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Original Research Article

Update: Primary immunodeficiency disorders among north Indian children

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ABSTRACT

Objective: Primary immunodeficiency disorders (PIDs) are a group of genetic abnormalities characterized by defectin one or more constituents of the immune system.This group of disorders are largely undiagnosed and unreported worldwide due to lack of awareness among the medical practitioners,parents as well as lack of state of art diagnostic facilities. Earlier we had reported the distribution pattern of various categories of PID in children of north India; in this report we are appending the data with current findings.

Materials and Methods: In this retrospective study we pooled data from PIDs workup of 706 children with suspected PIDs, below the age of 18Yrs, in the period of May 2017 October 2019. The clinical assessment and presentation of these children was suggestive of PID. The peripheral blood of these children was used for flow cytometry based immunophenotyping of immune cells. PIDs were classified according to the International Union of Immunological Societies' (IUIS) criteria.

Results: A total of 133 (18.38%) children were diagnosed with one or other form of PID with overall median age was 3.25 years (male: 2.3 and female: 4.2Yrs). Chronic infection, persistent diarrhea and retarded growth were the common warning signsin these patients. Combined humoral and cellular immunodeficiency was observed in 32%, phagocytic defect in 23%, antibody defect in 17%, dysregulated innate immunity in 19% and other well defined syndromes in 9% of total diagnosed PID children. Around 15.78% of PID cases were seen in coupleswithconsanguineous marriage, past family history of PID in 20.30% and families with sibling death of unknown cause in 24.06%. The cause of death of the sibling was not known. PID diagnosed children received prophylactic antibiotics and/or antifungals in addition tospecific therapy for the underlying immune deficiency.

Conclusion: The field of PID remainsunexplored worldwide. The awareness in the developed countries is more than that of developing countries like India. The developing countries face several challenges in the diagnosis of PIDs such as awareness among patients and medical practitioners, mostly in the rural settings, lack of sufficient number of tertiary care centres, lack of equipped immunological laboratory to diagnose the disease.

1. Introduction

Primary immunodeficiency disorders (PIDs) are a group of inherited single gene inborn genetic defects of immune system.[1] The product of defective gene is partly or completely missing due to which integrated immune

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system (innate immunity and/or adaptive immunity) are not functioning properly and that ultimately leads to the development of PIDs. More than 300 different types of PIDs are known[2] so far and their numbers are continuously increasing as the awareness amongst medical practitioners as well as among the parents' increases. The diagnosis of deficiency of immune components in early phase of life is more common.[3] However, it can be detected at any age of life.[4] The PIDs make individual more prone to a wide range of infections like: skin infection, intestinal infection, sinopulmonary infection, autoimmunity, inflammation, allergy, malignancy, physical disability, permanent organ damage, or even death etc.[5,6] Most common symptoms of PIDs has been seen in the form of recurrent infections; practitioners often treat the infections while missing the underlying cause.[7] Deficiencies in immunoglobulin (antibody) are the most commonly diagnosed type of PID.[7] PIDs are not uncommon and its prevalence rates are as high as 1:1,200 in general population.[8] In developing countries, the diagnosis of PIDs is often delayed or missed altogether due to high burden of infectious diseases and poor hygiene.[9] A US based assessment indicates that the onset of symptoms and diagnosis of PIDs takes an average of 12.4 years.[10]

The International Union of Immunological Societies (IUIS) Expert Committee categorized PIDs into 9 groups based on the genetic and molecular diagnosis of patients. Of 10 warning signs of PIDs, death of siblings with unknown cause, consanguineous marriage and past family history of PID in the family are the strong predictive indication of any form of PID. The distribution of PIDs in India varies with location (urban/rural), predominant type of marriage and the age of marriage. So there is an urgent need in India for the development of advance facility for diagnosis, awareness in parent for better management of PIDs.

Earlier we had report the distribution of PID since January2014 through December 2016; herein we report the finding between the periods of May 2017 to October 2019 based on flow cytometry based analysis.

2. Materials and Methods

Suspected children showing the well-established clinical and diagnostic feature(s) of PIDs of age 0.3 year to 16 years either in-patient, outpatient (OPD) or referral were enrolled for the diagnosis of the disease in the period of May 2017 to October 2019 in a tertiary care hospital. All suspected children were assessed for any infection (new/old), development of any new sign or symptoms and monitored for growth during each follow-up visit for every 12–16 week.PID was classified according to the IUIS criteria. The diagnosis was established based on characteristic clinical manifestations and corroborated by immunological markers expression using flow cytometry analysis and immunoglobulin levels.

Table 1:

Age (Mean ± SD)	3.25 ± 0.7 (Range; 0.3 – 8.4 years)
Sex (M/F	91/42
Ethnicity	Indian
Family status	
Consanguineous marriage:	21 (15.78%)
Past family history:	27 (20.30%)
Sibling death of unknown:	32 (24.06%)
Cause not known;	80 (60.15%)
Diagnosis	
T cell Deficiency	36
B cell Deficiency	21
NK cell Deficiency	20
Defect in NK cell activity	06
Defect in Leukocyte adhesion (LAD):	28
Defect in phagocytic function (CGD):	27
Antibody defect	41

Number of suspected primary Immunodeficiency (PIDs) cases: 706
Primary Immunodeficiency diagnosed: 133 (18.83%)
Period: May 2017 to October 2019

Laboratory diagnostic investigation included: complete blood cell count (CBC), Differential blood cell count, evaluation of immunoglobulin level (IgG, IgA, IgE and IgM) was done in almost all the cases. Peripheral blood lymphocyte subsets including T cell subsets (CD3, CD4 and CD8), B cells (CD19 and CD20), Leukocyte adhesion markers (CD11a/CD18 and CD11b/CD18), Phagocytic defect (DHR), natural killer cell markers (CD16/CD56), natural killer cell function ($CD16^+$ perforin and $CD56^+$Perforin) was done using flow cytometry as per medical practitioner's evaluation or the clinical manifestation of children observed during physical examination. Some children were also evaluated for IL-17/STAT-3, Btk protein expression and auto lymphocyte proliferative syndrome (ALPS).

We collected 2-3ml peripheral blood from children with suspected PIDs in heparin vials for flow cytometry based immune cell markers analysis. 1-1.5 ml blood in plain vial for the evaluation of immunoglobulin level was also collected. Fluorochrome tagged antihuman monoclonal antibodies were used for staining of PBMCs isolated from the collected blood. The stained cells were acquired in BD LSR Fortessa-X20 and further analysed with the help of flowjo software for the expression of immune cells markers. In suspected phagocytic defect cases, the collected peripheral blood was lysed with using red cell lysis buffer (RCLB) and after that cells were stained with dihydroxy rhodamine-123 (DHR) and further stimulated with phytohemagglutinin-A (5ng/ml) for 10 min. Finally, after washing, these cells were acquired immediately in BD LSR Fortessa-X20and further analysed in flowjo software.

The oxidative function of neutrophil was represented in the form of % neutrophil oxidation index (NOI) = MFI (stimulated cells/unstimulated cells) x 100.

3. Results

During a period of 2 and half year (May 2017 to October 2019), we received 706 suspected PIDs cases at our tertiary care centre from in-patient department, outpatient department (OPD) and referrals from Delhi based hospitals. We observed one or more types of abnormal immune cells or immune function deficiency in 133 (18.83%) children; 91 were boys (68.42%) and remaining 42 (31.57%) were girls out of total 706 evaluated. The median age of those children was 3.25 ± 1.7 (Range; 0.3 – 8.4 years). A large number of patients (approx. 15%) however, presented with severe clinical manifestations before the age of 6 months. The mean duration in delay of diagnosis from the onset of symptoms varied area wise as well as the type of PID. Children with suspected PID from urban areas were diagnosed in approx. 3 years; however, the children from rural areas were diagnosed in approx. 5 years or above. The symptoms for Common variable immunodeficiency (CVID) and Severe combined immunodeficiency disease (SCID) are more manifested so were diagnosed earlier than the other form of PIDs (such as leukocyte deficiency disease, phagocytic defect and NK cell function defect). 21 children (15.78%) diagnosed with PIDs had a family history of consanguinity. Further, 27 children (20.30%) had family history of previous sibling death (Table 1).

Lower respiratory tract infections i.e., pneumonia or pneumonia like symptoms was observed in most of the cases due to which suspected children were treated with multiple cycle of broad antibiotic treatment before confirmed PID diagnosis. Other most common symptoms that were noticed in suspected PID patients were persistentdiarrhoea, failure of thrives. The screening and diagnosis of PID was achieved by analysing the peripheral blood for absence or reduced expression of various immunological markers using specific monoclonal antibody through flow cytometry based assays. The diagnosed PID positive cases were divided into 4 broad categories.

3.1. Combined T and B cell deficiency

We observed 57 (31.84%) suspected PIDs are in this group. This group of patients were deficient for either T or B cell or deficient in both T and B cells. We also observed immature leukocytes ($CD45^+$) with negative for pan T (CD3) and B (CD19) cell markers in the blood of such children. Furthermore, in few cases we have also observed, CD3 double negative ($TCR\alpha\beta^+$ $CD4^-CD8^-$) cells which was indicative of autoimmune lymphoproliferative syndrome (Figure 1).

3.2. Phagocytic cell defect

It was diagnosed by Dihydrorhodamine-123 (DHR) flow cytometry assay. The chronic granulomatous disease (X-linked and carriers) is transmitted from the parents to children. In children with this defective group, the neutrophil oxidative index (NOI) was almost absent or less than 5% compared with the age matched control. Carrier females usually showed mosaic pattern with 2 subsets of neutrophils; one set of neutrophil with normal NOI and another set of neutrophil with reduced/negligible NOI. Leukocyte association deficiency (LAD) was also included in this group which was diagnosed with detection of expression of CD11a, CD11b and CD18 heterodimer expression on leukocytes. We have observed 55 (30.72%) suspected children in this group (Figures 2 and 3).

3.3. Diseases of immune dysregulation

Hemophagocytic lymphohistiocytosis (HLH), Familial hemophagocytic lymphohistiocytosis Type-2 (FHL-2) patients are known for normal degranulation activity (CD107a expression) but reduced NK cell function (perforin and/or granzyme-B expression)(Figures 4 and 5). We observed 26 (14.5%) of suspected PIDs are in this group.

3.4. Antibody deficiency

It was diagnosed by detection of reduced or absence of B cells, reduced expression of serum immunoglobulin (IgG, IgA, IgE and IgM), levels and also the lack of Brutons tyrosin kinase (Btk) protein expression of myeloid cells; these type of patients belong to the group of X-linked agammaglobulinemia (XLA) and we observed 41 (22.9%) suspected PIDs are in this group (Table 1). In some diagnosed children we have also observed the normal level of B cells; however, with significantly low level of immunoglobulin (IgG, IgA and IgM). Further workup revealed class switch memory ($CD19^+$ $CD27^+$ IgD^-) was absent in these children (Table 1).

4. Discussion

Developing countries still face impediments in the proper diagnosis of Primary immunodeficiency diseases, mostly due to dilemma in the clinical physician regarding the disease, lack of state of art facilities required for the diagnosis of disease as well as lack of awareness amongst the parents, and further PIDs sharing the common symptoms with infectious diseases.[9] India is picking up the pace in dealing with PIDs with the advancement and inclusion diagnostic techniques and clinical criteria. More clinicians and parents are being made aware of PIDs.[7]

In the present retrospective study carried out at India's apex hospital, we broadly divided the diagnosed PIDs

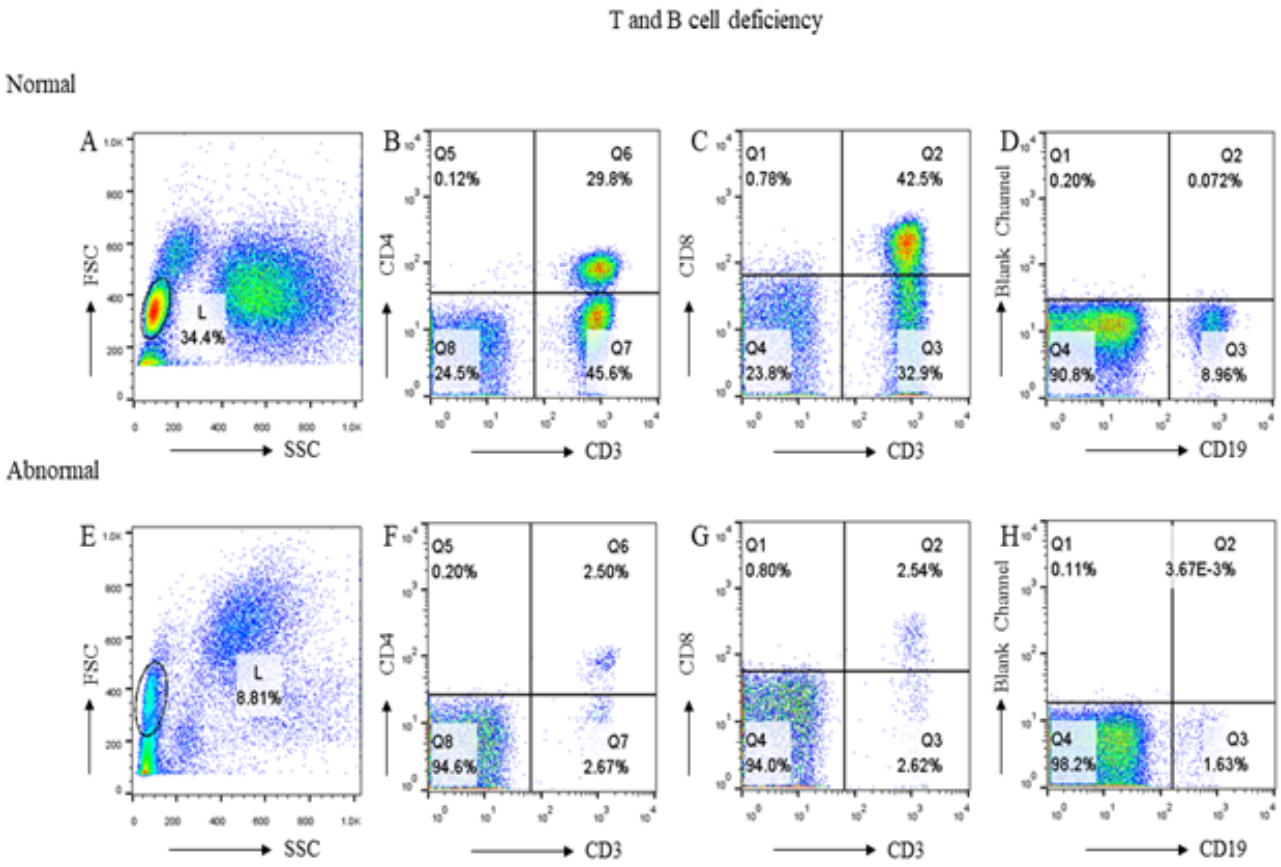

Fig. 1: Representative flow cytometry plots of T and B cell deficiency: PBMCs of age matched healthy control along with suspected PIDs was stained with fluorochrome tagged monoclonal antibodies of pan T cell marker (CD3) and broad T cell subsets (CD4 and CD8) markers and B cell marker (CD19). The upper panel **(A-D)**(Normal) shows normal expression of T and B cell markers in control; the immune markers are significantly deficient or absent in the lower panel (abnormal) insuspected PID sample **(E-F)**

into five categories. As per the IUIS classification of PID, Category-I include Combined T and B cell deficiency or Severe combined immune deficiency (SCID) which basically comprises of heterogeneous pathological conditions. This group of children are highly susceptible to infections. The immune restoring treatment like: bone marrow transplant, gene therapy, enzyme therapy can augment the immunity of this group of children; children without treatment have a shortened life span. More than 80% of SCID infants do not have a family history of the condition. Several genes have been identified to be associated with the development of SCID.[11,12] However, in more than 15% of children with confirmed SCID, the associated genes are unknown. Mostly the inheritance of SCID is recessive autosomal, in which the both copies of particular defective genes from the parents is responsible for the development of disease.[13]

The next highest number of PID category that our centre diagnosed was Phagocytic cell defect. It is Category-V in IUIS PID classification. Chronic granulomatous disease (CGD) is a rare inherited phagocytic defect caused by defect in oxidative burst in neutrophils and monocytes. The disease is caused by mutation in any one or more than one gene which is responsible for NAPDH oxidase complex in phagocytic cells.[13,14] These genes are normally induced during myeloid cell differentiation and are highly expressed in macrophages and neutrophils. The heterodimer ($gp91^{phox}$ and $p22^{phox}$) constitute the catalytic subunit of NADPH oxidase complex. Transcription factor PU.1 binds with ETS site (-57 to - 50) and activate gp91phox gene expression.[14] A single base pair mutation in ETS site interfere in binding of PU.1 to the activation site that leads to failure in expression of catalytic component of NADPH oxidase complex in phagocytic cells.[15] This type of mutation is reported in X-chromosome linked CGD patients. CGD patients have recurring life-threatening bacterial and fungal infections that require hospitalization including pneumonias and lung abscesses.[16]

Subsequent category of PID that we observed in high number was category-III of IUIS, which includes antibody

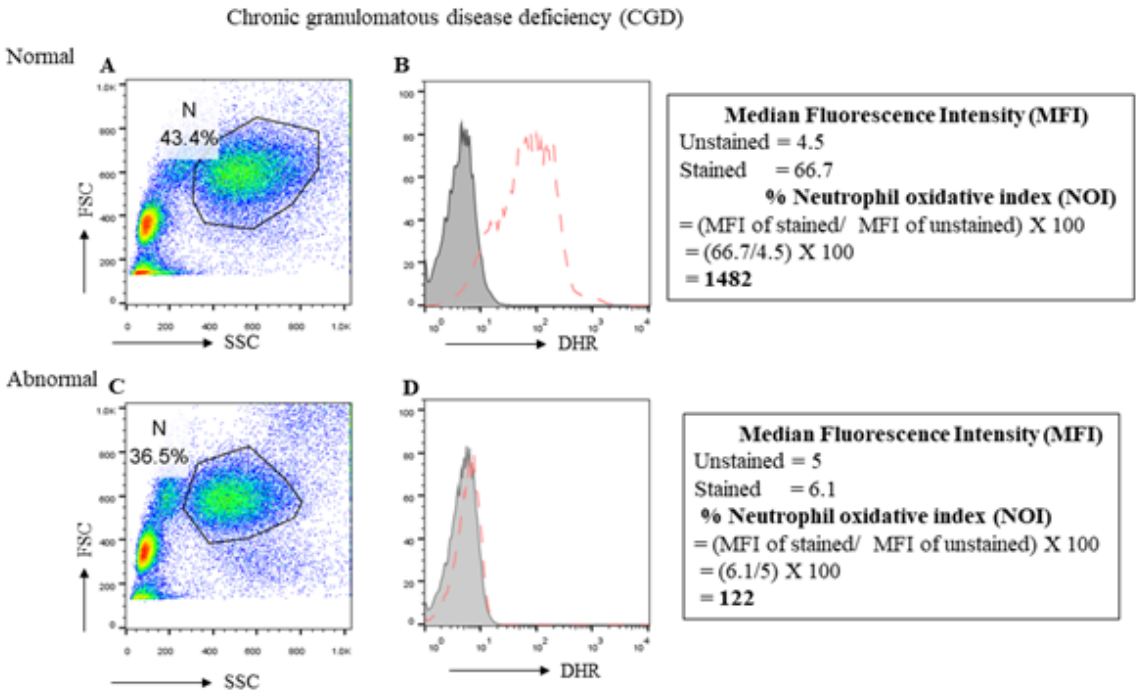

Fig. 2: Representative flow cytometry plots of Chronic granulomatous disease (CGD): The expression capability of reactive nitrogen species (ROS) in neutrophils was evaluated for the diagnosis of CGD. RBC lysedwhole blood were stained with Dihydroxy rhodamine-123 (DHR and were further stimulated with PMA. Stained cells without stimulation were used for comparative analysis. The analysis was expressed in terms of % neutrophil oxidative index (NOI) = MFI of (stained/unstained) X 100. The upper panel (**A-B**) in the figure (Normal) showing the normal expression of reactive oxygen species upon stimulation which was absent or compromised in patient with CGD (lower panel, **C & D**)

deficiency. The prevalence of this type of disease is 1 per 200,000 live birth.[17,18] The most common example of this is X-linked agammaglobulinemia. The appearance of this disease starts once the mother's IgG level starts decreasing usually at the children's' age of 7 to 9 months. In this disease the genetic abnormality interferes in the maturation of B cells in the bone marrow leading to the deficiency of antibody production. Mutation in gene (Btk; Xq21.3-Xq22) is responsible for encoding a cytoplasmic protein tyrosine kinase gene.[19] Btk is a signal transduction molecule required for pre-B-cell receptor (per BCR) and B cell receptor (BCR) development. Mutation in Btk gene results in defective, insufficient or absence of functional enzyme required for the development and maturation of B cell in bone marrow. Thereby children with such mutation have completely absenct B cells and plasma cells (antibody producing cells) due to arrest in maturation beyond pre-B cell stage.

In our retrospective PID workup, the immune dysregulation comprised of 26% of total diagnosed PIDs, lying in category-IV of IUIS classification, which also includes hemophagocytic lymphohistiocytosis (HLH) and autoimmune lymphoproliferative syndrome (ALPS). This condition is often caused by an over reactive abnormal response of immune system. The diagnosis of HLH patients is by observation of elevated level of soluble CD25 (Interleukin-2 receptor) and low or absent natural killer cell activity.[20] At least one gene out six (PRF1, UNC13D, STX11, STXBP2, RAB27, XLP) has been identified for the genetic predisposition of disease.[20]

The present retrospective study again reflects the importance of early detection of primary immunodeficiency diseases among children. The current treatment, especially in developing countries, is still biased towards the infectious disease symptoms. The onus thus lies on the awareness of clinicians and parents. Flow cytometry has proven to be an essential technique in the diagnosis of PIDs. This technique can be reliably used to evaluate the expression of several common immune markers. However, flow cytometry should be complimented with genetic studies to correlate PIDs with mutation of the causal gene. Furthermore, in addition to increasing awareness among clinicians, there is a need for better diagnostic techniques such as NGS and availability of SCIg replacement therapy and HSCT (common in developed countries such as USA) for treatment of PIDs in developing countries.

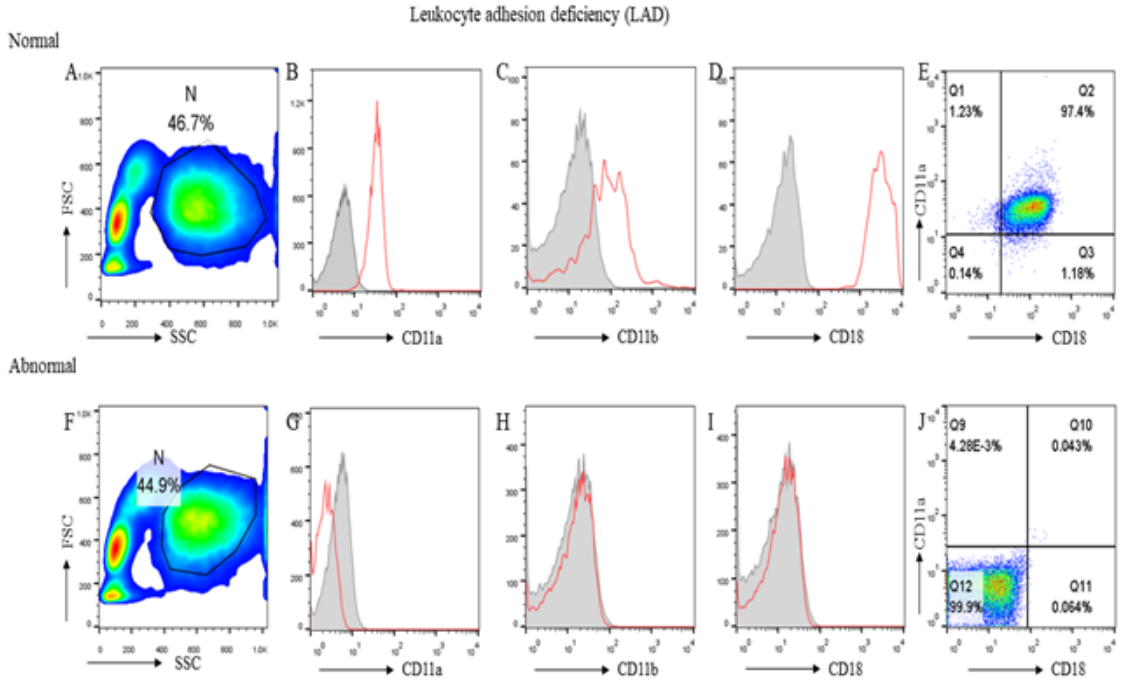

Fig. 3: Representative flow cytometry plots of Leukocyte adhesion deficiency (LAD): RBC lysed whole blood was stained with fluorochrome tagged monoclonal antibodies (CD11a, CD11b, CD18). The upper panel **(A-E)** in the figure (Normal) shows the normal expression of CD11a, CD11b and CD18 (lectin receptors) on neutrophils that was deficient in LAD patient (lower panel: **F- J**)

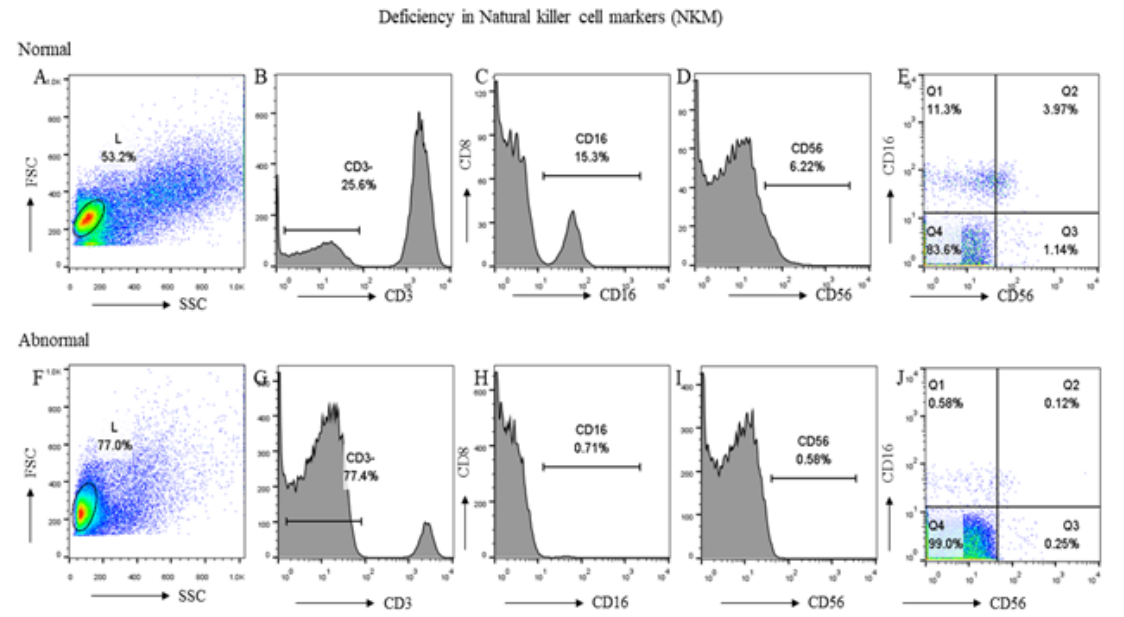

Fig. 4: Representative flow cytometry plots of Natural killer cell markers (NKM): Isolated PBMCs of age matched healthy control along with suspected PID sample was stained with fluoro chrome tagged monoclonal antibodies of CD16 and CD56 (NK cell markers). The upper panel (**A-E**) in the figure shows the normal expression of CD16 and CD56 which was significantly deficient or absent in the lower panel (abnormal) or suspected PID child (**F- J**).

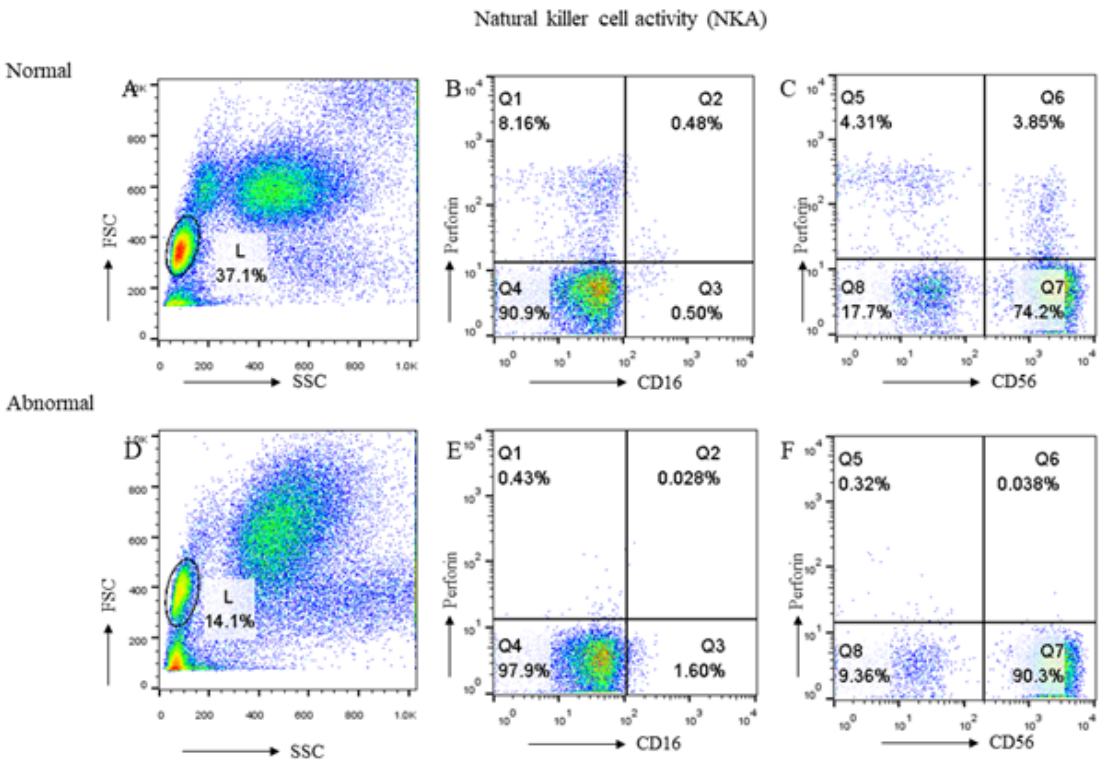

Fig. 5: Representative flow cytometry plots of Natural killer cell activity (NKA): Isolated PBMCs were stained with NK cell markers along with perforin (one of the marker of NK cell function). The upper panel (**A-C**) in the figure shows the normal activity of NK cells (perforin) expression which was significantly deficient or absent in abnormal or suspected PID (lower panel, **D- F**)

5. Abbreviations

PIDs: Primary immunodeficiencies, LAD: Leukocyte adhesion deficiency, DHR: Dihydroxy rhodamine-123. SCID: Severe combined immune deficiency.

6. Source of Funding

None.

7. Conflict of Interest

The authors declare no conflict of interest.

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