

Original Research Paper

# CD2 Shown to Facilitate CD4<sup>+</sup> Cell Differentiation during Visceral Leishmaniasis, a Receptor Based Study

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**Abstract:** This study addressed the role of CD2 on CD4<sup>+</sup> T helper cells in relation to the TCRV $\alpha$ 11 and CD70 in two groups of BALB/c mice *viz* control and infected ( $1 \times 10^7$  *L. donovani* promastigotes). The results showed with the downregulation of CD2 expression there is a pronounced decrease of TCRV $\alpha$ 11 and CD70 on CD4<sup>+</sup> cells in mice. An insight into the behavior of the cytokines *viz* IL-2, IFN- $\gamma$  and TNF- $\alpha$  also showed disturbance in the concurrent efficiency of these cells in responding to the *Leishmania donovani* infection. Hence it can be interpreted that probably CD2 plays an important role during maturation steps of CD4<sup>+</sup> T cell differentiation during VL infection.

**Keywords:** Visceral Leishmaniasis (VL), T Cell Receptor V $\alpha$ 11(TCR V $\alpha$ 11), Peripheral Blood Mononuclear Cells (PBMNCs), Cluster of Differentiation (CD70, CD2, CD4)

## Introduction

Visceral leishmaniasis is also known as Kala-azar (Hindi: Kala=black, azar=sickness) was first described in 1903 by Leishman and Donovan simultaneously but separately (Ross, 1903). The aetiological agents belong to the *Leishmania donovani* complex; *L. donovani*, *L. infantum* and *L. d. archibaldi* in the old world and *L. d. chagasi* in the new world. The old world species are transmitted by the sandfly vector *Phlebotomus sp.* Humans, wild animals and domestic animals are known to act as reservoir hosts. *Lutzomyia longipalpis* is the only sandfly vector that has been implicated in the transmission of the new world species of visceral leishmaniasis and wild and domesticated dogs are known to serve as reservoir hosts (WHO, 2000; Shiddo *et al.*, 1995a; 1995b; Arias *et al.*, 1996). The annual incidence of visceral leishmaniasis is estimated to be 500,000 cases with 90% of them occurring in Bangladesh, India, Nepal and Sudan. An estimated 59000 deaths occur annually due to VL (Hotez *et al.*, 2004). Moreover for each symptomatic case there are estimated to be 6-20 asymptomatic infections (Badaro *et al.*, 1986). In the Mediterranean basin 1.5-9% of AIDS patients develop visceral leishmaniasis and 25-70% of the adult VL cases are related to HIV infection. Both the new world and the old world form of the disease display similar symptoms and are often complicated by secondary infections. One of

the immunological hallmarks of kala-azar is a remarkable increase in the immunoglobulin levels, mostly of IgG and IgM classes (Ghose *et al.*, 1980) causing a reversal of the albumin/globulin ratio (Irunberry *et al.*, 1968). Much of this increase is nonspecific and appears to be the result of polyclonal B cell stimulation (Ghose *et al.*, 1980). The most striking pathological changes in visceral leishmaniasis are hyperplasia of the liver and spleen, pancytopenia and changes in plasma protein. The infection with visceral leishmaniasis promotes expansion of T<sub>H</sub> type 2 (T<sub>H</sub>2) of CD4 cells that arise as a result of IL-4 biased response, which severely abrogates IFN- $\gamma$  production in VL patients (Uzonna and Bretscher, 2001).

The inability of the VL subjects to produce cytokines such as IFN- $\gamma$ , particularly from CD4<sup>+</sup> cells is not only an immunological concern but also poses a major therapeutic obstacle as pentavalent antimonials and other antileishmanial drugs are also shown to require its presence to clear the infection. Recently we reported that most of the CD4<sup>+</sup> cell functions progressed also under the influence of CD2 which when increased on CD4<sup>+</sup> cells led these cells to proliferate and transduce efficient signaling necessary for the production of this cytokine in human VL patients (Bimal *et al.*, 2007). To gain further insight on how CD2 deficiency can have an impact on functional maturation of CD4<sup>+</sup> cells we examined the relation of CD2 on T cells with TCRV $\alpha$ -11, which

appears prior to the differentiation of DP T cells into single positive cells and CD70 in the subjects suffering from Visceral leishmaniasis and healthy controls which is an activation marker on T cells. While dealing with the mechanism it appeared that the presence or absence of CD2 had influenced the differential mechanism in CD4 cells with regard to their maturation following *Leishmania* infection. While the question still persisted whether such effect of CD2 on CD4 based immune response disturbed the concurrent efficiency of such cells in responding to the *Leishmania donovani* infection through displaying any form of infection. Furthermore an attempt was made to look into the cytokine profile of healthy and infected subjects by studying the release of IL-2, IFN- $\gamma$  from CD4 cells and TNF- $\alpha$  by macrophages.

## Materials and Methods

### Experimental Mice Category

Mice were obtained from animal house of Rajendra Memorial Research Institute of Medical Sciences, Patna. Inbred male six to eight weeks old BALB/c mice were injected subcutaneously with whole ( $1 \times 10^7$ /mL), *Leishmania* promastigotes suspended in 0.05 mL of PBS. Mice were challenged with  $1 \times 10^7$  promastigotes of WHO reference strain of *L. donovani* (MHOM/IN/80/DD8). The kinetics of the response was determined on day 35. One group was injected with saline suspension, which served as the control.

### Preparation of DAT Antigen

*Leishmania* parasites were harvested and washed in Locke's solution. Trypsinization was performed by adding 20 times (v/v) trypsin solution followed by incubation at 37°C for 45 min with gentle shaking. The trypsinized cells were washed with Locke's solution and then fixed using the formaldehyde suspension by incubation on ice for 20 h with gentle shaking. The fixed cells were washed thrice with citrate saline to remove traces of formaldehyde. 5 mL of promastigote suspension ( $200 \times 10^6$  cells/mL) was stained using equal volume of the staining solution by incubating at 4°C for 90 min with gentle shaking. A final thorough wash was given to remove all excess stain. A final stock suspension of the antigen was prepared in antigen storage solution at a concentration of  $50 \times 10^7$  parasites/mL. The stock solution was stored at 4°C (not under freezing conditions) and diluted using the antigen storage solution to working concentration before use.

### Experimental Procedure

#### Selection of Mice

One to two months old male BALB/c mice were chosen for the study. At the beginning of the study they were healthy and active. Five mice were taken in each category.

Groups	Evaluation scheme	No. of mice
1. Healthy control	5 weeks	5
2. Infected	5 weeks	5

### Parasite Challenge

- Day 1:  $0.5 \times 10^7$  early stationary phase promastigotes in 50  $\mu$ L N/S
- Day 2:  $0.5 \times 10^7$  early stationary phase promastigotes in 50  $\mu$ L N/S

### Assessment of Clinico Pathological Parameters

#### Weight of Mice

Mice were weighed on day 1 and before sacrificing on 35 days post challenge.

#### Blood Total Count/Differential Count

Blood was collected from the mice before sacrificing and smeared slides were prepared for TC/DC evaluation. The smears were air dried and stained followed by microscopic examination.

#### Serum Antibody Titer

Blood spots from mice were collected on what man paper (no.4) and air dried. Circular sections of the paper were obtained from the blood spotted regions using a punching device. The sections were submerged in Normal Saline (N/S), 1 circular piece immersed in 750  $\mu$ L of N/S, to obtain 1:50 diluted serum in a DAT dilution plate. Direct Agglutination Test (DAT) was performed as previously described (Harith *et al.*, 1988; Meredith *et al.*, 1995). In brief, the samples were diluted in physiological saline (0.8% NaCl) containing 0.78% 2-mercaptoethanol. Two-fold dilution series of the sera were made in a V-shaped microtitre plate, starting with a dilution of 1:50 and going upto a maximum of 1:25600. Negative and positive controls were added to two separate wells. 30  $\mu$ L of DAT antigen was added to each well containing 30  $\mu$ L diluted serum and results were read after 18 h of incubation at room temperature. Cut off titer was determined with the help of the titers obtained in healthy control mice.

### Assessment of Splenic Parasite Load

#### Limiting Dilution Assay (LDA)

96-well plate was prepared for LDA analysis by adding 100  $\mu$ L of brain heart infusion agar medium (supplemented with 20% defibrinated rabbit blood and antibiotics) overlaid with 100  $\mu$ L Locke's solution supplemented with 20% FCS and antibiotics). A weighed section of the spleen from its anterior end was homogenized in Locke's solution. The splenic homogenate was inoculated into the wells following a 10-fold serial dilution. The plate was incubated at

24(±2)°C and observed on the 7th day. The number of viable parasites was determined from the reciprocal of the highest dilution at which promastigotes could be detected after 7 days and was expressed as parasites per milligram of tissue (Lira *et al.*, 2000).

#### *Assessment of Immunological Profile*

##### *Isolation of Mononuclear Cells from Spleen in Mice*

The euthanized mice were dissected and their spleen was collected separately in PBS. The spleen was measured for its length and weighed. After removing 0.5 cm piece from the anterior end for the limiting dilution assay, the remaining portion was macerated in PBS to obtain a suspension. The suspensions of spleen were layered over Ficoll-Hypaque in separate tubes and the mononuclear cells were obtained following density gradient centrifugation (Boyum, 1968). The mononuclear cells fraction was collected carefully and washed once in PBS followed by counting of cells in a haemocytometer chamber. Cells to the order of  $10^6$  were obtained.

##### *Separation of Mice with Undersuppressed or Overexpressed CD2 Antigen on T cells*

PBMNCs ( $2 \times 10^6$ ) from control and experimental VL mice spleen were incubated with for 20 min at 4°C for *ex vivo* staining of lymphocytes for surface markers, CD2 PE and CD4 PE-Cy-Chrome. After staining, the preparations were washed with stain buffer (containing 0.09% sodium azide and 0.09% FCS), fixed with 200 µL of cytofix solution and kept at 4°C until data were acquired using a Fluorescence-Activated Cell Sorter (FACS).

To clarify the role of CD2 in maturation of T cells, we immunophenotyped mice from infected and control groups and evaluated the surface expression of CD2 antigen on T cells of their spleen suspension. In brief, mononuclear cells of their spleen suspension were double stained with PE anti CD2 and PE-Cy-Chrome anti CD4. The dot plot profiles of CD2 and CD4 expression was examined in 20,000 live cells by Flow cytometry. The number of CD2 and CD4 cells were calculated by quantifying the total leukocyte count with lymphocyte percentage divided by hundred which was further multiplied by percentage of CD2 as determined by FACS analysis. On the basis of respective number of CD2 mice were categorized into group with underexpressed (group-1) and overexpressed (group-2).

##### *Expression of Thymocytes in CD2 Underexpressed and Overexpressed Mice Experimental Group*

For this purpose FITC-conjugated rat antimouse Vα11.1,11.2<sup>b,d</sup> T cell receptor monoclonal antibody, clone RR8-1 stored in aqueous-buffered solution containing 0.09% sodium azide was used as a marker to study DP<sup>+</sup> T cells and their progression towards SP<sup>+</sup> T cells under the influence of CD2. In brief, mice splenic MNC-suspension

were consecutively co-incubated with PE conjugated anti CD2 and FITC conjugated Vα11.1,11.2<sup>b,d</sup> TCR along with CD70. Flow cytometry was performed on a FACS calibur.

##### *Intracellular Cytokine Studies*

For the evaluation of cytokine profile these were studied by using IL-2 (PE-Clone JES6-5H4), IFN-γ (FITC-Clone XMG1.2) from CD4 cells and TNF α (PE-Clone NP6-XT22) released by the macrophages was studied by using PE labeled antibodies. MNCs of the order  $5 \times 10^6$  cells were cultured Falcon tubes in 1 mL culture volume for 16h in RPMI-1640 (Himedia, India) supplemented with 10% FCS (Life Technologies, Gaithersburg, MD) in two groups viz: Control, infected at 37°C and 5% relative humidity. Brefeldin-A (BD Pharmingen) ( $1 \mu\text{g mL}^{-1}$ ) was added to the culture during the last 4-h of culture to impair protein secretion by the golgi complex. The cells were later harvested using ice cold PBS plus 0.09% sodium azide, stained for CD4 surface markers using anti-CD4-PE-CY Chrome antibodies (BD Pharmingen) and fixed with 4% paraformaldehyde. The fixed cells were permeabilized with a solution of saponin divided into three aliquots and stained for IFN-gamma-FITC, IL-2-PE respectively, using Fluorescent labeled antibodies (BD Pharmingen). The TNF-α released by macrophages was studied using PE labeled antibodies. Isotype control panels (negative control) were run in parallel to all experiments. Unbound antibodies were removed by washing the cells twice with wash buffer before each sample was resuspended in 450 µL stain buffer (containing 0.09% sodium azide) for examination by FACS Calibur.

## **Results**

The studies revealed the presence of TCRVα-11 positive T cells in very high proportion and this was shown particularly in those mice where T cells expressed CD2 in high quantity (Fig. 1-3). Of the total CD4<sup>+</sup> cells 75% were CD2<sup>+</sup> and among the total CD4<sup>+</sup> cells also positive for CD2, only 23% were CD70<sup>+</sup> (Fig. 4). Regression analysis confirmed the  $R^2 = 0.94$  i.e., the values are approaching to 1 at the p value of 0.02 clearly showing the significance of our results. Cytokine profile prior to infection in the VL subjects recorded lower IL-2 production in the sense that approximately 96% of the cells did not produce this cytokine. It was apparent that in the VL subjects where there was only 3.64% of the T cells produced IL-2 there was characteristic downregulation in the production of IFN-γ which was 1.2% only (Fig. 5). This indicated that greater than 98% of T cells were affected adversely by the *Leishmania donovani* parasite at this state of Leishmania dominance did not permit T cells to release IFN-γ, a proinflammatory cytokine which is known to be essentially required by the VL patients to clear the Leishmania parasite from infected macrophages.

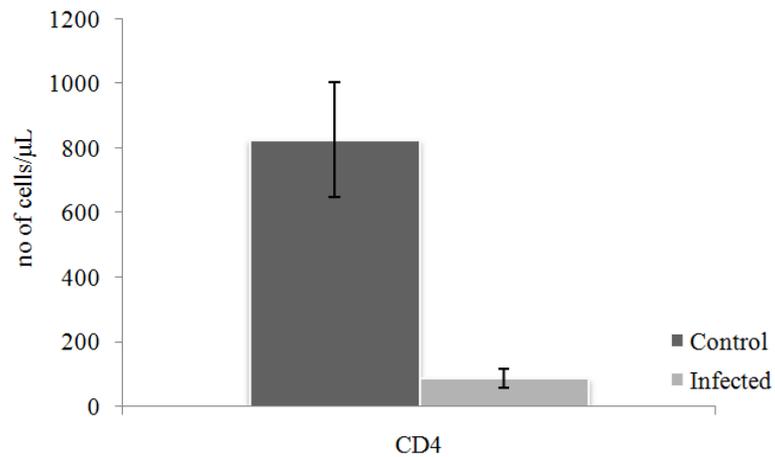


Fig. 1. Quantitative analysis of CD4 cells in healthy and VL induced BALB/c mice. Freshly isolated mononuclear cells were stained with CD4-PE Cy-Chrome and CD8-FITC. Flowdata were analysed using cell quest software of FACS. The means were confirmed with Mann Whitney T test. Comparison was made with  $p < 0.05$

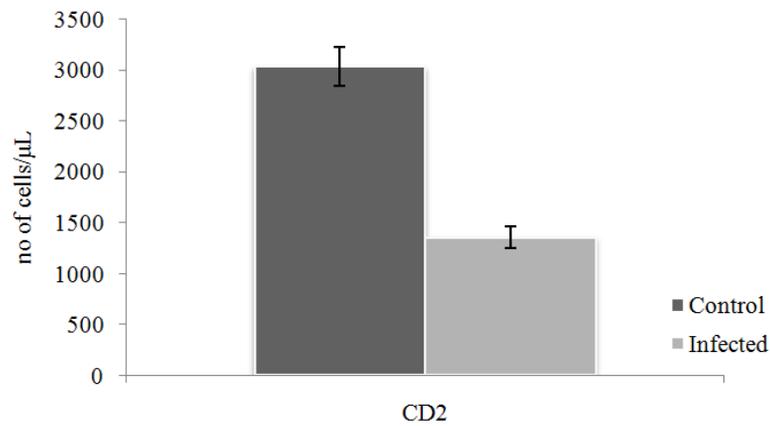


Fig. 2. Mononuclear cells isolated from spleen of *L. donovani* infected and control mice were stained with anti mice CD2 RPE and CD4 PE Cy.5 and acquired on FACS Calibur. Flowdata were analysed using cell quest software of FACS. The means were confirmed with Mann Whitney T test. Comparison was made with  $p < 0.05$

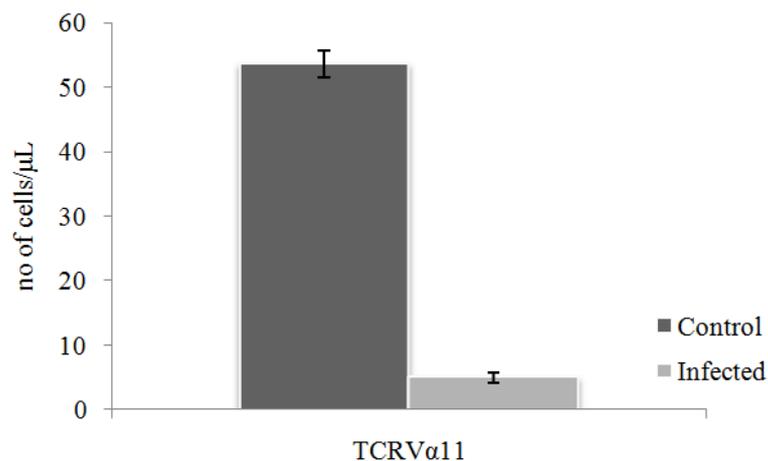


Fig. 3. Mononuclear cells isolated from spleen of *L. donovani* infected and control mice were stained with rat anti mice TCR V $\alpha$ 11 and CD4 PE Cy.5 and acquired on FACS Calibur using Cell quest software. The frequency of cells was analyzed using forward scatter and side scatter histograms

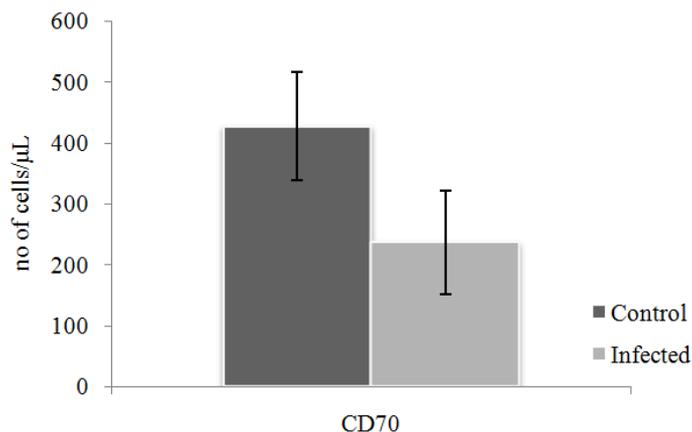


Fig. 4. Mononuclear cells isolated from spleen of *L. donovani* infected and control mice were stained with rat anti mice CD70PE and CD4 PE Cy.5 and acquired on FACS Calibur. Flowdata were analysed using cell quest software of FACS. The means were confirmed with Mann Whitney T test. Comparison was made with  $p < 0.05$

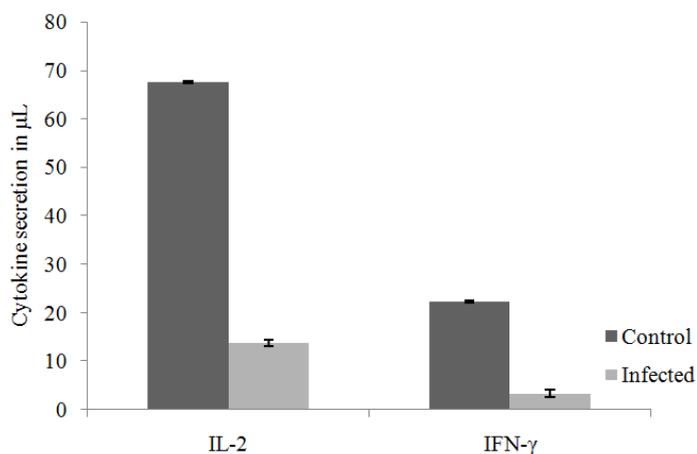


Fig. 5. IFN-γ and IL-2 secretion from CD4 T cells in control and *L. donovani* infected BALB/c mice. CD-4 cells were stained with CD4 PE-CY Chrome. For the evaluation of cytokine profile these were costained using IL-2 (PE-Clone JES6-5H4), IFN-γ (FITC-Clone XMG1.2). Flowdata was analysed using cell quest software of FACS. The means were confirmed with Mann Whitney T test. Comparison was made with  $p < 0.05$

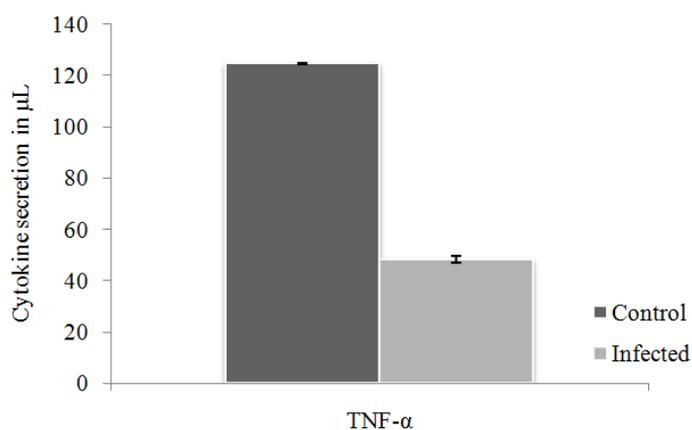


Fig. 6. TNF  $\alpha$  (PE-Clone NP6-XT22) released by the macrophages was studied by using PE labeled antibodies in control and infected BALB/c mice. Flowdata was analysed using cell quest software of FACS. The means were confirmed with Mann Whitney T test. Comparison was made with  $p < 0.05$

When TNF- $\alpha$  pattern was examined it was observed that only 6.75% macrophages in the infection group had produced this cytokine compared to 2.6% in the control subjects (Fig. 6). Approximately 93% of macrophages remain inactivated which might be due to the suppressed activity of CD4 cells arising as a result of subdued IL-2 and IFN- $\gamma$ .

## Discussion

It was shown that TCR V $\alpha$ -11 bearing T cells were mostly those which produced more CD2 on the surface of T lymphocytes. On the other hand lower expression of TCR V $\alpha$ -11 is found to be associated with reduced expression of CD2 on CD4 T cells. Thus most of CD4<sup>+</sup> T cells were inactivated as dictated by a low frequency of CD2<sup>+</sup>CD70<sup>+</sup> cells among the total CD4<sup>+</sup> cells in these subjects and at this stage the total T cells also did not express CD2 in high proportion. The cytokine profile which showed subdued activation of the protective cytokines to counter the infection revealed a state of over all immune suppression which might have originated due to the inactivation of CD4 cells which might be a result of the lowered expression of CD2 and TCR V $\alpha$ -11 under the influence of Leishmania infection. Perhaps a more pivotal role of IL-2 is its ability to induce the production of gamma interferons and tumor necrosis factors. This result is interesting for several reasons. The TCRs with V $\alpha$ -11 are associated with b and d haplotypes. These are mostly present on double negative thymocytes (CD3<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup>) in their last stage of maturation before appearing on DP cells with reduction in IL-2R $\alpha$  in thymus. Reasonably if a cell in PBMNC suspension shows a reduction in the number of such TCRs, one can easily anticipate that there would be a differentiation defect in T cells and may result in the less expression of single positive cells. This also suggests that under certain circumstances, CD2 would probably regulate the differentiation process. Other studies suggest that in absence of CD2, T cell development process is severely disturbed with reduction in DP as well as CD8 SP thymocytes and it has been shown that there is a blockade at CD25<sup>+</sup>CD44<sup>-</sup> to CD25<sup>-</sup> CD44<sup>-</sup> transition, which affects proliferation of the developing thymocytes as well. Taking our data into consideration it appears to have a major decision checkpoint for the maturation and differentiation of DP thymocytes towards SP CD4<sup>+</sup> cells.

## Conclusion

Collectively a critical role of CD2 was identified in maturation steps of CD4<sup>+</sup> T cell differentiation in Visceral Leishmaniasis.

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## Author's Contributions

**Sukrat Sinha:** Designed and performed experiments. Written the manuscript.

**Shanthy Sundaram:** Reviewed the manuscript.

**Sanjiva Bimal:** Designed the experiments, gave critical reviews and helped in writing the manuscript.

**Shubhankar Kumar Singh:** Feedbacks and suggestions.

**Pradeep Das:** Reviewed the manuscript.

## Ethics

No ethical issues are supposed to get originated after the publication of the manuscript.

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