Original Article

Flowcytometric Cytokine Profiling of
T- Lymphocytes in Pediatric Aplastic Anemia

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ABSTRACT

Background: Aplastic anemia is a result of complex immune mediated marrow injury. As activated T cells play a pivotal role in immune response, study of cytokine profile in patients of aplasticanaemia (AA) will be of use in understanding the initiation and propagation of the disease.

Methods: Thirty children with AA between ages 6 months to 18 years were included along with age & sex matched controls. Complete blood count (CBC) and bone marrow examination was done. They were grouped as Very severe aplastic anemia (VSAA), Severe aplastic anemia (SAA) and Non severe aplastic anemia (NSAA) as per standard guidelines. Intracytoplasmic expression of Type 1 cytokine (IFNγ) and Type 2 cytokine (IL4) was determined by Flow cytometry (FCM). CD8 and CD4 cell counts were also determined by FCM.

Result: CD8 cell count was higher than the CD4 counts with reversal of CD4:CD8 ratio in all patients. Mean intracytoplasmic IFNγ expression in CD8 and CD4 cells was higher in cases than in controls (p<0.0001) and showed graded positive correlation with the severity of the disease (p<0.0001). Type 2 cytokine (IL4) did not show any such correlation.

Conclusion: IFN gamma expression in T cells can be useful in predicting severity of the disease and also response to immunosuppression.

Keywords: Aplastic Anaemia, Cytokines, Flowcytometry, Immunosuppression, Interferon Gamma

Introduction

Aplastic anemia is a bone marrow failure syndrome, characterized by pancytopenia and hypocellular marrow in the absence of abnormal cellular infiltrate and with no increase in reticulin. [1]

The diagnosis usually requires the presence of pancytopenia with a (2/3 of the following) Neutrophil count less than 1500/µL (1.5×10^9/L), Platelet count less than 50,000/µL (50×10^9/L), Hemoglobin less than 10 g/dL (100 g/L),PLUS *Bone marrow aspirate/biopsy* showing, Marrow cellularity <25% Or 25–50% with <30% residual haemopoietic cells.[1]

Most cases of aplastic anemia are acquired; fewer cases are the result of an inherited disorder, such as Fanconi anemia, Shwachman-Diamond syndrome etc.[2] The acquired causes of aplastic anemia include exposure to chemical agents, drugs, viral infections and whole body irradiation. However in approximately 65 % cases, no cause is identified (idiopathic category). [3]

Most commonly accepted pathogenesis is immune mediated injury to marrow. T-cells are of CD4+ and CD8+ types. They are subdivided on the basis of specific cytokine secretion pattern. Type 1 T-cells secrete IL-2, IFN-gamma and TNF-α whereas type 2 T-cells secretes IL-4, IL-5 and IL-10. [4, 5] The relative balance between type 1 and type 2 cytokines secreted has been implicated in many immune responses such as infections, allergy and autoimmunity. Of these IFN – gamma and TNF-α are known to have suppressive and cytotoxic effects on haematopoietic progenitors. [6]

Dysregulated cytokine production and secretion may play a role in the initiation or propagation of the defect in aplastic anemia. Study of cytokines in Aplastic anemia patients is currently not being done routinely. Cytokine profile of aplastic anemia patients could be useful in planning treatment protocols especially to identify probable non-responders to immunosuppression at an early stage itself.

In the present study we aimed to conduct cytokine profiling of T-lymphocytes and analyze its correlation with severity of the disease.

Materials and Methods

The study included 30 newly diagnosed Aplastic anemia patients (6months to 18 years) and 30 age and sex matched controls. Patients were recruited between November 2012 and March 2014. Diagnosis of aplastic anemia was established by Bone Marrow biopsy and Peripheral Blood

counts according to the criteria of the International Study of Aplastic Anemia and Agranulocytosis Study and severity was classified by the degree of blood count depression.[1] [TABLE 1]

Known cases of aplastic anemia on immunosuppressive treatment were excluded from the study. CD8 and CD4 cell counts were determined by FCM (Beckman Coulter Cytomics FC 500). Intracytoplasmic expression of IFN-gamma and IL-4 in T-cells was determined using FCM.

Statistics: For the comparison between two group means, student T test was applied and for correlation between cytokine levels, Pearson's correlation was used. The statistical software used was SPSS v20. p value less than 0.05 is considered significant.

Result

The mean absolute CD4 and CD8 count was lesser in aplastic anemia patients. [TABLE 2]

In controls CD4: CD8 ratio was 1.2 whereas it was reversed in patients with aplastic anemia CD4: CD8 ratio is 0.65 signifying relatively higher number of circulating CD8 (Cytotoxic T cells). This difference was statistically significant (p<0.0001). [TABLE 2]

Intracytoplasmic cytokine expression in CD8 and CD4 cells was evaluated as percentage of cells expressing the cytokine. IFN γ expression by CD8 cells was higher (11.22%) in aplastic anemia patients, as compared to 1.31% in controls. This difference was statistically significant (p <0.0001).7.17% of CD4 cells expressed IFN γ in aplastic anemia patients, which was significantly higher as compared to 1.40% of CD4 cells in controls (p <0.0001). The healthy controls had higher population of type 1 cytokine-expressing CD4 cells (1.40%) when compared to CD8 cells (1.31%). In contrast, the aplastic anemia patients had a reversal in which the type 1 cytokine-expressing CD8 cells were higher (11.22%) when compared to CD4 (7.17%). [TABLE 3]

Mean intracytoplasmic expression of IFNγ in cells increased with the increase in severity of Aplastic anemia. [TABLE 4]

Lower number (1.66%) CD8 and CD4 (1.39%) cells expressed the Type-2 cytokine (IL4) in aplastic anemia patients compared to controls.[TABLE 5] Although CD4 IL4 and CD8 IL4 values show an increasing trend with severity of aplastic anemia, these differences were not statistically significant. [TABLE 6]

Table 1: Severity of Aplastic Anaemia.

Severe AA	BM cellularity <25%, or 25–50% with <30% residual haemopoietic cells 2/3 of the following: Neutrophil count <0.5 x 10^9/L Platelet count <20 x 10^9/L Reticulocyte count <20 x 10^9/L
Very severe AA	As for severe AA but neutrophils <0.2 x 10^9/L
Non-severe AA	Patients not fulfilling the criteria for severe or very severe aplastic anaemia

Table 2: CD8 & CD4 Absolute Counts (FCM).

	CD8 cells	CD4 cell	CD4:CD8 ratio
CASES	683.40+283.17	444.30+298.73	0.65
CONTROLS	783.63+156.52	942.53+140.41	1.2
P value	<0.0001	<0.0001	<0.0001

Table 3: Cytokines (Flow Cytometry).

	CASES (mean+ SD)	CONTROLS	p value
IFN GAMMA CD8 (%)	11.22 + 6.53	1.31 + 1.01	p <0.0001
IFN GAMMA CD4 (%)	7.17+ 5.61	1.40 + 0.79	p <0.0001

Table4: Cytokine Expression (CD8 & CD4 IFNγ) and Severity

CYTOKINE		CLINICAL SEVERITY		
		NSAA	SAA	VSAA
CD8 IFNγ	Mean	3.96	9.57	17.91
	Min-Max	1.81-6.09	6.66-12.48	15.45-20.50
CD4 IFNγ	Mean	2.79	6.07	11.36
	Min-Max	2.10-3.46	4.79-7.35	9.49-13.20

Table 5: CD8 and CD4 IL4 (Flow Cytometry).

	CASES	CONTROLS	p value
IL4 CD8 (%)	1.66 ± 1.00	1.73 ± 1.07	p = 0.005
IL4 CD4 (%)	1.39 ± 1.03	2.32 ± 1.11	p = 0.001

Table 6: Cytokine Expression (CD8 & CD4 IL4) and Severity.

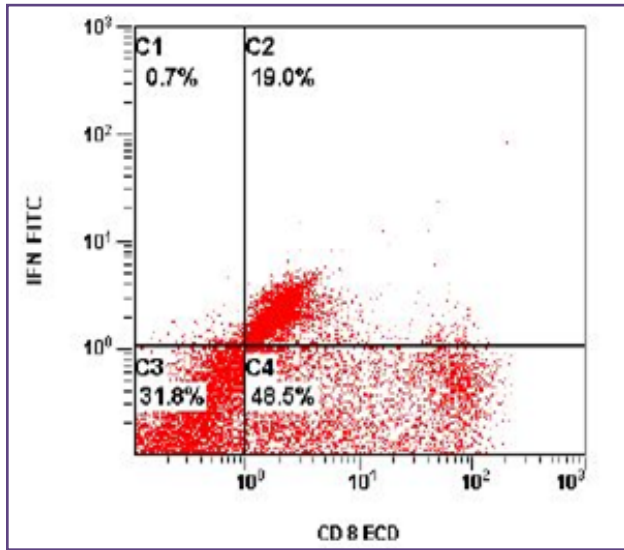| CYTOKINE		CLINICAL SEVERITY		
		NSAA	SAA	VSAA
CD8 IL4	Mean	1.23	1.66	1.91
	Min-Max	0.39-2.07	1.13-2.19	1.04-2.77
CD4 IL4	Mean	1.23	1.24	1.71
	Min-Max	0.39-2.07	0.72-1.74	0.57-2.70

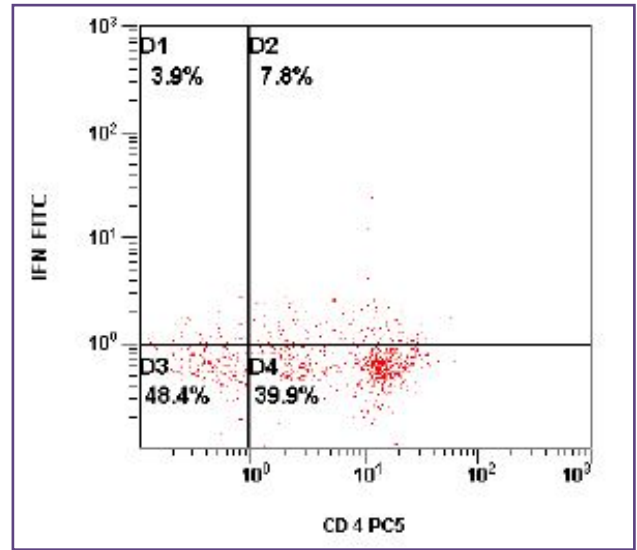**Fig. 1: 19% of CD8T- CELLS express IFN GAMMA (Flowcytometry).**

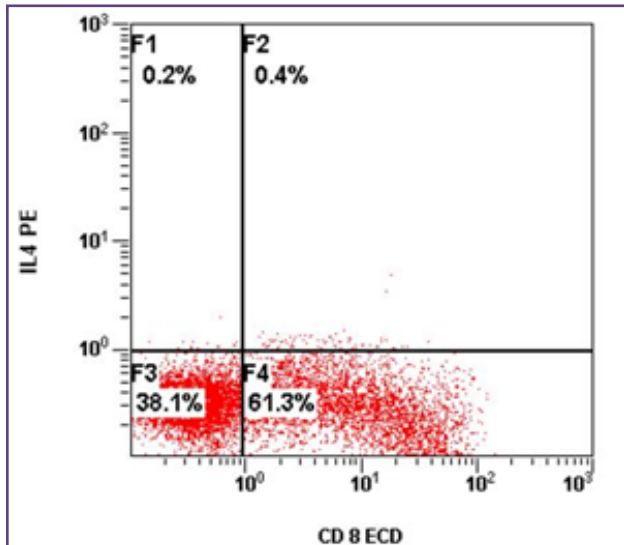**Fig. 2: Flow cytometry showing 7.8% of CD 4 T cells expressing IFN gamma.**

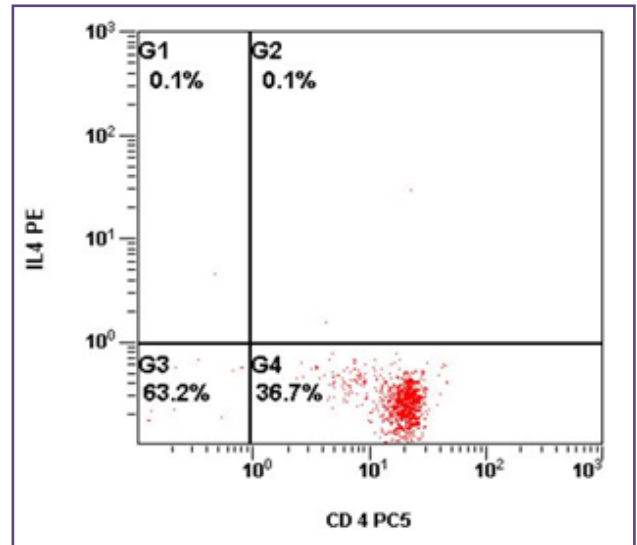**Fig. 3: Illustrates that 0.4% CD 8 T cells express IL4.**

Fig. 4: Illustrates that 0.1% of CD4 T cells express IL4.

Discussion

Aplastic anemia is defined as pancytopenia with hypocellular marrow in the absence of abnormal cellular infiltrate and without increase in reticulin. The bone marrow is replaced by fat as seen in biopsy specimen. The exact pathogenesis of aplastic anemia is still not completely understood. The response to immunosuppression implies that, an immune-mediated attack on bone marrow hematopoietic stem cell (HSC) could be the underlying pathogenic mechanism. But exact role of immune cells and cytokines in this immunological attack is not well understood. Certain patients do not respond to immunosuppressive therapy; in those, mechanisms other than immune-attack might play a role.

Currently, immunosuppression is the mainstay therapy for Aplastic Anemia. Yet, 30% of Aplastic Anemia patients fail immunosuppressive treatments. Reliable pre-treatment predictors of response are not currently available. Overproduction of IFN-γ by peripheral mononuclear cells (MNC) has been noted in patients with aplastic anemia.[6] Some studies have proposed predictive tests for response to immunosuppression such as, IFNγ mRNA measurements of marrow cells, the detection of ill defined marrow cells by flow cytometry, measurement of early progenitor cell numbers etc.,

Cytokines and chemokines are soluble low-molecular-weight proteins secreted by immune cells that mediate inflammatory responses and regulate hematopoiesis by modulating bone marrow microenvironment. Some are essential for the viability, proliferation and differentiation of hematopoietic stem cells.[7]

It was recognized that CD4+ and CD8+ Tcells can be subdivided based on specific patterns of cytokine secretion. Type I T-cells primarily secrete interleukin-2 (IL-2) and interferon γ (IFN-γ), whereas type II T- cells secrete IL-4, IL-5, and IL-10.

Cytokine-profiling holds key to identify possible responders/non-responders to immunosuppressive therapy. In our study, IFN gamma levels were elevated in all aplastic anemia patients (IFN gamma Levels also correlated with severity of the disease).

IFNγ is a 40-70 kDa homodimeric, 143-amino-acid protein mapped to chromosome 12. T cells and NK cells are the only known sources of IFNγ. T cells produce it in response to IL-2 or in presence of activated macrophages. In vitro, IFNγ is a strong stimulator of macrophages and strongly enhances their tumoricidal activity. It stimulates the release of other negative regulators like tumor necrosis factor and lymphotoxin. It enhances the susceptibility of cells to attack by cytotoxic lymphocytes by increasing major histocompatibility gene expression. In addition, it regulates antibody production by B cells and stimulates Th1 cells.[8]

In the year 2010 Xingmin Feng et al studied the levels of cytokines in 33 severe aplastic anemia patients and 57 MDS and 48 healthy controls, by immune-bead based multiplex. The following cytokines and growth factors were tested: CD40 ligand(CD40L), chemokine (C-X-C motif), ligand 5 (CXCL5), CXCL10,CXCL11, thrombopoietin (Tpo), TNF-α, G-CSF, epidermal growth factor (EGF), hepatocyte growth factor (HGF), chemokine(C-C motif), ligand 2 (CCL2), CCL3, CCL4, CCL5, CCL11, fibroblast growth factor basic (FGFb), GM-CSF, interferon-γ (INF-γ), IL-1α, IL-1b, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17, vascular endothelial growth factor(VEGF), and leptin . They found that aplastic anemia patients had a much higher level of Tpo and lower levels of CCL3, CXCL11, CCL5, CD40L, CCL11, TNFα, IL-1ra, IL-6, CXCL5, VEGF,and CCL4 than the patients with MDS. But IFN gamma and IL4 were undetectable.[9]

Aplastic anemia patients in our study expressed IFN gamma in higher percentage of CD8 cells (11.22%) as compared to controls (1.31%). The difference was statistically significant (p <0.0001). Similarly, expression of IFN gamma in aplastic anemia patients was found in 7.17% of CD4 cells, as compared to 1.40% of CD4 cells in controls (p <0.0001). The CD8 and CD4 cells expressing IFNγ had a positive correlation with severity of aplastic anemia (p <0.0001). The T – helper (Th1) subset of CD8 had higher expression of IFN gamma (11.22%) as compared to CD4 (7.17%) in patients with aplastic anemia (p<0.0001) in contrast to controls (CD4 1.40% and CD8 1.31%).

Our results are comparable to the results published by Tsuda H et al in 2000. They studied the IFN γ(Th 1) and IL4 (Th 2) levels in peripheral blood of 9 aplastic anemia patients. They found that the percentage of IFNγ positive CD4 cells, so-called Th1 cells(26.31% ±7.27%) and IFN-γ positive CD8 cells (Tc1 cells) (67.51% ± 16.71%) were significantly higher in AA than in normal controls (20.55% ± 7.64%).[10]

Our results were in contrast to the results published by Carlo Dufour et al in 2001. They analyzed the intracytoplasmic expression of IFN-γ and TNF alpha in bone marrow and peripheral blood of CD4 T and CD8 T cells by flow cytometry. They found that marrow (CD4 and CD8) T cells expressing IFNγ and TNF-alpha were significantly higher in the patients than in normal control subjects (p <0.05 to <0.0001 in the different cellular subsets). In their study the percentages of (CD4 and CD8) T cells in peripheral blood (PB) containing IFNγ and TNF-alpha from the patients

were comparable with those seen in PB from normal control subjects and were significantly lower than those found in the marrow of the same patients[11]

In a study by Soland E et al., most responding patients showed a marked decrease or disappearance of IFN-gamma cells with hematologic improvement. The detection of intracellular IFNγ by Flowcytometry is a powerful predictor of response to immunosuppression. In some patients whose blood was assayed serially, increased intracellular staining for IFN-g preceded a relapse in blood counts by weeks. [12]

Repeated unsuccessful attempts of immunosuppressive therapy in patients without increase IFN-γ levels can be avoided and referred for early marrow transplantation. Intracellular IFN-γ study using Flow cytometry could be done routinely in management of patients with Aplastic anemia for prognostic and therapeutic decisions. Large scale studies with more sample size are required to find if measurement of intracellular cytokines would have such prognostic utility.

Conclusion
CD8 and CD4 cell population expressing IFN γ, correlates with severity of aplastic anemia. Flow cytometry is a rapid tool to assess IFN γ expression, which could be useful in predicting response to immunosuppression.

Competing Interests
Nil

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