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Research Article

Cellular Immune Response Evaluation in Nepalese Patients with Cutaneous Leishmaniasis

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ABSTRACT

Cutaneous leishmaniasis (CL) remains a clinically and immunologically heterogeneous disease influenced by parasite species and host immune responses. In this study, seventeen CL cases were assessed through lesion characteristics, histopathological analysis and Kinetoplast DNA (kDNA) nested Polymerase Chain Reaction (PCR)figure. Lesions mainly appeared on exposed body parts, especially the face (64.70%) and most patients had a single lesion. kDNA PCR confirmed CL in 58.82% (n=10) of cases, identifying *Leishmania donovani* (720 bp) in 5 cases and *L. major* (560–590 bp) 5 cases as the circulating species. Immunological analysis showed reduction in T-lymphocytes in CL patients compared to Healthy Controls (HC), largely due to a significant decrease in $CD8^+$ T cells (p=0.036), while $CD4^+$ T cell levels remained stable. Further stratification based on CL PCR positivity showed that reductions in T-lymphocytes (p=0.045) were confined to patients with confirmed *Leishmania* infection, again attributed mainly to decreased $CD8^+$ T cells (p=0.040). No notable changes were observed in B cells, NK cells, or NKT cells. These findings highlight CL infection positivity status-dependent variations in host immune responses and emphasize the importance of $CD8^+$ T-lymphocytes in CL pathogenesis and disease outcome.

Keywords: Cutaneous leishmaniasis; $CD8^+$ T cells; Immune response; kDNA PCR

Introduction

Cutaneous leishmaniasis (CL) is a vector-borne disease caused by *Leishmania* parasites and typically presents with ulcerative or non-ulcerative lesions on the skin. Although the infection was once limited to endemic regions like the South and Central America, Northern Africa, the Middle East and parts of Asia (Kim et al., 2025; Scott & Novais, 2016), it has begun to emerge in previously non-endemic countries, including Nepal (Bhattarai et al., 2025; Pandey et al., 2021; Rai et al., 2023)

CL lesions often heal on their own, but in some cases, they progress into chronic, disfiguring forms. Due to their clinical similarity to conditions such as cutaneous malignancies, discoid lupus erythematosus, lupus vulgaris, and sarcoidosis, these lesions are frequently misdiagnosed, leading to suboptimal therapeutic interventions(Ermertcan, 2018). To enhance diagnostic precision, molecular techniques like PCR have been adopted. PCR-based tests provide faster and more sensitive, species-specific identification of *Leishmania* than conventional methods such as microscopy or culturing the parasite. In Nepal, *L. donovani* and *L. major* are the main etioligical agent causing CL (Rai et al., 2023).

In human leishmaniasis, T-lymphocytes play a key role in establishing protective immunity but they are also involved in sustaining the disease and contributing to the pathology of leishmaniasis. Both $CD4^+$ and $CD8^+$ T cells contribute to immune defense by producing key cytokines that activate monocytes and macrophages (Brelaz-de-Castro et al., 2012). In addition to cellular immunity, B cells have been shown to participate actively in the immune response to *Leishmania* infection, with increased frequencies of $CD19^+$ B lymphocytes and plasma cells as reported in cutaneous leishmaniasis patients presenting with lymphadenopathy (Bomfim et al., 2007).

Only limited data are available on the immunological events associated with human cutaneous leishmaniasis in Nepal. Immunophenotypic evaluation of the lymphocyte is helpful in demonstrating the host immune response. This study aimed to characterize the peripheral immune cell profiles particularly T-cell subsets and B cell subsets in patients with active cutaneous lesions and in healthy controls using flow cytometry, and to assess whether alterations in these immune cell populations are associated with disease activity and potentially influenced by infection with *Leishmania* parasite.

Materials and Methods

Study site

Samples were collected from two hospitals in Nepal: Sukraraj Tropical and Infectious Disease Hospital (STIDH), Teku, Kathmandu and Nepalgunj Medical College and Teaching hospital (NGMCTH), Kohalpur, Banke. These hospitals were selected as they serve as major referral centers, covering cases from the eastern to the western regions of Nepal, thereby ensuring a wide geographic representation.

Ethical approval and collection of biospecimen

The ethical approval for the research involving human participants was granted from Nepal Health Research Council (NHRC) (Reg. No. 45/2018). Anonymity of the patients has been secured in this research according to the NHRC guidelines. Informed consent was obtained before the sample collection from all participants and/or their legal guardians for publication of their clinical information, and laboratory findings.

For this study, 17 patients presented with cutaneous lesions with complaints of non-healing wounds were included. The sampling was done under sterile condition. Some tissue aspirate and freed tissue were withdrawn and collected in Rosewell Park Memorial Institute (RPMI) medium from all patients. Irrespective to treatment and duration of lesion appearance, 5 ml peripheral blood samples was drawn using a syringe with 22 gauze needle and collected in EDTA collection tube from 12 patients. After the collection, the samples were transported to Infectious and Viral Disease Research Laboratory (IVDRL) at Central Department of Biotechnology, Tribhuvan University under ambient condition for further processing.

DNA extraction and polymerase chain reaction

The DNA from the clinical specimen was extracted by using DNA extraction Kit (Quick-DNA™ Universal Kit, Zymo Research) according to the manufacturer's instructions. For the PCR assay, a kinetoplast minicircle DNA (kDNA) nested PCR was performed using CSB2F and CSB1R primers for first round PCR (PCR-1), and 13Z and LiR primers for second round PCR (PCR-2) as described by Noyes et al. (Noyes et al., 1998). The PCR-1 consists of 94 °C for 2 min, followed by 40 cycles of amplification (94°C for 30s, 54°C for 60s, 72°C for 90s), and final extension at 72°C for 10 min. In the PCR-2, 1 µl (1:10) diluted PCR-1 product was used as template and same PCR conditions of PCR-1 were used, except annealing temperature which was increased from

54 to 56 °C and extension time was 40s. PCR amplicons were analyzed on 1.5% agarose gels. The 100 bp DNA ladder (Solis Biodyne) was used as the DNA molecular marker.

Peripheral blood immunophenotyping

Fresh blood was collected from clinically diagnosed CL patients and healthy controls (HC). Fifteen microliter of T cell panel antibody cocktail (BD Biosciences, USA) and 15 µl of B cell panel antibody cocktail panel (BD Biosciences, USA), were mixed separately (Table 1) with 200 µl of fresh blood and incubated in dark for 15 mins at room temperature. Then, 2 ml of 1:10 diluted BD FACS lysing solution (BD Biosciences, USA) was added, vortexed briefly and incubated for 12 minutes at ambient temperature. After that, the tubes were centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and 2 ml of 1X Phosphate buffer saline (PBS) was added and mixed and centrifuged at 1200 rpm for 5 minutes. Again, the same procedure was repeated, supernatant was discarded and the cells were resuspended in 400 µl PBS. For unstained control, the same procedure was followed except the addition of antibodies.

Table 1: Cell markers and tagged fluorochromes of the T and B cell panel.

Channel	Fluorochromes	T-Cell panel	B-Cell panel
FL1	FITC	CD3	CD3
FL2	PE	CD8	CD56/16
FL3	PerCP	CD45	CD45
FL4	APC	CD4	CD19

Compensation of FL channels in flow cytometer

The four FL channels in the BD FACS Calibur machine are FL-1, FL-2, FL-3, FL-4 which collects the emission wavelength from the fluorochromes FITC, PE, PerCP and APC respectively. The compensation of the channels was done by using BD calibrate beads. Compensation was done by aligning the cells in their respective quadrant by manipulating the voltages, current and compensation of different channels. When compensation was performed properly, the further processing of samples were done with the same parameters.

Acquisition of stained cells and analysis

After the completion of compensation of FL channels, the processed cells were acquired. The cells immunophenotypic and morphometric parameters were determined by flow cytometry (FACSCalibur-BD), using the software CELLQuest alies™ (BD Bioscience) for acquisition (50,000 events/tube) and FlowJo software version 10 for analysis of data. The statistical analyses were performed in statistical software R version 4.5.0.

Results and Discussion

Clinical presentations of cutaneous lesions

Out of 17 patients, 8 patients were histo-pathologically confirmed as CL and other 9 patients were diagnosed clinically by the characteristics of lesions. The lesions were found in various parts of the body like face, neck and limbs (Figure 1). Most of the patients (64.70%) had lesions on their face followed by neck (17.64%), lower limb (11.76%) and upper limb (7.69%). Most of the patients had single lesion (n = 14), three patients had multiple lesions; two patients had 2 lesions and one patient had 3 lesions and the lesions were most common in the facial parts. These findings suggest that the exposed body parts are prone to the bite of sandfly (Ghimire et al., 2018).

Figure 1: Clinical feature of CL patients: Nodulo-ulcerated lesion on face (left), Nodulated lesion of upper lip (middle) and Ulcerated lesion of right thigh with central depression (right).

Molecular confirmation: PCR positive CL cases

Molecular level confirmation using kDNA nested PCR revealed that 10 patients (58.82%) were CL positive while histopathological analysis showed LD bodies in 8 cases. Two samples tested negative by microscopy were found to be positive by PCR. Diagnosis of CL with conventional technique is challenging due to extremely diverse clinical presentation and the involvement of different *Leishmania* species (Bailey & Lockwood, 2007; de Monbrison et al., 2007); however, PCR offers a solution, it provides much higher sensitivity than other diagnostic methods. (Pourmohammadi et al., 2010). Moreover the PCR approach makes possible the fast identification at the *Leishmania* species and subspecies level compared to conventional techniques like microscopy and parasite culture (Yehia et al., 2012). Based on comparing the PCR-2 amplicon band size with 100bp DNA ladder, it was found that the band size of 720 bps was comparable with that of *L. donovani* (Bastola et al., 2020) in 5 cases and band size of 560-590 bps was comparable with that of *L. major* (Oryan et al., 2013) in 5 cases (Figure 2). PCR-negativity in 7 samples may reflect low or resolved parasitic burden.

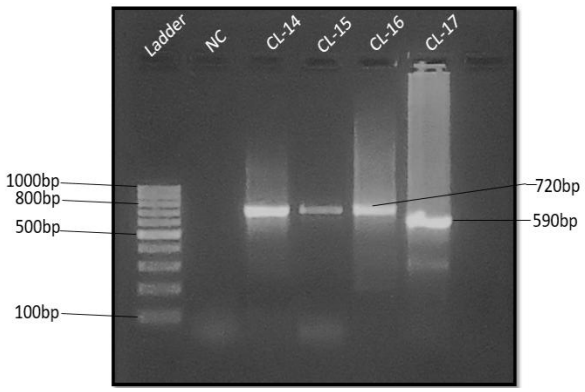

Figure 2: PCR-2 amplicon in 1.5% agarose gel electrophoresis. Ladder: 100 bp ladder, NC: Negative control, CL-14,15,16,17: CL samples.

Analysis of T-Cell and B-Cells population: A flow cytometry study

T-Cell population

Fresh blood samples were collected and stained with T cell panel from 12 CL patients (9 PCR positive and 3 PCR negative). Samples from the remaining five patients were unavailable for the study. The gated mean lymphocyte population of the CL patients in the FSC vs. SSC plot was 21.9 ± 7.73%. The cells positive for CD45 and CD3 were identified as T cells and mean percentage were of 60.5 ± 9.61%. The gated population of the T cells were further categorized to T sub-type cells into $CD4^+$ and $CD8^+$ T cells, the mean percentage of which were respectively 52.00 ± 7.17% and 37.59 ± 6.06% (Table 2 and Supplementary Table 1). Shapiro–Wilk normality testing indicated that all immune cell variables followed a normal distribution in both groups ($p > 0.05$), and none of the percentages are close to 0 or 100 (Table 2), validating the use of parametric Welch's t-statistical analyses. Representative acquisition of flow cytometry is below in Figure 3.

Similarly, the fresh blood samples collected and stained from 6 healthy controls with T cell panel antibodies showed the 68.0 ± 5.48% frequency of T cells and the relative proportion of $CD4^+$ and $CD8^+$ cells among the T cells in healthy control population were 43.4 ± 10.02% and 49.3 ± 10.12% respectively (Table 2 and Supplementary Table 2).

To compare T-lymphocytes between independent two-sample CL patients and healthy individuals, boxplots were generated, and Welch's t-test p-values were calculated. A p-value less than the significance level of 0.05 was considered statistically significant difference in mean between the groups. As depicted in Figure 4, the relative proportion of T-lymphocytes among total lymphocytes in the peripheral blood of CL patients and healthy controls was visualized using box plots. The total lymphocytes in CL patients were found to be higher compared to those in Healthy controls (21.92 ± 7.73% vs. 15.6 ± 3.71%, $p = 0.032$) suggesting immune activation and inflammation and the T-lymphocytes in CL patients were found to be lower compared to those in HC (60.49 ± 9.61% vs. 67.97 ± 5.48%, $p = 0.053$). The percentage of $CD4^+$ T cells was not significantly different among the CL patients and HC. However, there was approximately 24% reduction in $CD8^+$ T cells in CL patients compared to HC (37.59 ± 6.06% vs. 49.28 ± 10.12%, $p = 0.036$).

We infer the reduction in T cell compartment was contributed by the reduction in $CD8^+$ T cells. This observation further strengthens the possibility of protective function of $CD8^+$ cells in cutaneous leismaniasis. $CD8^+$ T cells actively move from blood to infected skin and they participate in parasite killing and tissue inflammation (Bertho & Coutinho, 2005) leading to lower circulating $CD8^+$ T cells and T lymphocytes in peripheral blood.

Table 2: Summary statistics and normality assessment of immune cells (T cells) in peripheral blood from CL patients and healthy controls.

Cells	CL patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
Lymphocytes	21.9	7.73	20.9	15.6	3.71	15.8	0.584	0.795
T-lymphocytes	60.5	9.61	63.3	68.0	5.48	68.6	0.126	0.949
$CD8^+$ T cells	37.6	6.06	35.8	49.3	10.12	50.5	0.794	0.714
$CD4^+$ T cells	52.0	7.17	52.7	43.4	10.02	40.2	0.877	0.183

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous leishmaniasis, SD= Standard deviation, HC= Healthy control

It has been shown that the host immune cells are involved in the progression or healing of the CL lesions primarily based on the types of the immune cells activated. Th1 mediated immune response is considered protective while Th2 mediated immune response support parasite growth and dissemination (Ajdary et al., 2000). Th1 mediated arm of adaptive immune response involve the potent cytokines such as IFN-γ produced by Th1 cells and $CD8^+$ cells which activate macrophages to phagocytose the parasites effectively. The mechanisms by which $CD8^+$ cells limit bacterial and viral infection is well understood.

However, their effector roles in parasitic diseases like cutaneous leishmaniasis is gradually being unfolded (Novais & Scott, 2015).

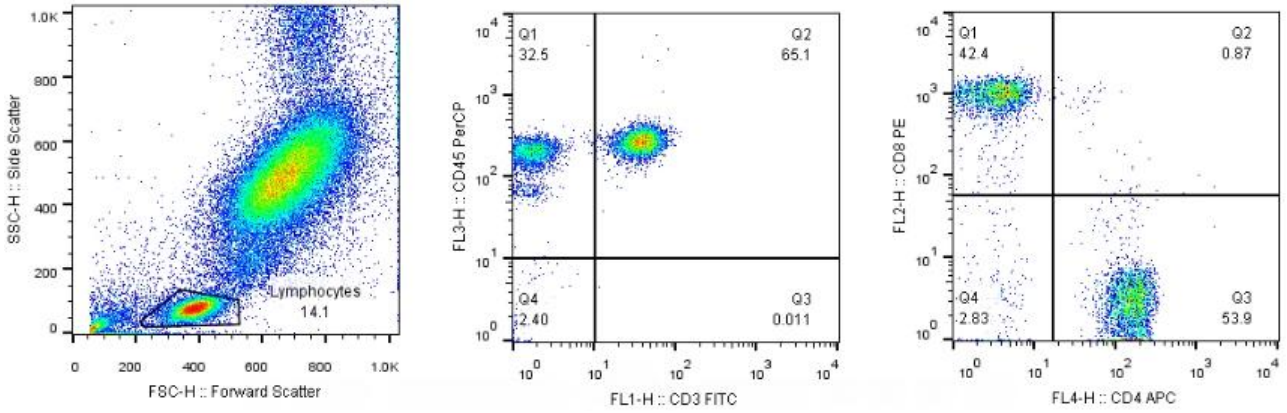

Figure 3: Gating strategy for identification of T cells, CD4+ and CD8+ cells from peripheral blood sample.

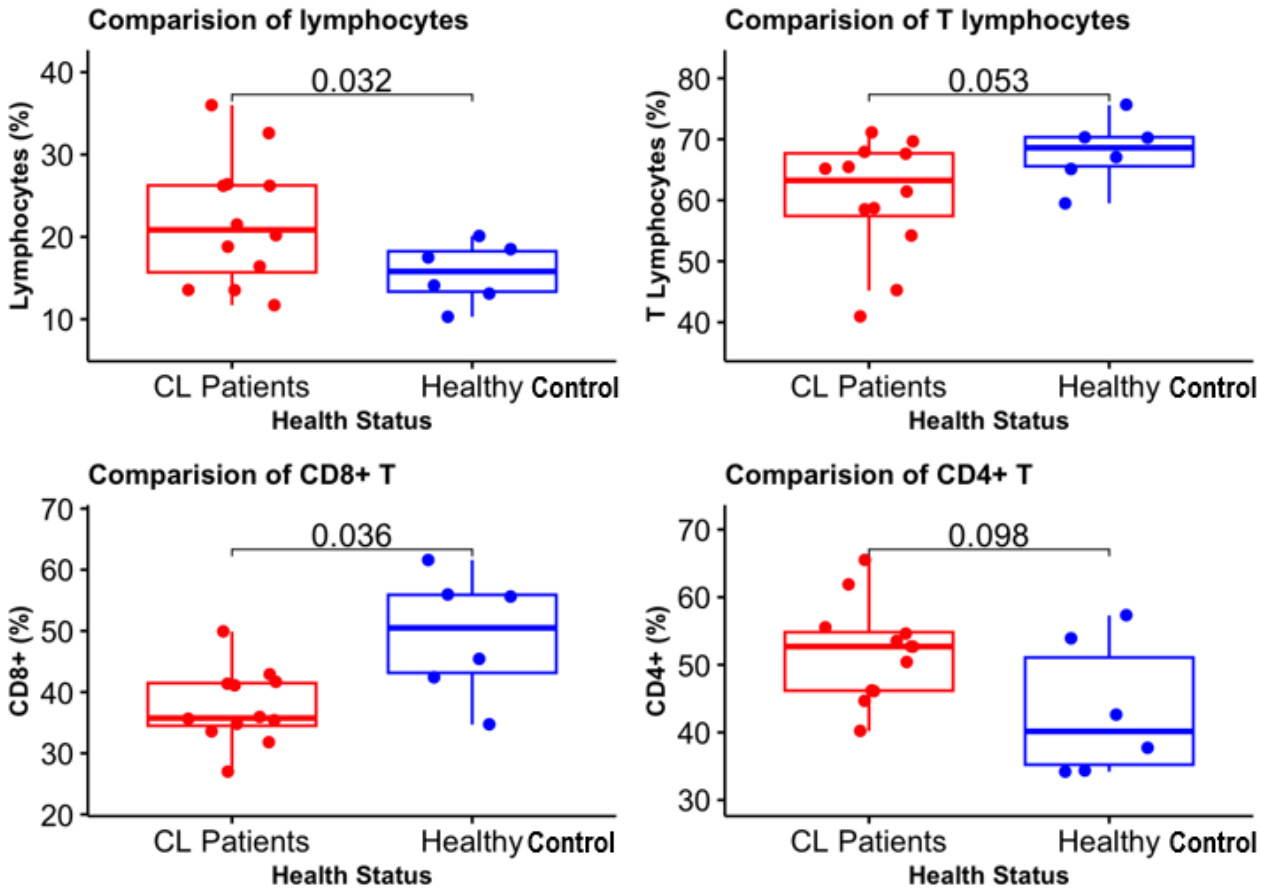

Figure 4: Box plot showing the comparison of T-lymphocytes and its subsets in the peripheral blood of CL patients and Healthy controls (HC) with Welch t-test p-value. Percentage of T-lymphocytes staining positive for $CD45^+$ $CD3^+$ were calculated out of the total lymphocyte gate. Similarly, percentages of $CD4^+$ and $CD8^+$ T-lymphocytes were calculated relative to the gated population of T-lymphocytes (n = 12 for CL, n = 6 for HC).

In our study, we reported decrease in $CD3^+$ T-lymphocytes in patients with active CL disease compared to healthy controls. In a study done by Campanelli et al, there were not significant differences in $CD3^+$ T cells, $CD4^+$ T cells or $CD8^+$ T cells in the peripheral blood of CL patients and the healthy control (Campanelli et al., 2006). In another study done in Brazil by Af et al, it was shown the T cells decreased in active CL cases by approx. 3% compared to control patients which later increased upon chemotherapy. In the same study, the authors did not find significant differences in the $CD4^+$ and $CD8^+$ T cells between active CL cases and controls, which contrasts with our findings. However, in the same study upon chemotherapy, both $CD4^+$ and $CD8^+$ cells were found to be increased which suggest of protective functions of $CD4^+$ and $CD8^+$ T cells (Af et al., 2011). Discrepancy in the composition of immune cells compared to other studies might be due to sampling time as blood was withdrawn irrespective of treatment and also might be because of inherent pathological mechanism associated with different *Leishmania* spp. that cause cutaneous

leishmaniasis. In the previous studies (Af et al., 2011; Campanelli et al., 2006), *L. vianna brazilensis* was implicated in the active CL cases while in our study we found the CL cases could be attributed to infection by *L. donovani* and *L. major*. The reduced $CD4^+$ T-cell proportion relative to $CD8^+$ T cells observed in healthy controls may be related to the inclusion criteria, as controls were defined by the absence of leishmaniasis without assessment of other health factors.

B-Cell population

Fresh blood samples from 9 CL patients and 5 Healthy control (HC) were stained for B cell panel antibodies. The lymphocytes plotted in the FSC vs. SSC further identified as the population of leukocytes as $CD45^+$ cells. B cells ($CD19^+CD3^-$) and T cells ($CD19^-CD3^+$) were identified in CD19 vs. CD3 plot. Likewise, the NK cells and NKT cells in the gated lymphocyte population was identified as NK cells ($CD56^+CD3^-$) and NKT cells ($CD56^+CD3^+$) in the CD56 vs. CD3 plot (Figure 5). Supplementary Table 3 and Table 4 showed the percentage of T cells, B cells, NK cells and NKT cells in CL patients and HC respectively. The frequency of T cells (59.4 ± 6.05%), B cells (9.4 ± 4.21%), NK cells (14.2 ± 7.38%) and NKT cells (3.6 ± 2.57%) were identified in CL patients (Table 3). Shapiro–Wilk testing indicated normal distribution for NK cells, NKT cells, and T cells in both groups ($p > 0.05$), but for B cell CL patients, data deviated from normality ($p = 0.036$). So, to compare two independent sample means, the Welch t-test for NK cells, NKT cells, and T cells, and for B cell data, we use non-parametric Mann-Whitney U-test. The average percentage of B cells in CL patients (9.4 ± 4.21%) was not significantly different from those in HC (8.8 ± 3.96%). Similarly, the NK cells constituted 14.2 ± 7.38% in CL patients which was not statistically different from 17.8 ± 8.73% in the HC. The NKT cells constituted a small portion with an average of 3.6 ± 2.57% in the CL patients and 3.7 ± 1.49% in the HC. Although NK cells, NKT cells and B lymphocytes showed minor variations between groups, these differences were not statistically significant.

In contrast, T cells ($CD3^+$ T) cells differed significantly between CL patients and healthy controls ($p = 0.024$) (Figure 6), highlighting a predominant alteration in the T-cell compartment during CL. The absence of significant differences in B cells, NK cells, and NKT cells significant alteration in T cells in our study suggests that CL predominantly alters the T-cell compartment, consistent with the central role of T-cell mediated immunity in intracellular parasite control. CL is not mainly a humoral (antibody-driven) disease. So B-cell percentages often remain stable (Novais & Scott, 2015). NK cells are rapidly and transiently activated during infection, provide an early source of IFN-γ, and then their activity often wanes. Consequently, detectable alterations in peripheral NK or NKT cell levels may not always be observed in blood samples (Bogdan, 2012). Furthermore, the duration of lesion appearance was not taken into account during sample collection, which may have reduced the ability to detect minor variations in these immune cell population ability.

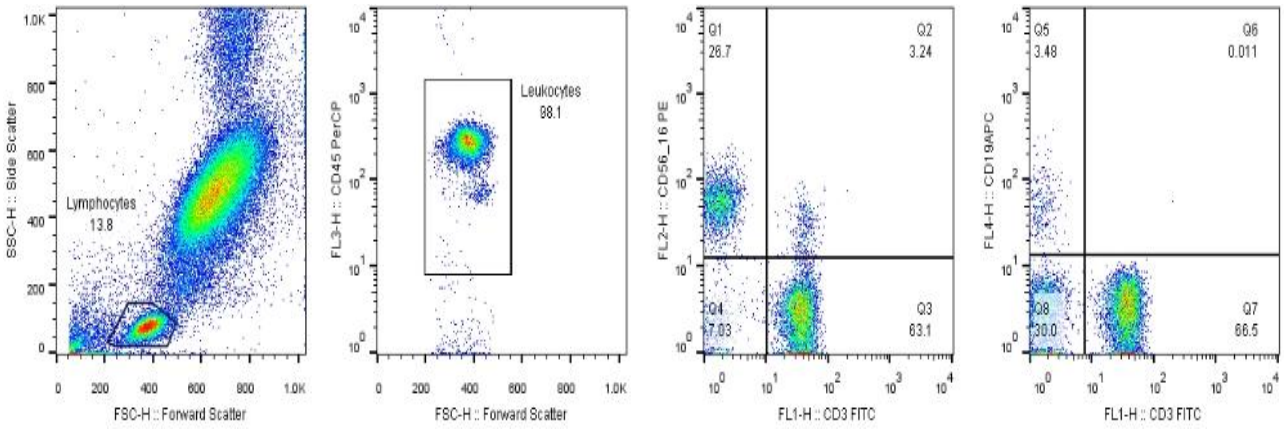

Figure 5: Gating for identification of T cells, B cells, NK cells and NKT cells.

Table 3: Summary statistics and normality assessment of immune cells (NK, NKT, T and B) in peripheral blood from CL patients and healthy controls.

Cells	CL patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
NK cells	14.2	7.38	13.2	17.8	8.73	16.2	0.479	0.513
NKT cells	3.6	2.57	2.6	3.7	1.49	3.2	0.100	0.456
T cells	59.4	6.05	58.5	65.7	3.09	66.8	0.864	0.429
B cells	9.4	4.21	8.2	8.8	3.96	9.5	0.036	0.977

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous Leishmaniasis SD = Standard deviation, HC = Healthy control

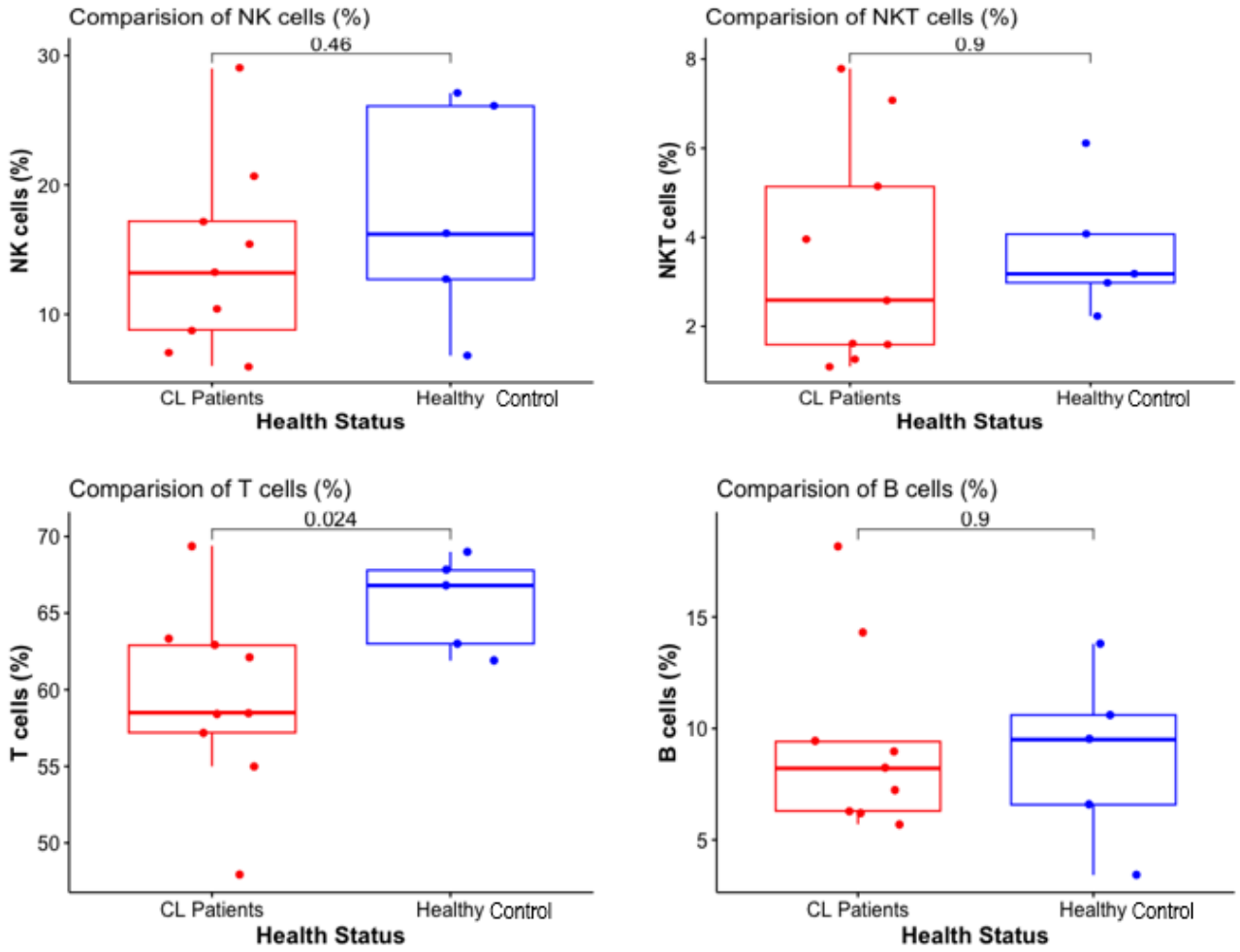

Figure 6: Comparison of the immune cells in the peripheral blood of CL patients and Healthy Controls (HC). Peripheral blood samples were stained for B cell panel the data was acquired in BD FACS Calibur. The relative percentages of NK cells ($CD3^{-}CD16/56^{+}$) and NKT cells ($CD3^{+}CD16/56^{+}$), T cells ($CD19^{-}CD3^{+}$) and B lymphocytes ($CD3^{-}CD19^{+}$) were calculated out of the total lymphocyte gate (n = 9 for CL and n = 5 for HC).

Comparison of cell population between PCR +ve and PCR –ve samples

Interestingly, when we further analyzed the relative proportion of T cell subsets based on PCR positivity for *Leishmania* spp. DNA, there was a significant reduction of T-lymphocytes ($p = 0.045$) in patients who tested positive for *Leishmania* spp. DNA by PCR compared to healthy controls (Table 4). We could infer that reduction in T-lymphocytes was contributed by the significant reduction in $CD8^{+}$ T-lymphocytes ($p = 0.040$) but not by $CD4^{+}$ T-lymphocytes (Figure 7). However, no significant difference was found in any T cell subsets when PCR negative samples were compared with both PCR positive samples and healthy control (Table 5). The lack of significant T-cell changes in PCR-negative individuals likely reflects low or absent parasitic burden, indicating that systemic T-cell modulation is mainly associated with active infection when parasite antigens stimulate immune responses (Novais & Scott, 2015). This further supports the *Leishmania* infection-dependent nature of $CD8^{+}$ T-cell reduction observed in PCR-confirmed cases. Additionally, the limited sample size of PCR-negative cases may have reduced the statistical power to detect minor immunological differences. The Shapiro–Wilk test was performed to assess normality. All variables were normally distributed except $CD8^{+}$ T cells in PCR-negative patients, deviated from normality ($p = 0.034$). Welch's t-test was used for mean comparisons when the normality assumption was satisfied; otherwise, the nonparametric Mann–Whitney U test was applied.

As observed previously, when the patient samples were analyzed without subdivision into PCR +ve or PCR –ve, no significant difference could be observed in the relative proportion of B cells, NK cells or NKT cells between CL patients and HC (Table 6), while significant changes observed in T cells ($p = 0.024$). The similar result was obtained when further compared with PCR+ CL patients and HC (Figure 8). There is only one case with PCR negative for B-cell panel so further comparison could not be made. Moreover, small study population included in the study may have limited the ability to detect subtle changes in these cell populations. To the best of our knowledge, this is the first study the immune cells in peripheral blood from cutaneous leishmaniasis by the application of flow cytometry.

Table 4: Summary statistics and normality assessment of immune cells (T cells) in peripheral blood from PCR positive CL patients and healthy controls.

Cells	PCR positive CL patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
Lymphocytes	22.0	8.84	21.5	15.6	3.71	15.8	0.367	0.795
T-lymphocytes	58.9	10.29	61.4	68.0	5.48	68.6	0.382	0.949
$CD8^+$ T	37.9	5.70	35.6	49.3	10.12	50.5	0.099	0.714
$CD4^+$ T	51.2	6.37	52.7	43.4	10.02	40.2	0.925	0.183

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous leishmaniasis, SD = Standard deviation, HC = Healthy control

Table 5: Summary statistics and normality assessment of immune cells (T cells) in peripheral blood from PCR negative CL patients and healthy controls.

Cells	PCR negative CL patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
Lymphocytes	21.8	4.04	20.2	15.6	3.71	15.8	0.332	0.795
T-lymphocytes	65.4	6.04	68.0	68.0	5.48	68.6	0.270	0.949
$CD8^+$ T	36.7	8.40	41.4	49.3	10.12	50.5	0.034	0.714
$CD4^+$ T	54.3	10.49	52.7	43.4	10.02	40.2	0.747	0.183

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous leishmaniasis, SD = Standard deviation, HC = Healthy control

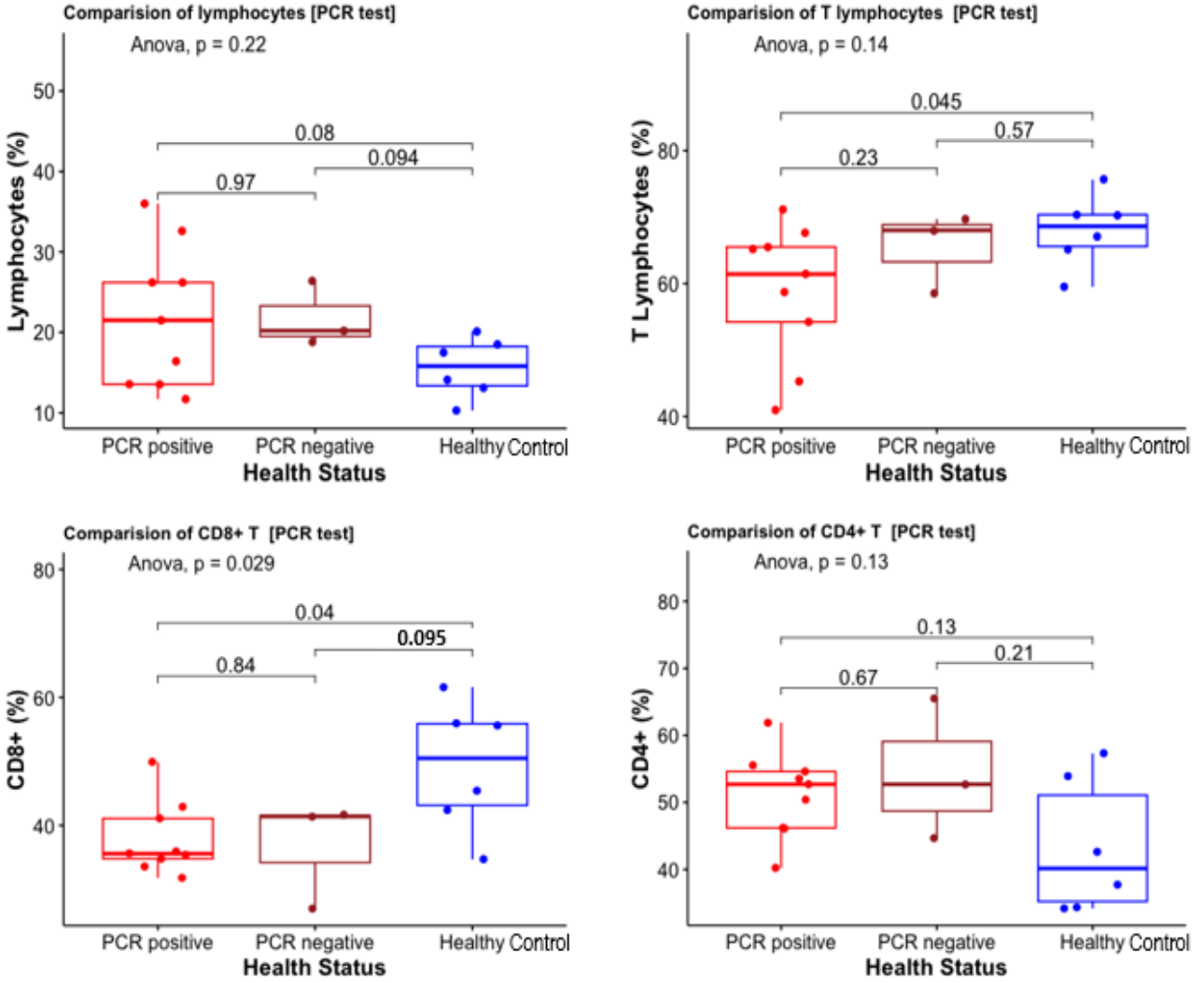

Figure 7: Comparison of T-lymphocytes and its subsets in the peripheral blood of patients with PCR positive (CL PCR +), PCR negative (CL PCR -) and Healthy Controls (HC). Peripheral blood samples were stained with T cell antibody panel and the data was acquired in BD FACS Calibur. Percentage of T-lymphocytes staining positive for CD45+CD3+ were calculated out of the total lymphocyte gate. Similarly, percentages of CD4+ and CD8+ T-lymphocytes were calculated relative to the gated population of T-lymphocytes. (n = 9 for CL PCR +, n = 3 for CL PCR -, n = 6 for HC).

Table 6: Summary statistics and normality assessment of immune cells (NK, NKT, T and B) in peripheral blood from PCR positive CL patients and healthy controls.

Cells	CL Patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
NK cells	13.8	7.79	11.8	17.8	8.73	16.2	0.288	0.513
NKT cells	3.0	2.17	2.1	3.7	1.49	3.2	0.120	0.456
T cells	59.7	6.41	60.3	65.7	3.09	66.8	0.823	0.429
B cells	9.8	4.32	8.6	8.8	3.96	9.5	0.103	0.977

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous Leishmaniasis, SD= Standard Deviation, HC=Healthy Control

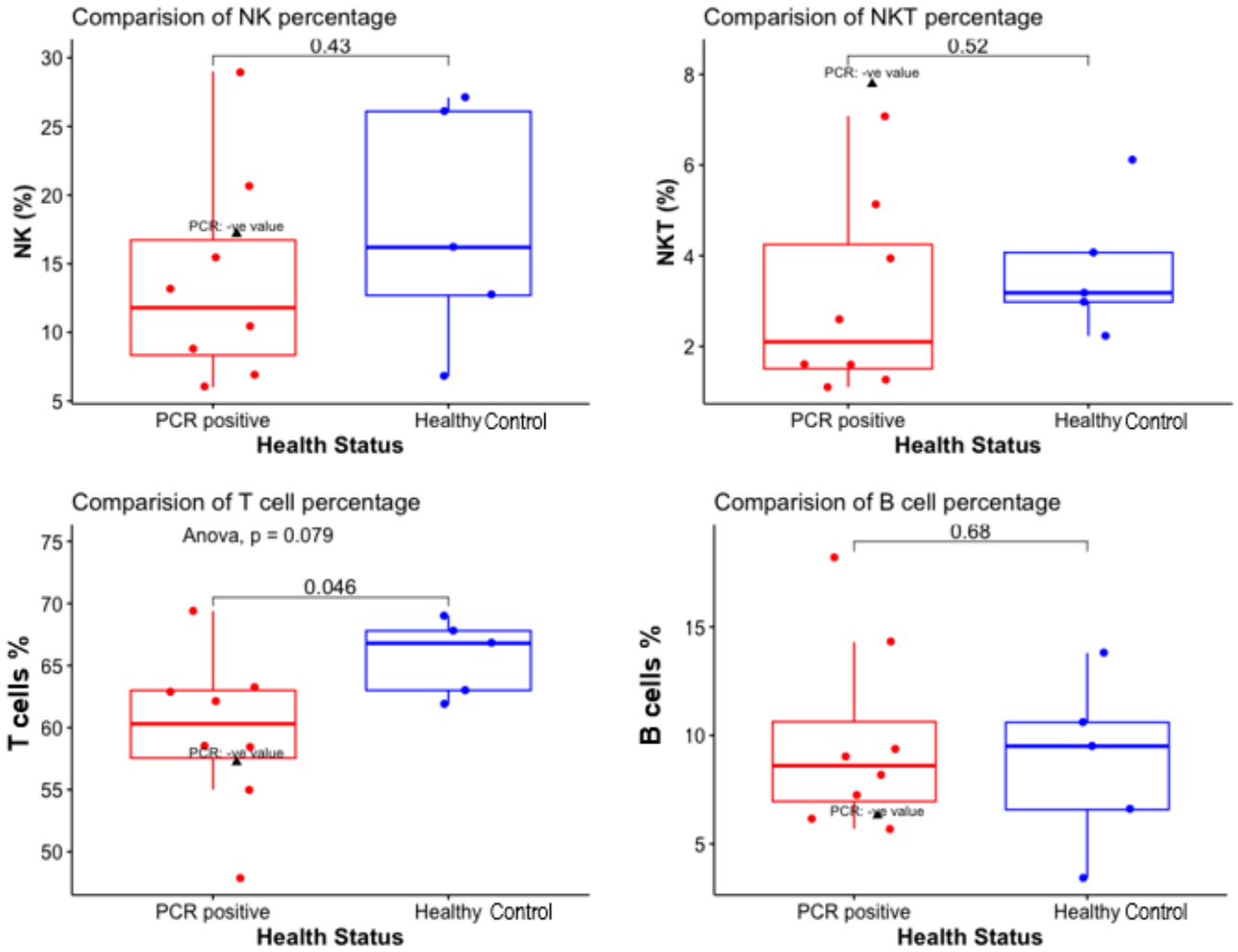

Figure 8: Comparison of the immune cells in the peripheral blood of CL patients with PCR positive (CL PCR +) and Healthy Controls (HC). Peripheral blood samples were stained for B cell panel the data was acquired in BD FACS Calibur. The relative percentages of (A) B-lymphocytes ($CD3^-CD19^+$) (B) NK cells ($CD3^-CD16/56^+$) and (C) NKT cells ($CD3^+CD16/56^+$) were calculated out of the total lymphocyte gate. (n = 8 for CL PCR +, n = 1 for CL PCR -, n = 5 for HC). PCR-negative single case value is represented by small black triangle.

Conclusion

This study highlights the clinical and immunological complexity of cutaneous leishmaniasis in the study population. Molecular analysis confirmed *L. donovani* and *L. major* as the circulating species, emphasizing the coexistence of multiple etiological agents. Immunophenotyping revealed a significant reduction in $CD3^+$ and $CD8^+$ T-lymphocytes in active and PCR-positive CL cases, suggesting an important protective role of $CD8^+$ T cells in controlling infection. In contrast, $CD4^+$ T cells, B cells, NK cells, and NKT cells showed no significant differences between patients and controls. Overall, these findings indicate active immune responses specific to *Leishmania* infection and reinforce the relevance of T-cell–mediated immunity in CL pathogenesis. Further studies with larger cohorts are needed to better define immune alterations and their implications for diagnosis, prognosis, and therapeutic strategies.

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Supplementary Table 1: Percentage of T-lymphocytes in the fresh blood samples drawn from patients.

Sample No.	PCR	% of Lymphocytes	% of T-lymphocytes	% of $CD8^+$ T cells	% of $CD4^+$ T cells	Ratio (CD4/CD8)
CL5	+	36	65.5	33.6	61.9	1.84
CL8	+	16.4	45.2	35.9	53.5	1.49
CL10	+	26.2	40.9	49.9	40.2	0.81
CL12	+	32.6	67.6	31.8	54.6	1.72
CL13	+	11.7	54.2	35.4	52.7	1.49
CL14	+	13.55	65.1	35.6	55.5	1.57
CL15	+	13.53	71.1	41.1	46.2	1.12
CL16	+	26.2	61.4	42.9	50.4	1.17
CL17	+	21.5	58.7	34.8	46.1	1.32
CL4	-	18.8	58.5	27	65.5	2.43
CL9	-	20.2	69.7	41.7	52.7	1.26
CL11	-	26.4	68	41.4	44.7	1.08
Mean		21.92	60.49	37.59	52.00	1.44
SD		7.73	9.61	6.06	7.17	0.43

Supplementary Table 2: Percentage of T-lymphocytes in the fresh blood drawn from healthy controls.

Sample no.	% of Lymphocytes	% of T-lymphocytes	% of $CD8^+$ T cells	% of $CD4^+$ T cells	Ratio (CD4/CD8)
HC1	13.1	75.6	34.7	57.3	1.65
HC2	20.1	67.0	45.4	42.6	0.94
HC3	14.1	65.1	42.4	53.9	1.27
HC4	18.5	70.2	55.6	37.7	0.68
HC5	10.3	70.4	56	34.4	0.61
HC6	17.5	59.5	61.6	34.2	0.56
Mean	15.60	68.0	49.3	43.4	0.95
SD	3.71	5.48	10.12	10.02	0.43

Supplementary Table 3: Percentage of T cells, B cells, NK cells and NKT cells in the fresh blood samples drawn from CL patients.

Sample No.	PCR	% of NK cells	% of NKT cells	% of T cells	% of B cells
CL5	+	8.8	1.1	69.4	18.2
CL8	+	29	5.14	47.9	8.21
CL12	+	6.01	2.59	58.5	9
CL13	+	20.7	7.08	55	5.7
CL14	+	6.98	3.95	63.3	9.41
CL15	+	13.2	1.61	62.9	6.17
CL16	+	15.4	1.59	62.1	7.22
CL17	+	10.4	1.26	58.4	14.3
CL 11	-	17.2	7.79	57.2	6.3
Mean		14.2	3.6	59.4	9.4
SD		7.38	2.57	6.05	4.21

Supplementary Table 4: Percentage of T cells, B cells, NK cells and NKT cells in the fresh blood samples drawn from Healthy Controls

Sample No.	% of NK cells	% of NKT cells	% of T cells	% of B cells
HC1	16.2	4.07	67.8	9.5
HC2	26.1	3.18	61.9	3.42
HC3	12.7	6.11	66.8	6.58
HC4	6.81	2.98	69	10.6
HC5	27.1	2.23	63	13.8
Mean	17.8	3.7	65.7	8.8
SD	8.73	1.49	3.09	±3.9