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CD8^{dim} but not CD8^{bright} cells positive to CD56 dominantly express KIR and are cytotoxic during visceral leishmaniasis

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ABSTRACT

This study reports a structural and functional heterogeneity of CD8$^+$CD56$^+$NKT cells, which usually decrease quantitatively during visceral leishmaniasis. Based on fluorescence intensity of CD8 receptors on CD56$^+$NKT cells, two populations of CD8$^+$CD56$^+$NKT cells have been identified. These cells were recognized as CD8^{dim}CD56$^+$NKT and CD8^{bright}CD56$^+$NKT cells. We further analyzed the functional nature of CD8^{dim} and CD8^{bright} positive CD56$^+$NKT cells. In comparison to CD8^{bright}CD56$^+$NKT cells, a significantly higher percentage of CD8^{dim}CD56$^+$NKT cells expressed KIR during VL. The percentage of CD8^{dim}CD56$^+$NKT cells expressing KIR was found 4 fold higher in VL as compared to healthy subjects. But, the difference was insignificant in case of CD8^{bright}CD56$^+$NKT cells. CD8$^+$CD56$^+$NKT cells release granzyme B to kill the infected cells. A categorical difference was also observed in the function of CD8^{dim}CD56$^+$NKT and CD8^{bright}CD56$^+$NKT cells during visceral leishmaniasis. The percentage of granzyme B expressing CD8^{dim}CD56$^+$NKT cells was 2.83 fold higher in VL compared to healthy subjects. But, there was no significant difference in granzyme B expressing CD8^{bright}CD56$^+$NKT cells in samples from healthy and VL subjects. However, within VL subject, the percentage of granzyme B expressing CD8^{dim}CD56$^+$NKT cells was 5.7 fold higher in comparison to CD8^{bright}CD56$^+$NKT cells. This study concludes that CD8^{dim}CD56$^+$NKT cells are more cytotoxic than CD8^{bright}CD56$^+$NKT cells during VL.

1. Introduction

Visceral leishmaniasis (VL) is a deadly disease caused by the parasitic protozoan *Leishmania donovani*. India is one of the six countries bearing 90% of total VL in world. Within India, VL is mostly restricted to North-Eastern parts, particularly in Bihar state, which bears about 80% cases. Even after serious efforts of the government as well as various national and international agencies for VL elimination programme, about 5700 cases has been reported last year from this area. It is serious as the VL is fatal if left untreated and the fatality rate can rise 100 percent within two years if left unattended [1]. Increasing rates of resistance to anti-leishmanial drugs are the most challenging issue as there is no vaccine [2]. Further, sterile cure from VL needs immune support, but *Leishmania* down modulates proinflammatory and effector cytokine [3], which results into a gross alteration of Th1/Th2 cytokine ratio and immunosuppression. Natural Killer T (NKT) cells being a connecting link between innate and adaptive immune cells play a vital role in immunoactivation [4]. NKT cells are naturally occurring T-cells that express receptors of both T and Natural Killer (NK) cell [5]. It is a distinctive subset of T cells, which produces both type 1 and type 2 cytokines [6,7]. There are three types of NKT cells: type 1-NKT cells express invariant T cell receptors, which are reactive to CD1d, type 2 NKT cells express semi invariant T Cell Receptor (TCR) and reactive to CD1d restricted glycosphingolipid (GSL) and phospholipid antigen and type 3 NKT cells express CD56 and not restricted to CD1d expressing antigen [8]. Here, we focused our study on type 3 NKT cells positive to CD8 and CD56. Previously, we have reported the protective efficacy of CD8$^+$CD56$^+$NKT cells [9]. Normally, CD8$^+$T memory cell subsets exhibit specific responses based on the expression of Killer Cell Immunoglobulin-like Receptors (KIRs). It is used to discriminate unhealthy or infected cellular subjects from the healthy host cells [10,11]. KIR is also involved in the regulation of natural killer cell cytotoxicity [12]. The presence of KIR receptors on CD8$^+$T cells were referred as KIR$^+$CD8$^+$T cells [13]. KIR$^+$CD8$^+$T cell which is expressing NKG2A, is recognized as an effector cell capable of innate

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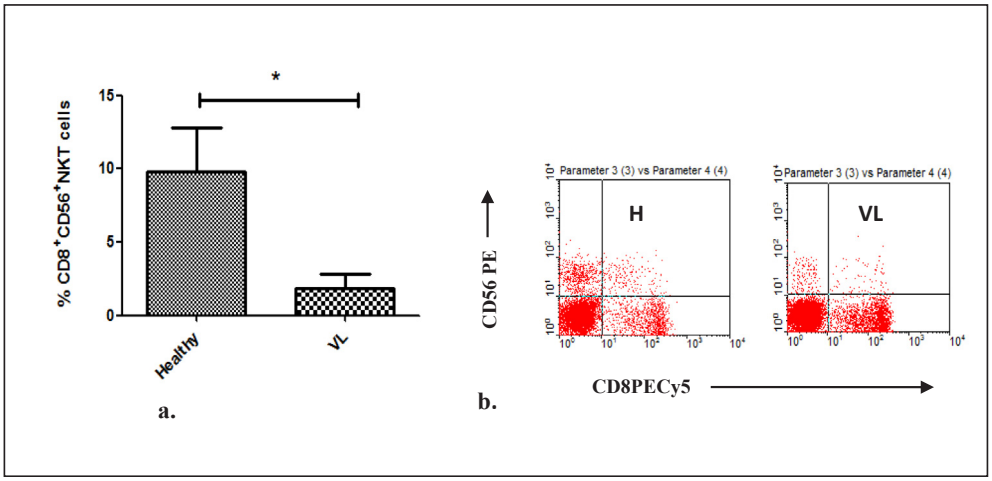

Fig. 1. (a) Flow diagram showing down regulation of $CD8^+CD56^+$ NKT cells in VL as compared to healthy subjects. (b) Comparative bar diagram showing percentage of $CD8^+$ cells expressing CD56 ($CD8^+CD56^+$ NKT cells) in peripheral blood of healthy and VL subjects (n = 8). $^*P < 0.05$.

and adaptive immune functions [14]. Granzymes are serine proteases present in the form of cytotoxic granules. Both the cytotoxic T lymphocytes ($CD8^+$T cells) and NK cells are known for its granzyme producing capacity [15]. Based on the intensity of CD8 expression on T cells, two subsets of $CD8^+$T cells have been identified: $CD8^{bright}$ and $CD8^{dim}$ T lymphocytes. The functional role of dim and bright $CD8^+$T cell is also unclear. Therefore, the objective of the present study was to explore the function of $CD8^{dim}CD56^+$NKT cells and $CD8^{bright}CD56^+$NKT cells during VL.

2. Material and methods

Fifteen healthy and twelve active VL cases were selected for this study. The recommendations outlined in the Helsinki Declaration 1975 and later revised in Edinburgh 2000 on human rights were followed and the ethical approval was acquired from the Ethical Review Committee of ICMR-RMRIMS, Patna (India). Those who were apparently healthy, had no past history of VL and no complain of illness during last six months were selected for collection of healthy blood sample. Those subjects who were positive for HIV, malaria, heart disease, liver disease, asthma, rheumatic arthritis, tuberculosis etc. were excluded from this study. The VL subjects were selected from institute's OPD. For this, VL suspects (having fever for more than two weeks and coming from VL endemic area) were tested using rapid diagnostic kit (rK39 strip test, InBios, India) and the positive cases were further confirmed by microscopic examination of Giemsa stained splenic or bone marrow aspirate smears for presence of *L. donovani* amastigote. Peripheral blood was collected from healthy and VL subjects in Na_2 heparin vacutainer (Becton Dickinson, Gurgaon, India) after getting written informed consent. First of all the samples were prepared for expression of $CD8^+CD56^+$NKT cell subsets from healthy and VL peripheral blood samples. Heparinized peripheral blood (100 µl) was collected into Falcon™ tubes (12 × 75 mm, Becton Dickinson, India). Samples were stained with anti CD56 PE, CD8 PECy5 and prepared for flow cytometry analysis following the protocol used earlier [16]. Briefly red blood corpuscles (RBCs) in sample were lysed using FACS lysis buffer (BD, USA), washed and acquired with a flow cytometer (FACS Calibur™, Becton Dickinson, San Diego, USA) and analyzed using Cell Quest Pro and WINMDI software. For functional nature of the dim and bright population of $CD8^+$NKT cells, expression of KIR and granzyme B was evaluated. For this, Peripheral Blood Mononuclear Cells (PBMCs) were isolated by density gradient centrifugation (800 × *g*, 15 min) of heparinized peripheral blood over Histopaque–1077 (Sigma, USA). The PBMCs were stained with antihuman monoclonal antibodies to CD8 (clone HIT8a), CD56 (clone B159) and KIR (clone DX27) conjugated to PECy5, PE and FITC respectively (procured from BD biosciences, USA). Granzyme B staining was performed using antihuman granzyme B FITC (BD biosciences, USA) antibody, cytofix/cytoperm™ plus kit (BD, USA) and manufacturer's instruction manual.

Statistical analysis was performed and statistical significance was evaluated by two tailed *t*-test.

3. Result

$CD8^+CD56^+$NKT cells were decreased by 5.2 fold in active VL patients as compared to healthy subjects (P = 0.04, Fig. 1a and b). Two populations of dim and bright $CD8^+$ cells were found after optimal use of antibodies. This study explored the functional role of both $CD8^{dim}CD56^+$NKT cells and $CD8^{bright}CD56^+$NKT cells in VL. The expression of KIR was studied on both $CD8^{dim}$ and $CD8^{bright}$ NKT cell population in VL and healthy subjects by flow-cytometry (Fig. 2a) $CD8^{dim}CD56^+$NKT cells shows 4.05 fold higher expression of KIR in VL as compared to healthy subjects (P = 0.0008, Fig. 2b) whereas $CD8^{bright}CD56^+$NKT cells shows 1.58 fold elevated expression of KIR in VL compared to healthy subjects (P = 0.225, Fig. 2c). In comparison to $CD8^{bright}CD56^+$NKT cells, a significantly higher percentage (2.84 fold) of $CD8^{dim}CD56^+$NKT cells expressed KIR during VL (Fig. 2d). Granzyme B productions by $CD8^{dim}$ and $CD8^{bright}$ $CD56^+$NKT cells were examined. Our results shows that $CD8^{dim}CD56^+$ NKT cells from VL patients produced 2.83 fold higher granzyme B in comparison to healthy subjects (P = 0.0079, Fig. 3b). While, there was no significant difference in granzyme B expressing $CD8^{bright}CD56^+$NKT cells from healthy and VL subjects. However, within VL subjects, the percentage of granzyme B expressing $CD8^{dim}CD56^+$NKT cells was 5.7 fold higher in comparison to $CD8^{bright}CD56^+$NKT cells (Fig. 3d).

4. Discussion

$CD8^+CD56^+$NKT cells decrease in peripheral blood during active VL, which is otherwise protective in nature [9]. It was also reported