pathology of T1R, was identified by both host gene profiles as well as analysis of perforin$^+$granulysin$^+$ T-cells. For cellular host defense in leprosy one pathway includes exocytic granule mediated killing involving cytolytic granules containing perforin (cytolitic molecule), granzymes and the microbicidal molecule granulysin [36]. Thus, increased expression of GZMA, GZMB, GNLY and PRF1 indicated a potential biomarker profile for transition from LL to a reactional state. In line with these increased gene expression profiles, more perforin$^+$granulysin$^+$ CD8$^+$ T-cells were induced by *M. leprae* WCS in PBMC of BT leprosy patients than in healthy donors.

Finally, during T1R decreased expression was observed for IGF, KIF1B and LRRK2. The latter is consistent with a TT/BT-like state during T1R since significantly higher levels of LRRK2 were also found in BL/LL compared to TT/BT (Geluk et al., in preparation). Additionally, alterations in the IGF system have been proposed as possible biomarkers for ENL [37], whereas mutations of KIF1B are associated with Charcot-Marie-tooth neuropathy [38], which is characterized by progressive loss of muscle tissue and touch sensation.

Although n=1 studies like this can contribute in a noteworthy manner due to its intrinsic ability to identify clinical phenotype with measurable biomarkers [39], it is obvious that large scale longitudinal studies of leprosy patients are required to identify robust biomarker profiles that are indicators of developing leprosy reactions. This study, however, is the first longitudinal case report on the identification of immune– and genetic biomarkers for T1R and accentuates the potential of blood derived analytes as biomarkers for a skin/nerve disease

such as leprosy. Besides, this patient represents a "real-life" case that emphasizes the complexity of leprosy disease and prediction of leprosy reactions of (treated) leprosy patients.

In conclusion, our results provide potential T1R-specific host biomarkers candidates associated with inflammation, cytotoxicity, vasculoneogenesis, T-cell regulation as well as IFN-induced genes. Thus, these findings clearly encourage longitudinal cohort studies analyzing blood samples of leprosy patients in order to evaluate and extend biomarkers for leprosy reactions (T1R) in multiple regions where leprosy is endemic.

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Conflicts of Interest The authors declare that they have no conflict of interest.

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