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Research article

Unveiling the role of NK cells, NKT-like cells, and γδ cells in pathogenesis of type 1 reactions in leprosy

Vinay Kumar Pathak [a,c], Itu Singh [a,*], Bhawna Sharma [b], Ravindra P. Turankar [a], Mamta Arora [d], Shoor Vir Singh [c], Utpal Sengupta [a]

[a] Stanley Browne Laboratory, TLM Community Hospital, Nand Nagari, Delhi, India
[b] Department of Immunology, National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Tajganj, Agra, India
[c] Department of Biotechnology, GLA University, Mathura, UP, India
[d] Clinical Division, National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Tajganj, Agra, India

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ABSTRACT

Leprosy is a disease with spectral clinical manifestations along with two types of reactions, type 1 reaction (T1R) and type 2 reaction (T2R). T1R especially occurs because of the defensive upgradation of cell-mediated immunity (CMI) to *M. leprae* antigens. T1R is the main cause of disability in leprosy. The role of conventional adaptive T cells has been well studied to understand T1R. A comprehensive understanding of the role of unconventional T cells in the manifestation of inflammation during T1R is crucial and has not been studied. In our study, we found significantly higher plasma levels of TNFα, IL1β, IL17, and IP10 in T1R when compared to non-reaction (NR). Gene expression for cytokines in blood circulation by qPCR showed significantly higher expression of IFNγ, IP10, TNFα, IL6, IL17A and chemokines CCL3, CCR1, CCR5, and CXCR3 in T1R as compared to NR. Frequencies of NKT-like cells (48.7 %) and NK cells (22.3 %) were found significantly higher in T1R in comparison to NR (36.9 %, 18.3 %, respectively) ($p = 0.0001$). Significantly lower levels of γδT cells (3.32 %) were observed in T1R in comparison to NR (5.16 %). The present study has provided evidence for the first time on the role of plausible unconventional T cells in the immunopathogenesis of T1R in leprosy.

1. Introduction

Leprosy is a chronic infection caused by an obligate intracellular bacterium, *Mycobacterium leprae*. In this disease, peripheral nerves, skin, and mucous membranes are particularly found to be affected. In this era of multidrug therapy (MDT), the global prevalence of leprosy has dropped to less than 1 in 10,000, but new cases are emerging between 100,000 and 200,000 cases every year in the world and India is contributing >50 % of these cases indicating the existence of active transmission of infection in the community, as reported by WHO 2022 [1]. According to this report, A total of 75,394 new cases have been reported in India in 2021 out of which 1863 were with grade 2 disabilities. Two primary routes of *M. leprae* exit from the human body have been documented: the skin and the nasal mucosa. Concrete evidence indicates that there is an elevated risk for transmission of leprosy who are near leprosy patients, likely

* Corresponding author.
E-mail addresses: pathakv.vp@gmail.com (V.K. Pathak), itusingh@gmail.com, itu.singh@leprosymission.in (I. Singh), bhavnamicrobio@gmail.com (B. Sharma), turankarr83@gmail.com (R.P. Turankar), mamtaarora_aroram@rediffmail.com (M. Arora), shoorvir.singh@gla.ac.in (S.V. Singh), usengupta2002@yahoo.com (U. Sengupta).

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due to exposure to infectious aerosols generated through coughing and sneezing, and possibly through direct contact as well. The nasal mucosa is considered the primary route of transmission [2].

It is well established that host immunity decides the outcome of the clinical manifestations of leprosy. Strong host cell-mediated immunity (CMI) limits the growth of *M. leprae* in the tuberculoid pole while weak CMI allows the overgrowth of *M. leprae* at the lepromatous pole of leprosy. Leprosy is a disease that occurs in a wide spectrum having five clinical spectrums with two polar forms, tuberculoid and lepromatous poles with three borderline forms namely, borderline tuberculoid (BT), borderline-borderline (BB) and borderline lepromatous (BL) in between these poles depending on the immune status of the patient. Borderline clinical forms are known to occur because of the fluctuating immune status of the host between the two poles of leprosy. The classification of leprosy has been further standardized based on the clinico-histopathological, bacteriological, and immunological parameters [3]. However, excruciating inflammatory episodes within these borderline spectral clinical manifestations may also occur during and after treatment, known as "reactions in leprosy" and have been named type 1 reaction (T1R) and type 2 reaction (T2R). T1R especially occurs in borderline groups of leprosy with exacerbations of the pre-existing skin and nerve lesions usually due to up-gradation of CMI frequently during and after MDT [4]. However, it has been also reported to manifest before the start of treatment [5]. T1R occurring in the nerve may lead to nerve damage leading to disability. Several attempts to explain the mechanism of T1R reactions have been made and it has been demonstrated that there is a spontaneous increase of in-situ Th1 type of immunity with infiltration of $CD4^+$ T cells in skin and nerve lesions along with an increase of TNF-α and IFN-γ in the local mielu [6]. In T1R, CMI up-regulation appears to be spontaneous and it is a primary cause of neuritis, which frequently results in nerve damage. According to a study, entry of CD3+TCRγδ+ T cells expressing a transient rise in the level of FOXP3 with a low level of TGF-β, in both types of reactions has been reported [7]. It has been determined recently that Th17 cells have a role in the initiation of type 1 reactions in leprosy [8,9]. Many reports are available to explain the possible role of conventional T cells in the occurrence and pathogenesis of type 1 reaction in leprosy [6–9]. However, there is a scarcity of published evidence on the role of unconventional T cells in the occurrence and/or immuno-pathogenesis of type 1 reactions. Since type 1 reaction occurs due to a sudden increase in CMI in leprosy patients, the mechanism causing the abrupt increase in CMI remains mostly unclear and needs more evidence to explain the exact mechanism. Interaction of conventional and unconventional T cells and their various cytokines, and chemokines may contribute to the key events in dictating the state of immunity during type 1 reaction. Because histopathological changes often fail to differentiate between relapse and reactivation, the diagnosis of T1R is still primarily based on clinical characteristics. Previously, a cohort study failed to associate the levels of TNF-α, antibody levels against S100, LAM, PGL-I and ceramide with T1R [10]. Although earlier studies established a significant rise in CXC ligand 10 [CXCL10 (IP10)] in tissues and serum during reactions [11,12], however, it is still unclear whether a rise in the blood level of IP10 can predict the development of reaction in leprosy patients.

It has been reported earlier that NK cells, NKT cells and γδT cells are involved in innate immunity and help in immuno-activation and/or immune-regulation of adaptive immunity. Activated natural killer (NK) cells release a variety of cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), granulocyte macrophage colony-stimulating factor (GM-CSF), along with chemokines including CCL1, CCL2/MCP-1 (Monocyte Chemoattractant Protein-1), CCL3/MIP-1α (Macrophage Inflammatory Protein-1 alpha), CCL4/MIP-1β (Macrophage Inflammatory Protein-1 beta), CCL5/RANTES (Regulated upon Activation, Normal T Cell Expressed and Secreted), and CXCL8/IL-8. These molecules have the potential to modulate the activity of various innate and adaptive immune cells [13–15]. Moreover, the NKT cells release a variety of cytokines into their surroundings, comprising IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, TNF-α, IFN-γ, transforming growth factor-beta (TGF-β), and GM-CSF. These diverse signalling molecules collectively contribute to shaping immune responses, mediating cellular interactions, and influencing the dynamic equilibrium of the immune system in various physiological contexts. The γδ T cells have been described as polyfunctional, they produce an array of cytokines, including IL-17A, IL-17F, IFN-γ, IL-10, IL-22, IL-21, GM-CSF, and TNF-α [16–19]. The role of conventional T cells like Th1, Th17, and Treg cells has been well studied in T1R however, the functionality of various sporadic, yet significant lymphocyte subsets, which subsequently regulate the host's cellular immune response and consequently pathogenesis of leprosy has not been yet worked out.

It is critical to diagnose T1R symptoms in leprosy early so that protective treatment, such as steroids, can be administered early and damage to nerve and tissue can be avoided [20]. Leprosy reactions occur due to the dynamic nature of immunity which could be reflected by changes in the levels of certain circulatory molecules like cytokines and chemokines, by differences in the circulatory and skin-homing patterns of various conventional and unconventional T cell subsets that play a crucial role in the control of peripheral inflammations, and further by differences in the expression levels of various cytokine mRNAs. One of the above conditions when analysed on a case-control basis may throw some light on the pathogenesis of Type 1 reactions in leprosy. Our aim of the study is to find out the frequencies of unconventional T cells in the peripheral blood of T1R group of leprosy. Further, circulatory level and gene expression of cytokines and chemokines were measured to understand their role in the occurrence/progression of T1R.

Table 1
Demographic characteristics of leprosy patients and healthy controls.

Characteristics		Non-reaction (n 50)	Type 1 reaction (n 50)	Healthy Controls (n 20)
Age (Mean ± SD)		34.29 ± 15.96	38 ± 12.12	33.65 ± 11.02
Gender (%)	Male	36 (72 %)	38 (76 %)	12 (60 %)
	Female	14 (28 %)	12 (24 %)	8 (40 %)
Bacillary index (Mean ± SD)		1.86 ± 1.62	2.36 ± 1.39	–

2. Methodology

2.1. Study subjects

After obtaining informed consent from patients, a total of 100 clinically confirmed cases (based on cardinal signs) of leprosy patients, 50 T1R and 50 non-reactional (NR) leprosy cases were recruited from the outpatient department of TLM community hospital, Nand Nagari, Shahdara, Delhi. A total of 20 healthy controls (HCs) were used, all of whom had no indications or symptoms of leprosy or any other illness (Table 1).

The study was approved by the Institutional Ethical Committee based on the guidelines of the Indian Council of Medical Research, Delhi (Dated September 17, 2012) and informed consent was obtained from each subject after explaining them about the study in local language.

2.2. Sample collection

Peripheral blood samples ≈ of 8 ml were aseptically collected from all the study subjects including healthy controls. Peripheral blood ≈ of 6 ml was collected in a heparinized vacutainer and 2 ml in an EDTA vacutainer. Blood in the EDTA vial was processed for plasma collection and RNA extraction and then stored at −20 °C till further use. Blood in the heparinized vial was processed for peripheral blood mononuclear cells (PBMCs) isolation.

2.3. RNA extraction

RNA was extracted from whole blood (n = 30) in each group by using RNeasy blood kit (Qiagen, Germany) as per the manufacturer's instructions. RNA concentration was estimated by Qubit 4 Fluorometer (Thermo-Fisher Scientific) and purity at 260/280 from 1.8 to 2.0 was considered for further processing. The cDNA was prepared from 1 μg of total RNA from each sample by using a High-Capacity cDNA Reverse Transcription Kit (Thermo-Fisher Scientific) according to the manufacturer's instructions.

2.4. ELISA for estimation of plasma levels of proinflammatory cytokines and chemokines

Proinflammatory cytokines (IFNγ, TNFα, IL1β, IL6, IL7, IL17) and chemokines (IP10) were measured by ELISA kits as per manufacturers' instructions (R&D Systems, Inc. USA), across T1R (n = 50), NR (n = 50) and HC (n = 20) groups. Each test was done in triplicate. Absorbance was measured at 450 nm by Multiskan FC microplate photometer (Thermo-Fisher Scientific, USA).

2.5. PBMCs' Isolation and lympho-proliferation

PBMCs were isolated from 6 ml heparinized venous blood from all study subject groups (n = 20) by Ficoll–Hypaque (Sigma-Aldrich, USA) density gradient centrifugation [21]. Isolated PBMCs were cultured (1×10^5 cells/well) in 96-well tissue culture plates (NUNC, USA) in triplicate wells at 37 °C and 5 % CO_2 for 48 h in complete RPMI 1640 medium supplemented with 10 % foetal calf serum (FCS) (Gibco, Thermo Fisher Scientific, USA), 2 mM antibiotic antimycotic solution (Sigma-Aldrich, USA) and 2 mM of L-glutamine (Sigma-Aldrich, USA).

The *Mycobacterium leprae* Cytosol Fraction (MLSA) (Catalog No. NR-19330) was kindly provided by BEI resources (NIH, leprosy research support contract) which were used for stimulation in the lympho-proliferation assay. The provided lyophilized MLSA was reconstituted in sterile PBS (pH 7.2), and added to the PBMC culture in 96-well tissue culture plates to give a final concentration of 10 μg/ml in the culture medium. Phytohaemagglutinin (PHA) was included as a positive control at a final concentration of 5 μg/ml. Thereafter the culture plates were incubated at 37 °C and 5 % CO_2 level. The MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed according to Mosmann [22]. A 10 μl of MTT dissolved in PBS at a concentration of 5 mg/ml was added to each well of 72-h cultured lymphocytes and incubated for 4 h at 37 °C. Followed by the incubation, unspent media along with the suspension of cultured cells was pipetted out the spent and the Formazan crystals thus formed were dissolved with 100 μl of lysis buffer made up of 10 % sodium dodecyl sulfate (SDS) in a mixture of dimethyl sulfoxide (DMSO) and ethanol (1:1). After a few minutes of incubation at RT, the OD values were taken in the dual-wavelength measuring system, at a test wavelength of 540 nm and a reference wavelength of 630 nm using MULTISKAN FC ELISA Reader (Thermo Fisher Scientific, USA). The stimulation index (SI) was calculated by using the formula given below.

$$\text{Stimulation Index (SI)} = \frac{Absorbance\ of\ stimualted\ cells}{Absorbance\ of\ unstimulated\ cells}$$

S.I. > 2 was taken as significant stimulation.

2.6. Flow cytometry

Whole blood was collected by venepuncture from all the study subject groups, T1R (n = 15), NR (n = 15), HC (n = 15) in heparinized tubes. The peripheral blood mononuclear cells (PBMCs) were isolated according to the protocol described in the section PBMCs isolation and culture. Further, the PBMCs (1×10^5 cells) were incubated overnight in a 5 % CO2 incubator at 37 °C. For surface

staining the cells were tagged with fluorochrome labelled surface antibodies viz., PerCP-Cy™5.5 Mouse Anti-Human CD3, PE-Cy™7 Mouse Anti-Human CD4, APC Mouse Anti-Human CD56, FITC Mouse Anti-Human TCRγ/δ, with respective isotype controls (BD Biosciences, USA) and incubated at 4 °C for 30 min. Cells were suspended in a staining buffer containing 2 % paraformaldehyde and acquired by using the flow cytometer (FACSAria, BD Biosciences, USA). Analysis of the acquired flow cytometry data was done with the help of FlowJo v7.6 software (FlowJo LLC, OR, USA). Individual marker expression and cellular subsets (NK subsets, NKT-like and γδ T cell subset) were evaluated for statistically significant differences by making paired comparisons between different study groups using the Mann-Whitney *U* test. Comparisons were done between the groups and P values < 0.05 were considered as significant. The data were analysed using GraphPad Prism software (GraphPad Software Inc., version 5, CA, USA).

2.7. Gating strategy

The flow cytometry analysis of peripheral blood using gating strategy for the NKT-like cells stained with monoclonal antibodies to CD3 and CD56 [Fig. 2 (A-C)] and γδ T cells stained with antibodies for CD3 and γδ [Fig. 4 (A-D)], respectively. The lymphocytes were gated on a forward scatter (FSC)/side scatter (SSC) dot plot. Further, lymphocytes were gated to establish double positives like $CD3^{+}CD56^{+}$ and CD3+γ/δ+ were also gated on lymphocytes.

2.8. Real time PCR analysis

qPCR was performed in Rotor-Gene Q real-time PCR machine (Qiagen Pvt Ltd, USA) by using SYBR green qPCR master mix (Agilent). qPCR was done for cytokines (IFN γ, TNF α, IL 6, IL 10, IL17A), CCRs/chemokines (CCL3, CCR1, CCR3, CCR5, CXCR3, IP-10) mRNA gene expression in each group (n = 30) by using specific primers and reaction conditions published earlier [23–28]. Each reaction was set up in triplicate. For each sample, mRNA abundance was normalized by the human gene GAPDH. Primer sequences are mentioned in Table 2.

The mRNA expression levels for cytokines and chemokines were calculated after determining the primer efficacy for all the genes using the Pffafl method [29] by a standard curve with 6-fold dilutions of cDNA. A standard graph was prepared for each cytokine, chemokines, and housekeeping gene GAPDH gene by serially diluting the cDNA of each cytokine and chemokine gene from 1 μg/reaction to 10ng/reaction. The percentage efficiencies of the primers were determined as 95 % for IFNγ, 97 % for IP10, 99 % for TNFα, 96 % for CCL3, 97 % for CCR1, 99 % for CCR3, 98 % for CCR5, 99 % for CXCR3, 99 % for IL6, 98 % IL10, 99 % for IL17A and 99 % for GAPDH, from the standard graphs. The fold difference in expression was determined using the following formula:

$$Ratio = \frac{\left(E_{target}\right)^{\Delta CTtarget\ (Control-Sample)}}{\left(E_{reference}\right)^{\Delta CTref\,(Control-Sample)}}$$

Individual expression ratios were calculated using the aforementioned formula for T1R and NR leprosy cases, with the average ratio of NR as the control value for all the cases. The statistical differences were calculated by using the non-parametric Mann-Whitney Test.

Table 2
Primer sequences used for mRNA expression by using real-time PCR.

S. No.	Gene Name	Primer sequence
1.	IFN γ	F 5'-ATGAAATATACAAGTTATATCTTGGCTTT-3' R 5'-GATGCTCTTCGACCTCGAAACAGCAT-3'
2.	TNF α	F 5'-CGGGACGTGGAGCTGGCCGAGGAG-3' R 5'-CACCAGCTGGTTATCTCTCAGCTC-3'
3.	IL 6	F 5'-CCAGCTATGAACTCCTTCTC-3' R 5'-GCTTGTTCCTCACATCTCTC-3'
4.	IL 10	F 5'-TGTGAAGGGTTTGGTCCCAG-3' R 5'-TGCTCTGAGACCCTCTCTACC-3'
5.	IL 17A	F 5'-GCAATGAGGACCCTGAGAGA-3' R 5'-TGGATGGGGACAGAGTTCAT-3'
6.	CCL3	F 5'- CAGGTCTCCACTGCTGCC-3' R 5'-CACTCAGCTCCAGGTCACT-3'
7.	CCR1	F 5'- CTCTTCCTGTTCACGCTTCC-3' R 5'- GCCTGAAACAGCTTCCACTC-3'
8.	CCR3	F 5'- GTCATCACCAGCATCGTCAC-3' R 5'- TCATGCAGCAGTGGGAGTAG-3'
9.	CCR5	F 5'- GGGATAGCACTGAGCAAAGC-3' R 5'- GTTTTAGCCATCCCCCAAAT-3'
10.	CXCR3	F 5'- CCACCCACTGCCAATACAAC-3' R 5'- CGGAACTTGACCCCTACAAA-3'
10.	IP 10	F 5'- TGACTCTAAGTGGCATTCAAGG-3' R 5'- GATTCAGACATCTCTTCTCACCC-3'

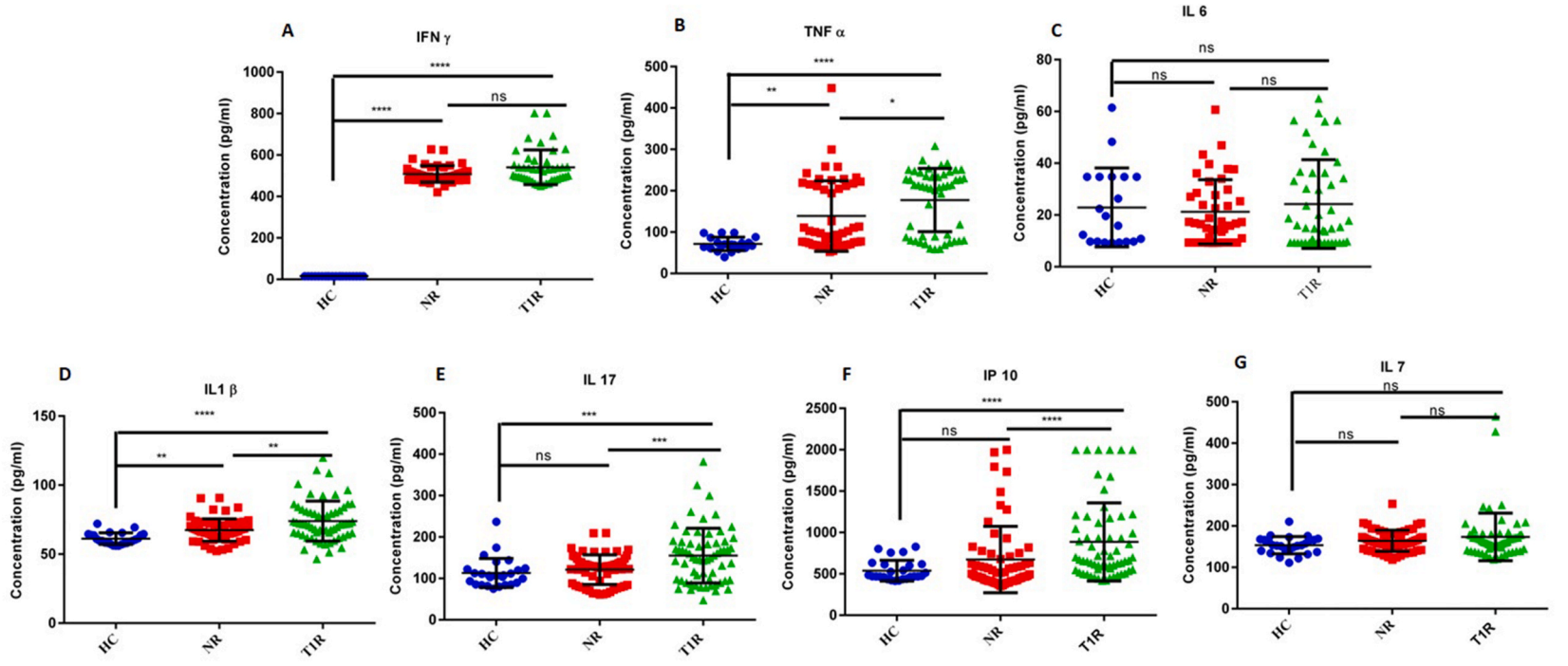

Fig. 1. Scattered graph showing the plasma level of cytokines in healthy controls, NR and T1R group of leprosy patients. (A) showing plasma level of IFN γ, (B) showing plasma level of TNF α, (C) showing plasma level of IL6, (D) showing plasma level of IL 1β, (E) showing plasma level of IL17, (F) showing plasma level of IP10, (G) showing plasma level of IL 7. Each dot represents the OD value obtained from individuals. Smooth vertical lines along with horizontal lines represent the mean concentration and SD of each group (**$p < 0.001$, ***$p < 0.0001$, ns = not significant).

2.9. Statistical analysis

Data were analysed using GraphPad Prism software version 5.0 (GraphPad Prism, La Jolla, CA). ELISA data was expressed as mean +2SD in the graphs (Fig. 1). The outliers of ELISA were removed by the ROUT method. The differences between O.D. values obtained in the sera of HCs, NR, and T1R leprosy patients were determined using one-way ANOVA (non-parametric test) and multiple comparisons (Kruskal-Wallis test). A two-tailed *t*-test was performed (Mann – Whitney test) for comparison between NR and T1R leprosy patients. The p value < 0.05 was considered statistically significant (****p value < 0.00001, ***p value < 0.0001, **p value < 0.001, ns = not significant). The statistical differences across each subject groups for the flow cytometry and gene expression data were calculated by using the non-parametric Mann-Whitney Test.

3. Results

3.1. High plasma circulatory levels of pro-inflammatory cytokines in T1R

Significantly higher plasma circulatory levels of IP10 (Fig. 1F), IL1β (Fig. 1D), IL17 (Fig. 1E) and TNFα (Fig. 1B) were observed in the T1R group in comparison to the NR group. We observed no significant difference in the plasma levels of IFN-γ (Fig. 1A), IL6 (Fig. 1C) and IL7 (Fig. 1G) between T1R and NR. Significantly higher levels of IFN-γ (Fig. 1A), TNF-α (Fig. 1B), IL 1β (Fig. 1D), IL17 (Fig. 1E) and IP10 (Fig. 1F) were also observed in leprosy patients when compared to healthy controls (Fig. 1). Further, it was observed that plasma circulatory levels of IL17 (Fig. 1E) and IP10 (Fig. 1F) were significantly higher in T1R in comparison to NR group however, there was no significant difference between HC and NR groups.

3.2. High frequencies of CD3 + $CD56^+$ NKT-like Cells and $CD3^-CD56^+$ NK cells in T1R

Frequency of γδ T cells, Natural Killer T-like cells (NKT-like cells) and Natural Killer cells (NK) were measured in healthy controls (n = 5), NR leprosy patients (n = 15) and leprosy patients with T1R (n = 14) by using flowcytometry. A representative dot plot across the subject groups for NKT-like cells and NK cells has been shown in Fig. 3 (A, B and C). We observed the highest mean frequency of $CD3^+CD56^+$ NKT-like cell subsets in the T1R group (53.72 %) of leprosy followed by HC (50.73 %) and NR group (31.38 %) (Fig. 3D). The population of $CD3^+CD56^+$ NKT-like cells was found significantly higher in the T1R group as compared to the NR group ($p = 0.0003$). It was observed that the level of NKT-like cell subsets was significantly higher in HC in comparison to the NR group ($p = 0.0013$). Additionally, no significant difference was observed between the T1R group and HC (Fig. 3D).

We observed the highest mean frequency of $CD3^-CD56^+$ NK cell subsets in the T1R group of leprosy (18.69 %) followed by the NR group of leprosy (12.94 %) and HC (11.66 %). The level of $CD3^-CD56^+$ NK cells was also found significantly higher in the T1R group as compared NR group ($p = 0.0023$) (Fig. 3E). The level of $CD3^-CD56^+$ NK cells was found to be significantly higher in T1R group of leprosy in comparison to HC ($p = 0.0019$). No significant difference was observed between the NR group of leprosy and HC (Fig. 3E).

3.3. Low frequencies of γδT cells in T1R

The mean frequency of γδ T cells was examined across subject groups and a representative dot plot has been shown in Fig. 4 (A, B and C). We observed the highest frequency of CD3+TCRγδ+ T cell subsets in HC (6.42 %) followed by NR (4.28 %) and T1R (2.8 %). However, a significantly lower level of γδ T cells was found in the T1R group when compared with the NR group ($p = 0.0072$). We

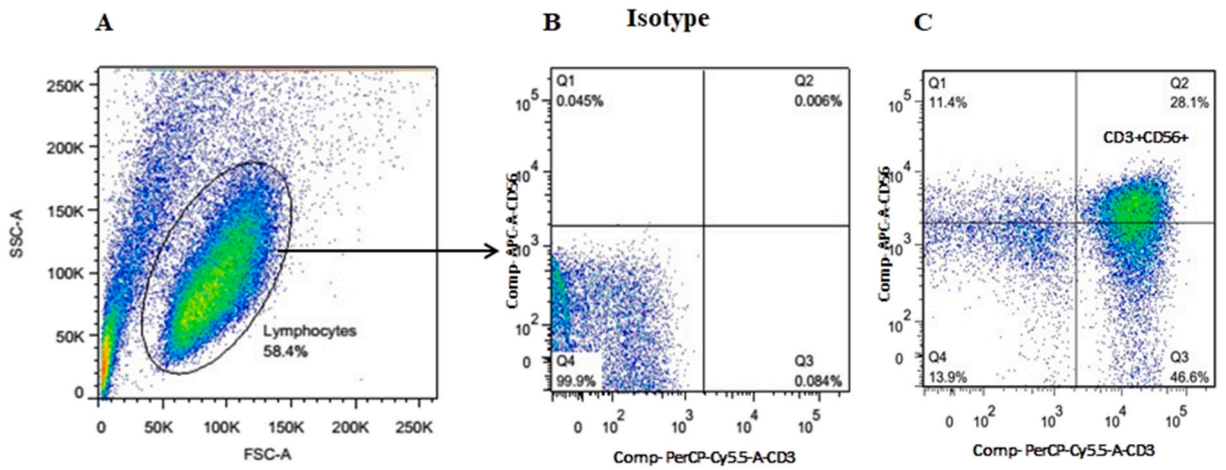

Fig. 2. Gating strategy for defining NKT-like cell subsets. Representative flow cytometry plots of patients. (A) Gated lymphocytes on a forward scatter (FSC)/side scatter (SSC) dot plot, (B) Isotype control, and (C) Lymphocytes were then gated to determine $CD3^+CD56^+$ double positive T cells.

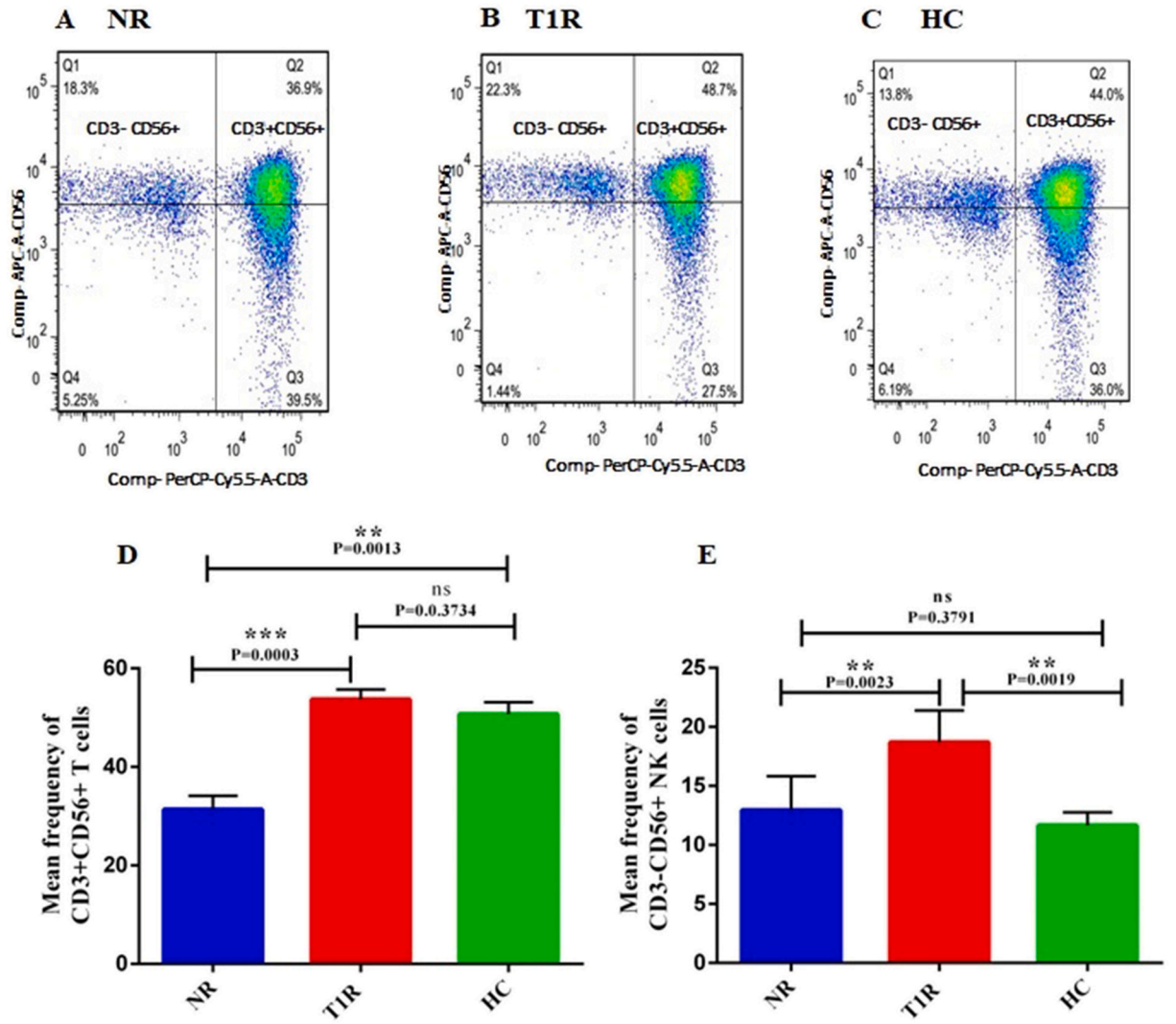

Fig. 3. Expression of NKT-like and NK cell subset in healthy individuals and in Leprosy patients with and without reactions. Representative flow cytometry plots showing $CD3^+CD56^+$ NKT-like cell frequency in leprosy patients without reactions (A), Patients with type 1 reactions (B), and healthy individuals (C). The baseline frequency of the $CD3^+CD56$ double positive cell subset and CD3-D56+ cell subset was determined by the healthy control group. Differential expression (in terms of percentage) of $CD3^+CD56^+$ double positive (D), $CD3^-CD56^+$ (E) in all three study groups. The comparison between different study groups was made using Mann-Whitney *U* Test. NR- Leprosy patients without reactions, T1R- Leprosy patients with type 1 reactions and HC- Healthy controls, ns-not significant.

observed a significantly higher frequency of $CD3^+$ TCRγδ+ T cell subsets in HC than in NR ($p = 0.019$) and T1R ($p = 0.0022$) groups of leprosy (Fig. 4E).

3.4. High T cell proliferative response in T1R with MLSA

We observed the highest lympho-proliferation with MLSA in the T1R group (mean ± SD; 8.8 ± 4.1) of leprosy followed by NR (mean ± SD; 3.42 ± 1.19) and HC (mean ± SD; 1.96 ± 1.2). Significantly high lympho-proliferation was observed in the T1R group in comparison to the NR group ($p < 0.0001$) and HC ($p < 0.001$). However, significantly high lympho-proliferation was observed in the NR group of leprosy in comparison to HC ($p < 0.05$) (Fig. 5).

3.5. mRNA gene expression of cytokines and chemokines in T1R

Individual expression ratios were calculated for T1R and NR leprosy cases taking the average ratio of HC as the control value for all the cases. The statistical differences were calculated using the non-parametric Mann-Whitney Test. We observed mean level of mRNA expression ratio of IFNγ/GAPDH (T1R vs NR; 12.9 ± 19.96 vs 1.559 ± 2.749) (Fig. 6A), of IP10/GAPDH (T1R vs NR; 3.157 ± 1.609 vs

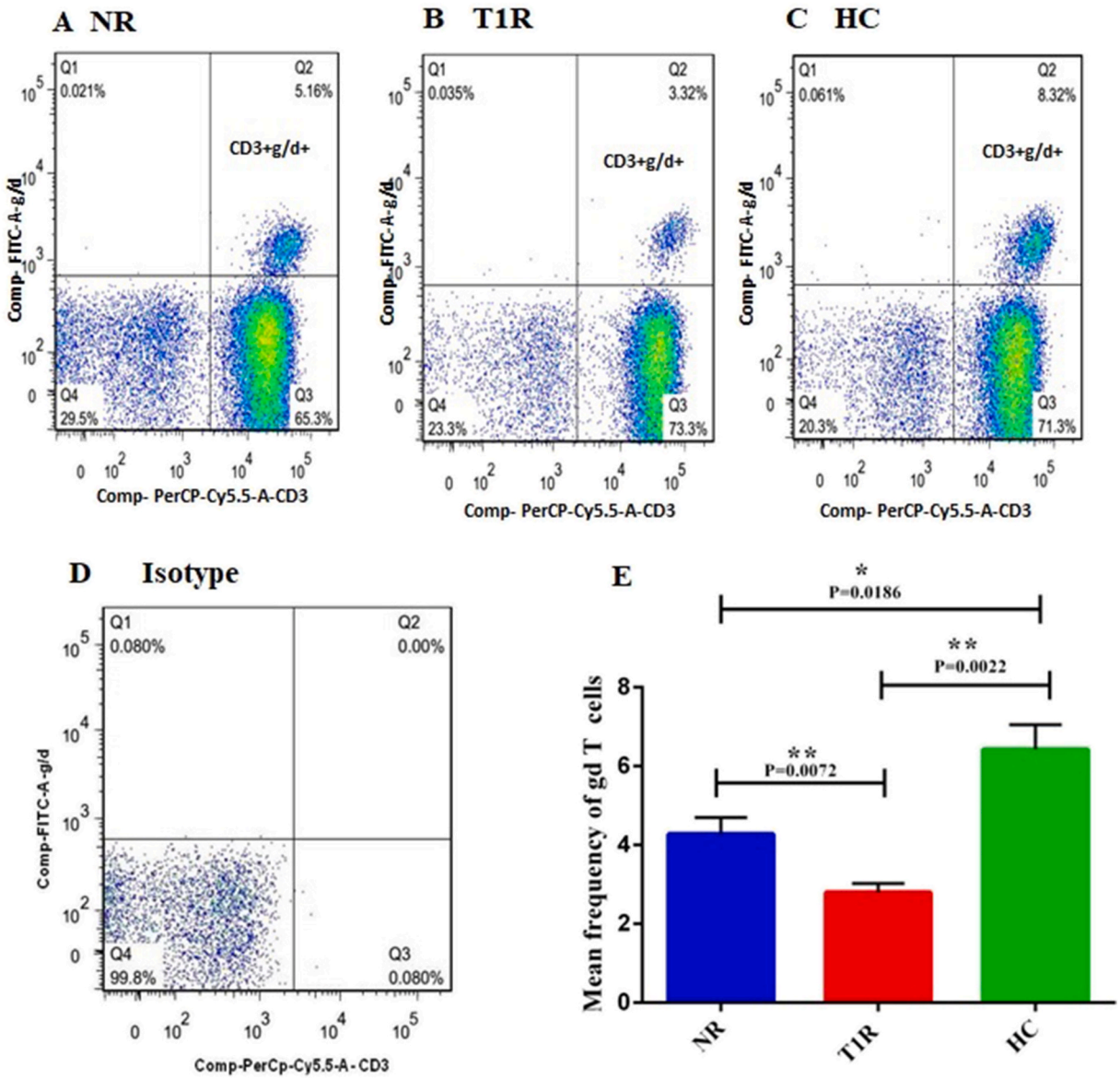

Fig. 4. Expression of gamma delta T cell subset in healthy individuals and in Leprosy patients with and without reactions. Representative flow cytometry plots showing $CD3^{+}$ gamma delta + T cell frequency in leprosy patients without reactions (A), Patients with type 1 reactions (B), and healthy individuals (C). The baseline frequency of the CD3+gamma delta + double positive cell subset was determined by healthy group (D) and Differential expression (in terms of percentage) of CD3+gamma delta + double positive in all three study groups, (E). The comparison between different study groups was made using Mann-Whitney *U* Test. NR- Leprosy patients without reactions, T1R- Leprosy patients with type 1 reactions and HC- Healthy controls, ns-not significant.

1.328 ± 1.020) (Fig. 6B), TNFα/GAPDH (T1R vs NR; 26.38 ± 57.51 vs 0.9378 ± 2.571) (Fig. 6C), CCL3/GAPDH (T1R vs NR; 0.3312 ± 0.3362 vs 0.04866 ± 0.037) (Fig. 6D), CCR1/GAPDH (T1R vs NR; 0.5694 ± 0.3341 vs 0.3749 ± 0.2341) (Fig. 6E), CCR5/GAPDH (T1R vs NR; 0.1549 ± 0.067 vs 0.1085 ± 0.064) (Fig. 6G), CXCR3/GAPDH (T1R vs NR; 406.1 ± 299.2 vs 238.7 ± 230.4) (Fig. 6H), IL6/GAPDH (T1R vs NR; 251.9 ± 232.9 vs 22.40 ± 20.51) (Fig. 6I) and IL17A/GAPDH (T1R vs NR; 82.37 ± 32.09 vs 64.50 ± 23.57) (Fig. 6K) were found to be significantly higher in T1R as compared to NR groups of leprosy (Fig. 6). While mean level of mRNA expression ratio of CCR3/GAPDH (T1R vs NR; 5.136 ± 4.841 vs 9.689 ± 10.63) (Fig. 6F) was found to be lower (not significant) and mean level of mRNA expression ratio of IL10/GAPDH (Fig. 6J) was found to be significantly lower in T1R (0.002386 ± 0.0036) as compared to NR (0.7372 ± 0.9695) group of leprosy (Fig. 6).

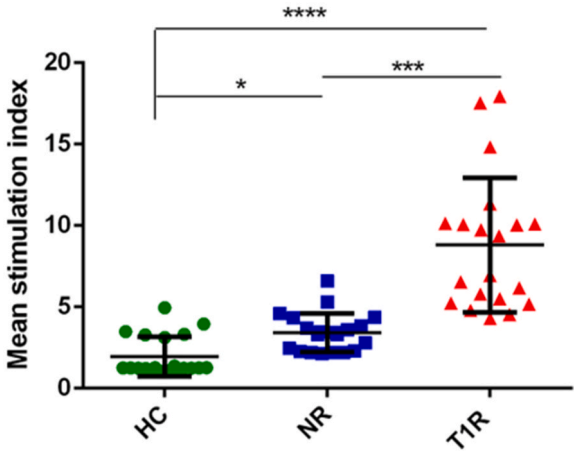

Fig. 5. Scatter graph showing the level of stimulation index across healthy control (HC) (n 20), Non-Reaction (NR) leprosy patients (n 20) and Type 1 Reaction (T1R) leprosy patients (n 20). Each dot represents the stimulation index of individuals. Smooth horizontal lines along with error bars represent the mean stimulation index and SD of each group. (*P < 0.05, ***p < 0.0001, ****p < 0.00001).

3.6. Correlation between NK cells, NKT cells with chemokines and cytokines

It was observed that NK cells were positively correlated with IP10 (r = 0.64; 95 % CI = 0.16 to 0.87; p = 0.01) and negatively correlated with IL 17A (r = −0.59; 95 % CI = −0.86 to −0.07; p = 0.029) in T1R group of leprosy. NKT cells were found to be negatively correlated with CXCR3 (r = −0.64; 95 % CI = −0.87 to −0.15; p = 0.01) in the T1R group. However, in the NR group NKT cells were found to be negatively correlated with CCR3 (r = −0.54; 95 % CI = −0.84 to 0.01; p = 0.05). Spearman correlation coefficient was calculated by GraphPad prism software.

4. Discussion

It is known that the clinical manifestations of leprosy are determined by host immunity. It has been found that T1R occurs during or after treatment when the bacterial load in patients is remarkably lower in comparison to polar lepromatous patients. A suitable explanation for the occurrence of T1R at this stage of having a lower bacterial load has not been clearly understood. The present study was therefore planned to dissect the pattern of various unconventional T cell subsets along with the circulatory plasma levels of cytokines and chemokines that might play a crucial role in the precipitation of inflammatory reactional episodes during T1R. Despite many studies carried out to date which mainly focused on the functional analysis of the response of classical T cells to *M. leprae* in leprosy and leprosy reactions, the extent and the involvement of the unconventional T cells are largely unknown. These unconventional T cells might likely have an equally important role in determining the clinical outcome of leprosy disease. Unconventional T cells are a diverse and unappreciated group of relatively rare lymphocytes that are distinct from conventional T cells, and that mainly recognize antigens in the absence of classical restriction through the major histocompatibility complex (MHC). Unconventional T cells respond to a wide range of microbial pathogens and may have either protective or pathogenic roles [30].

We observed a significantly higher frequency of NKT-like cells and NK cells in the blood circulation of the T1R group when compared to the NR group of leprosy patients. A recent report demonstrated a significant accumulation of γδT cells within the skin lesions of the tuberculoid (TT) group of leprosy patients [31]. However, we observed the frequency of γδ T cells in blood circulation was found to be lower in T1R in comparison to the NR group. Furthermore, we observed the frequency of $CD3^{-}CD56^{+}$ NK and $CD3^{+}CD56^{+}$ NKT-like cells higher in the blood circulation of the T1R group in comparison to the NR group of leprosy. It was noted that the mean frequency of NK cells ($CD3^{-}CD56^{+}$) was highest in T1R (18.69 %) followed by NR (12.94 %) and HC (11.66 %). Further, the mean frequency of NKT-like cells ($CD3^{+}CD56^{+}$) was observed to be highest in T1R (53.72 %) followed by HC (50.73 %) and NR (31.38 %) groups. The present finding of significantly higher frequencies of NK cells and NKT-like cells in circulation might be due to the inclusion of $CD3^{+}CD56^{+}$ T cells as an NKT-like cell subset. Further, the $CD3^{+}CD56^{+}$ cell population includes "true" CD1d-restricted NKT cells. Conventional T cells have also been reported to express CD56 [32,33]. Since it is unclear whether all $CD3^{+}CD56^{+}$ cells are CD1d-restricted, this population is often referred to as "NKT-like" cells and therefore, a higher percentage might be due to the inclusion of the conventional T cells expressing the CD56 marker. Earlier, it was concluded by Converse et al. (1986) [34] that in acute reversal reactions, higher NK activity could be a consequence instead of a cause for reversal reactions. It has been established from earlier studies that NK cells produce both Th1 and Th2 types of cytokines [35]. These cells have been suggested to assist in developing antigen specific T cell mediated immune responses to *M. leprae*. NKT cells have a potential role in connecting innate with adaptive immune responses. Peripheral NKT cells can promptly produce huge amounts of pro-inflammatory cytokines upon antigenic stimulation. In this study, we evaluated the level of NK cells, NKT-like cells and γδT cells in the T1R group and NR group of leprosy patients after stimulation with MLSA. We observed NK cells and NKT-like cells were in significantly higher frequency in the T1R group in comparison to the NR group of leprosy patients. Interestingly, in the present study, a significantly lower level of γδT cells in the T1R group in

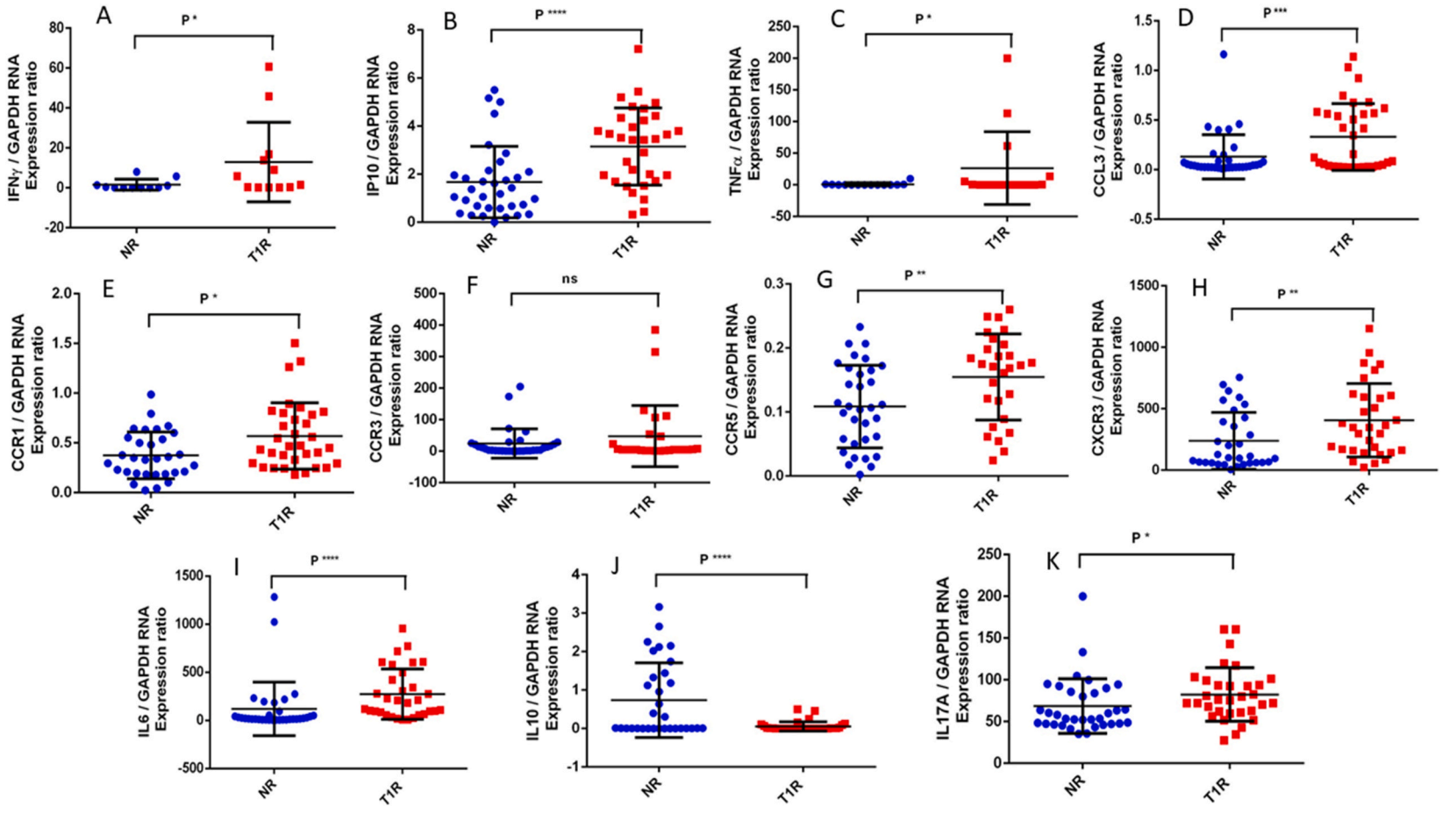

Fig. 6. Scattered graph showing mRNA expression ratio of cytokines and chemokines with GAPDH. mRNA expression ratio (A) of IFNγ/GAPDH, (B) of IP10/GAPDH, (C) of TNFα/GAPDH, (D) of CCL3/GAPDH, (E) of CCR1/GAPDH, (F) of CCR3/GAPDH, (G) of CCR5/GAPDH, (H) of CXCR3/GAPDH, (I) of IL6/GAPDH, (J) mRNA expression ratio of IL10/GAPDH, (K) mRNA expression ratio of IL17A/GAPDH. Each dot shows the gene expression ratio of individuals. Smooth horizontal lines along with error bars represent the mean gene expression ratio and SD of each group. (*P < 0.05, **p < 0.001, ****p value < 0.00001, ns = non-significant).

comparison to the NR group of leprosy patients has been noted. This finding of a lower number of γδT cells in T1R patients is not in concordance with an earlier finding of a higher frequency of these cells in T1R [7]. Thus, the present study clearly demonstrated a regulatory imbalance of NK cells, NKT-like cells, and γδT cells during T1R which could be responsible for the occurrence of inflammation during T1R. Gamma delta (γδ) T cells, NK cells and NKT cells are known to play a pivotal role in the early stages of infection and act in synchrony with innate immune cells [36]. These lymphocytes are non-MHC restricted and characteristically recognize non-peptide antigens. Prior observations have indicated a rise in γδT cells during instances of mycobacterial diseases [37]. T cell clones derived from skin lesions have been observed to encompass CD4+/CD8+ cells as well as TCRγδ cells, regardless of the clinical status of the patients. Nonetheless, reports indicated that T cell clones originating from skin lesions of patients experiencing a reversal reaction exhibited phenotypes characterized by CD4-/CD8-/TCR γδ expression [38]. Though we noted significantly lower circulatory levels of γδ T cells and due to the limitation of the present study, we were unable to investigate the levels of NK, NKT-like and γδ T cells in situ in the local milieu in the skin. It is established that prior to the in situ peripheralization of immune cells within the skin, these cells undergo acquisition of the skin-homing receptor CLA (Cutaneous Lymphocyte Antigen), leading to the recruitment of cells within the circulatory system [39] and in the context of the inflammatory reaction (T1R), there is a potential occurrence of in situ peripheralization of γδT cells within skin lesions might contributing to a decline in their circulatory abundance during T1R observed in present study. Mucosal-associated invariant T cells (MAIT cells) are a subset of T lymphocytes that sit at a bridge between innate and adaptive immunity, called as unconventional T cells. These cells are particularly present in liver and blood but also inhabit mucosal sites such as skin, oral, intestinal, respiratory, and urogenital tracts that are in direct contact with the environment and microbiota of their host. MAIT cells were found to be involved in safeguarding the mucosa against external microbial threats. Simultaneously, mucosal MAIT cells have been implicated in immune and inflammatory pathologies affecting these organs. Hence, it would be an important addition to observe the role of MAIT cells in leprosy reactions in future.

Further, the development of different phenotypes of immune cells is crucially dependent on various cytokines. We next investigated the relationship between cytokines and chemokines which could have implications for the development and differentiation of NK cells, NKT-like cells, and γδ T cells. In the present study, we observed a significant increase in the plasma levels of proinflammatory cytokines like TNFα, IL17, IL1β and IP10 in the T1R group in comparison to that of the NR group of leprosy. We also observed similar plasma levels of IFNγ, IL6 and IL7 in the T1R group and NR group. Saini et al. (2020) have reported that IL6 induces Th17 cell differentiation along with TGF-β in leprosy reactions in stimulated PBMC culture supernatant. However, we could not find a significant difference in basal plasma levels across the subject groups for IL6 [9]. Many studies have proven heightened expression of proinflammatory cytokines TNFα, IFNγ, IL-6 and IL-12 in skin lesions of reactional leprosy cases along with the proof of macrophage activation [38,40]. Previously documented evidence reveals that these cytokines elicit a suitable immune response as necessitated during that particular timeframe. Earlier in a longitudinal study, an upregulation of IP10, IL1β along with pro-inflammatory cytokines has been observed during the reactions [41]. Proinflammatory cytokines play a critical role in the regulation of T1R in leprosy. Increased levels of IP-10 and IFNγ released by several cell types in reactions could be due to various inflammations. Furthermore, the mRNA gene expression levels of IFNγ, IP10, TNFα, CCL3, CCR1, CCR5, CXCR3, IL 6 and IL 17A were observed significantly higher in the T1R group when compared to the NR group by qPCR analysis. However, we found a lower mRNA gene expression level of IL10, and CCR3 in T1R in comparison to the NR group. IL 10 was found to be significantly lower in T1R while CCR3 was not significantly lower in the T1R group. Based on previous studies and concepts developed [7,42,43].

In conclusion, we are proposing that the distinct modulation of cytokines and chemokines, in conjunction with the involvement of NK cells, NKT-like cells, and γδ T cells, potentially underlies the pathogenesis of T1R in individuals with leprosy. Whilst the responses observed could consist of a notable portion of conventional or adaptive T cell reactions because of the presence of *M. leprae* proteins in the antigens employed (specifically, the MLSA cytosolic fraction), the phenotype data obtained from flow cytometry analysis, it appears that unconventional T cells might constitute a substantial portion of this immune response. To address this concern, we propose conducting studies in future. These studies could encompass the incorporation of more precise cell markers for unconventional cells, in addition to employing the MLSA lipid fraction as an antigenic stimulus. Furthermore, we present a novel observation of unconventional T cell imbalances, accompanied by alterations in the concentrations of cytokines and chemokines, which may contribute to the onset of inflammatory processes during T1R.

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Data availability statement

The authors declare that all the data of the present study has been included in the manuscript.

CRediT authorship contribution statement

Vinay Kumar Pathak: Writing – original draft, Investigation, Formal analysis, Data curation. **Itu Singh:** Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Bhawna Sharma:** Investigation, Formal analysis, Data curation. **Ravindra P. Turankar:** Writing – review & editing, Formal analysis. **Mamta Arora:** Writing – review & editing, Investigation. **Shoor**

Vir Singh: Writing – review & editing, Resources. **Utpal Sengupta:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

M. leprae	Mycobacterium leprae
LPs	leprosy patients
BT	borderline tuberculoid
BB	borderline borderline
BL	borderline lepromatous
LL	lepromatous leprosy
T1R	type 1 reaction
T2R	type 2 reaction (erythema nodosum leprosum)
HC	healthy controls
MLSA	M. leprae sonicated antigen
ELISA	enzyme linked immunosorbent assay

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