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Leishmania donovani: Role of CD2 on $CD4^+$ T-cell function in Visceral Leishmaniasis

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Abstract

In this study, we investigated whether alteration in the CD2 mediated coordination of an immune response was associated with down regulation of CD4 associated Th1 cell response during Visceral Leishmaniasis (VL). *Leishmania donovani* (*Ld*) infection in VL patients markedly reduced expression of CD2 cell surface antigen on $CD4^+$ cells. T-cells of VL patients were mostly in G0/G1 stage of the cell cycle (98.20%) with little or no activity of protein kinase C-α (PKC-α) isoform. However, pre-incubation with activating anti-CD2 monoclonal antibody (MAb) resulted in a corresponding increase up to 2.52-fold in T-cells of G2/M population supported by both activity and expression of PKC-α isoform. Furthermore, we observed that co-incubation of T-cell with anti-CD2 increased the lymphocyte-blast population in patients in whom the CD4 cells became more antigen responsive ($CD4^+$ $CD69^+$ cells). Consistent with these observations, it was shown that 59.3% of CD4 cells from patients responded to *Ld* by producing IFN-γ. Even in the culture condition, when the T-cells from patients were depleted of APC, IFN-γ production was noticed after CD2 activation. On the other hand, IL-4 production became low in the anti-CD2 antibody supplemented peripheral blood mononuclear cells (PBMNCs) culture. These findings imply that infection with *L. donovani* induces less CD2 on the surface of $CD4^+$ T-cells, which once activated orchestrate the protective IFN-γ dominant host defense mechanism via PKC-mediated signal transduction and cell cycle.

Index Descriptors and Abbreviations: VL, Visceral Leishmaniasis; *Ld*, *Leishmania donovani*; PBMNCs, peripheral blood mononuclear cells; APC, antigen presenting cells; anti-CD2, anti-CD2 antibody; anti-PKC-α, anti-protein kinase C-α; IFN-γ, interferon-gamma; IL-10, interleukin-10; IL-4, interleukin-4; r-IL-4, recombinant interleukin-4; IL-12, interleukin-12; PBS, phosphate buffered saline; FCS, fetal calf serum

1. Introduction

Visceral Leishmaniasis (VL) caused by *Leishmania donovani* (*Ld*) in India is one of the several clinically important infections where Th1 sub-populations of CD4 cells fail to produce interferon-gamma (IFN-γ) which activate macrophages and coordinate the immune response to intracellular *Leishmania* species. On the other hand, expansion of an interleukin-4 (IL-4) and interleukin-10 (IL-10)-biased Th2 response inhibits the immune control *in vivo* and leads to severe disseminated forms of the disease (Ghalib et al., 1993; Babaloo et al., 2001; Uzonna and Bretscher, 2001). Cell surface adhesion molecules of the immunoglobulin super-family-lymphocyte function associated antigen-1 (LFA-1), CD2 and intracellular activation motifs (ICAM-3) can mediate T-cell adhesive interactions which is later extended, through support of co-stimulatory receptor CD28 on T-cells, to enable an immune response to occur (Janeway, 1992; Moingeon et al., 1989; Harris et al., 2000; Pulendran et al., 2001). As a result, antigen responsive lymphocytes are shown to express CD69, an activation marker during *in vitro* antigen stimulation (Schlossman et al., 1995). A study by Gallob et al. (1996) documented a key role of CD2 antigen on T-cells in mediating

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the ability of monocytes to enhance T-cell activation by IL-12 during the ongoing process of antigen presentation by APCs. Several studies have also documented the role of CD2 on T-cells through thymic selection events and signal transduction (Sasada and Reinherz, 2001; Yang and Reinherz, 2001), which promotes proliferating T-cells to transduce efficient signaling to affect IFN-γ production even in the absence of APCs (Meuer et al., 1984; Green et al., 2000; Meinl et al., 2000; Sasada et al., 2002). The role of IFN-γ in forms of leishmaniasis is very important for resistance and cure and we previously reported a deficiency of CD2 cell surface expression on T-cells in VL patients, which we presume might be a critical factor in activation of Th1 response of $CD4^+$ T-cell during VL (Bimal et al., 2005). As the role of CD2 in infectious diseases has been relatively little studied, the present study was undertaken to determine the immuno-modulatory effect of CD2 antigen co-receptor in the reversal of T-cell non-responsiveness to *L. donovani* antigen in acute Visceral Leishmaniasis.

2. Materials and methods

2.1. Subjects

Twenty subjects of both sexes aged between 18 and 27 years (10 patients with acute VL in their pre-treatment stage and 10 controls) were studied. Amastigotes of *L. donovani* had been found in bone marrow and/or spleen aspirates from each of the VL cases investigated and, when blood samples were collected, each of these cases had splenomegaly and had been suffering with continuous or intermittent fever for at least two weeks. The controls had no splenomegaly or recent history of illness. Potential subjects who were unwilling to give their informed consent or were positive for tuberculosis (TB), kidney, heart and liver diseases, malaria and HIV were excluded.

2.2. Phenotype cell analysis

Peripheral blood mononuclear cells (PBMNCs) isolated from blood samples by centrifugation (800*g*, 15 min) over Histopaque®-1077 (Sigma, USA) were washed in phosphate buffered saline (PBS). Cells (1×10^6 in 50 μl) were stained with labeled antibodies specific for different human cell surface antigens (BD Pharmingen, San Diego, CA, USA) for 30 min, according to manufacturer's instructions. The following monoclonal antibodies were used: FITC labeled anti-human CD2 (clone: RPA-2.10), FITC or PE labeled mouse anti-human CD3 (clone: UCHT1) or PE labeled mouse anti-human CD4 (clone: RPA-T4) or FITC labeled anti-human CD8 antibodies (clone: RPA-T8). The un-reacted antibodies were removed by washing the cells twice with PBS containing 2% FCS, pH 7.2 before each sample was re-suspended in 450 μl stain buffer containing 1% freshly prepared formaldehyde (MERCK BDH, India) for examination of fluorescence in a FACS-Calibur Flow-Cytometer (Beckton Dickinson, San Jose, CA, USA). Flow data were evaluated on Cell Quest software. Negative control samples were incubated with irrelevant isotype matched antibodies (FITC and PE labeled IgG; BD Pharmingen, USA) in parallel with all experimental samples.

2.3. Cell cycle studies

Cycle Test™ plus DNA reagent kit (BD Pharmingen, San Diego, CA, USA) was used to compare the progression of T-cells in the cell cycle with and without stimulation with mouse anti-human-LFA-2 (anti-CD2), clone S5.2 (BD Pharmingen, San Diego, CA, USA) after pre-incubation of cells in *L. donovani*. In brief, the PBMNCs suspension (5×10^6 cells) was pulsed with *L. donovani* antigen (10 μg/ml) and cultured in a water saturated air atmosphere with 5% CO_2 at 37 °C for 2 h. Later, T-cells were fractionated from the PBMNCs suspension using nylon wool column. Following washing (400*g*, 5 min at room temperature) cells were treated with 250 μl of a solution containing trypsin in a spermine tetrahydrochloride detergent buffer (kit content, BD Pharmingen, San Diego, CA, USA) for 10 min at RT to digest cell membrane and to stabilize the nuclear chromatin. The cells were later treated with 200 μl of a solution containing trypsin inhibitor and RNase buffer (provided with the kit) for 10 min at RT. These cells were incubated with 200 μl of propidium iodide (PI, provided with the kit) stain solution for 10 min in the dark on ice to allow PI to bound to isolated nuclei. The stained cells were finally run on a FACS-Calibur (BD, San Diego, CA) with FL-2 detector using a 585/42-band pass filter. Finally, the FL-2-A DNA histogram was analyzed using Modfit software (Becton Dickinson) on the FACS-Calibur. An additional sample tube of PBMC mixed with experimental tubes was also prepared and used as a control.

2.4. Expression of PKC-α proteins in T-cells

Leishmania donovani primed PBMNCs (2 h) stimulated in presence or absence of anti-CD2 (clone S5.2) for 16 h (as described above) were fixed with freshly prepared 2% formaldehyde (MERCK BDH, India) at 37 °C for 10 min. Cells were washed and permeablized by cold 90% methanol on ice (30 min). After washing in stain buffer, we performed a co-staining of T-cells (1×10^6) using 20 μl of anti-CD4 PE-conjugated antibody (BD Pharmingen, USA) and 1 μg of mouse IgG 2b anti-PKC-α antibody, Mol. Wt. 82 kDa, clone 3 (BD Pharmingen, USA) and 20 μl mouse anti-human IgG antibodies conjugated to FITC (BD Pharmingen). Washed cells were re-suspended in 500 μl of stain buffer and production of PKC-α by $CD4^+$ T-cells of patients and control samples were determined by flow cytometry. Further, cellular proteins of T-cells of patients and healthy control either stimulated or not stimulated with anti-CD2 (unstimulated) were analyzed by SDS–PAGE and Western blotting for the expression of PKC-α following method as described by Laemmli