Original Article

Importance of IL-10 and IL-17 cytokines in human asthma as studied by ELISPOT

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ABSTRACT

Background: ELISPOT assays are important part of many basic immune monitoring research and clinical trials. Detection of cytokines and other functional assays can be used to evaluate an immune response. **Aim**: The low frequencies of the consumption of specific T cells and cellular receptor hamper the detection of cytokines secreted by ex vivo-stimulated peripheral blood mononuclear cells (PBMNC) by ELISA. Hence it would be ideal to study the cytokines by Elispot assay which shows the actual number of cells secreting the cytokine. **Methods:** IL-10 and IL-17 cytokines in 12 asthmatic patients were studied and compared to 12 healthy controls by ELISPOT assay. **Results:** Significant difference in IL-10 cytokines by elispot on stimulation by mitogen was observed, but no difference in IL-17 cytokine seen.There was spontaneous production of IL-17 cytokines in asthmatic patients. Increase in IL-10 cytokines seen only after the trigger of and by the allergens that may act as mitogens. **Conclusion:** ELISPOT gives information about how much cytokine these cells secreted, but no quantitative cytokine concentrations.

Key words: Allergy, asthma, Elispot, interleukins, T-cell cytokines

INTRODUCTION

Asthma is a major public health problem, affecting 300 million people worldwide, and has increased considerably in prevalence over the past three decades, particularly in Western countries.[1] It has been clearly shown that asthma is caused by multiple environmental factors associated with several major and minor susceptibility genes,[2] which can give origin to many different forms or phenotypes of disease period.[3] These phenotypes include allergic asthma, the most common form of the disease, severe steroid-resistant asthma,[4] asthma induced by air pollutants,[5] obesity,[6] aspirin, and exercise.[7] Cumulative evidence suggests that Th2 cytokines play important roles in inducing eosinophilic airway inflammation and bronchial hyper-responsiveness (BHR) characteristic of asthma.[8] Expression of Th2 cytokines is increased in allergic inflammation and asthma.[9] These atopic allergies are characterized by an immunological

overreaction (hypersensitivity) to common components in our environment such as pollen, animal hair, certain types of food.[10,11] The allergic reaction is initiated via the interaction of a particular allergen with IgE antibodies that in turn, triggers the release of pro-inflammatory mediators from IgE receptor bearing mast cells and basophils, IgE antibodies are normally found at low concentrations but are increased in allergic individuals and the detection of allergen-specific IgE antibodies can serve as diagnostic tools.[10,11] Besides, the T-cells are of great importance. IL-4 is the prototype cytokine and T cells produce IL-4, IL-5 as well as IL-13 and can be easily detected by Elispot.[10,11] Due to its high sensitivity and easy performance, the Elispot assay has proven to be an extremely useful method for measuring antigen-specific T-cell responses in a variety of situations.[12]

There are some cytokines such as IL-10 of the Th2 and IL-17 of the Th series whose role has not yet being established.[13] Interleukin-10 (IL-10) is a cytokine derived from CD4+ T-helper type 2 (TH2) cells identified as a suppressor of cytokines from T-helper type 1(TH1) cells.[13] Its role in the pathogenesis of asthma has not been well documented. Some evidence suggests that IL-10 production is reduced in patients with asthma compared with non-asthmatic control subjects and murine studies provide evidence that IL-10 suppresses development of eosinophilic inflammation in the airways and knockout of the IL-10 gene augments allergen-induced eosinophilic airway inflammation.[13] Nevertheless, the role of IL-10 in the pathogenesis of BHR is not known.

Little or none is known about the role of Th-17 in lung disease. It is known that IL-17 is increased in BAL fluid, sputum and blood from patients with asthma.[14,15] The role of IL-17 in asthma is an area of intense current investigation. As the role of IL-17 in neutrophil recruitment to the airways is well known, in the last years several studies tried to find an association between Th17 lymphocytes and asthma.[16] Identifications of these cytokines by elispot opens up a new methodology in research.[17] The enzyme-linked immunospot or ELISPOT assay allows the detection of low frequencies of cells secreting various molecules.[17,18] ELISPOT can be used in many areas of research and, because of its high sensitivity, has the potential to become a valuable diagnostic tool.[18] Based on the same "sandwich" immunochemical principles as enzyme-linked immunosorbent assay, ELISPOT is easy to perform and quantify the results.[18,19] At the same time, ELISPOT remains a state-of-the-art technique that requires accuracy, thorough selection of antibodies and detection reagents, and an understanding of the principles of data analysis.[18]

ELISPOT is a sensitive method. ELISPOT allows for detection of a single cell that secrets a protein of interest (cytokine, effector protein, receptor, surface marker, antibody) among 1,000,000 cells in the culture.[18,19] In ELISA, detection of proteins secreted by a single cell is impossible.[18,19] Sometimes, cytokine production by hundreds of stimulated cells remains undetected because of the insufficient sensitivity of ELISA and/or the ongoing consumption of the cytokine by cultured cells.[20] In many situations, detection of interleukin 2 (IL-2) in cell cultures with ELISA appears impossible, because cultured cells instantly consume the secreted IL-2. In contrast to ELISA, ELISPOT is not affected by cytokine consumption, and thus very well suitable for the detection of cells secreting IL-2.[21] Elispot measures the frequencies of antigen specific T-cells within freshly isolated cell material such as peripheral blood mononuclear cells (PBMNC). It measures the quality of T-cell immunity of a single cell.[14]

METHODOLOGY

The study group consisted of 12 patients of asthma who attended the OPD of our private hospital. They were carefully examined by our Chief Allergologist and the symptoms suggestive of asthma were noted. These patients were off treatment during the study. Immunological proforma was filled and details regarding name, age, gender and duration of symptoms were recorded. A detailed history about the type, duration of symptoms, and frequency of exacerbations was taken for each patient. The questionnaire including respiratory symptoms, previous medical history, and smoking habits was noted.

Age and sex-matched 12 healthy volunteers were selected as controls. Exclusion criteria

included diabetes, smoking and any heart problems, parasitic infections, tuberculosis and other lung diseases.

This was a pilot project for initial standardization of ELISPOT assay for our main project that was approved by the Institutional Scientific Advisory and Ethical Committee with the consent taken from the patients before collection of the blood.

Blood sample

Blood samples were collected in plain tubes without any anti-coagulant for the separation of serum via centrifugation. The serum was stored in eppendorf tubes at -70^0C until ELISA was performed for the detection of Total IgE.

Total and differential count

Mediators influencing bronchial asthma such as T-cells, eosinophils, mast cells, basophils mononuclear phagocytes and platelets were determined. Total and differential count was done in all the patients using Wright's stain for differential count.

Total IgE in serum by Elisa

Serum IgE levels were estimated in all the subjects taken for our study using kits from RADIM diagnostics (Italy). The standard protocol as mentioned in the kits was thus followed for the quantitation of IgE in IU/ml.[22]

Cell Isolation and cultures

Peripheral blood mononuclear cells (PBMNC) were separated from heparinised blood with density gradient separation by overlaying blood diluted in normal physiological saline in equal proportions on Histopaque (Sigma density of 1.077) in conical tubes and centrifuging the same at 400g (1520rpm) for 20 minutes.[22] Cells separated were then suspended at 5-10 x 10^6cells per tube and then cultured with and without PHA for 24 hrs. These cells were then used for the Elispot assay.

IL-10 and IL-17 detection by Elispot

All the wells in the microplate were filled with 200ul of sterile culture media and incubated for approximately 20 minutes at RT. When the cells were ready to be plated culture media was aspirated from the wells. 100 ul of appropriate cells from patients or controls added to each wells in duplicates. Cells were incubated in humidified 37^0C in CO_2 incubator for overnight (12-16 hrs). Optimal temperature for incubation was standardized. Cells were not disturbed during incubation period. Each well was aspirated and washed, the procedure was repeated for 3-4 times for total of 4 washes. The plate was cleared of excess wash buffer by tapping it upside down and 100ul of diluted antibody (specific for IL-10 or IL-17) was added into each well and incubated at $2-8^0$C overnight. This was followed by 4 washes to remove the excess detection antibody. To this was added 100ul of diluted Streptavidin –Alkaline Peroxidase conjugate into each well and incubate for 1 hr at RT. This was followed by 4 washes with buffer and then 100ul of BCIP/NBT chromogen added into each well and incubated for 1 hr at RT in dark to prevent exposure to light. After incubation, the chromogen was removed by rinsing the microplate with water. Inverted and tap for the excess water and allowed to dry at 37^oC for 30 minutes

Calculation of results

The development of microplate was analysed by counting spots on a Elispot reader (CTL-Corporation, USA) and read as number of spots per well. Thus quantitation of results can be done on CTL Elispot reader.

Statistical analysis

SPSS package of version 16 was used for analysis of all the samples and statistical significance was calculated. Pearson correlation was also used.

RESULTS

The study involved 12 asthmatic patients between the age of 30-70 yrs and 12 healthy individuals taken as controls between the ages of 23-55 yrs. as represented in Table 1. The ratio of male to female was 3:9 in patients and 10:2 in controls.

Table 1: Age-sex pattern of subjects

Subject	Age Mean +(Range) yrs	Male:Female
Patient (N=12)	48.83 ± 2.86 (30-70)	3:9
Controls (N=12)	36.75 ± 3.64 (23 – 55)	10:2

Table 2: Analysis of differential count in asthmatic and controls

Subject (N=12)	Neutrophils%	Eosinophils%	Lymphocytes%	Basophils%	Monocytes%
Patient	54.5 + 5.28	8.75 + 1.76	35.75 + 5.02	0.00 + 0.00	2.75+ 2.49
Controls	59.41 + 8.08	3.00 + 1.41	35.41 + 2.90	0.00 + 0.00	0.58+ 0.51
P value	0.052	0.001 *	0.37	1.00	0.001*

Table 3: Comparison of total IgE in asthmatic and controls

Parameter	Patient (N=12)	Controls (N=12)	*P*-value
IgEIU/ml	1070.64 ±314.44	102.42 ±35.06	0.001 *

Table 4: IL-10 levels by Elispot

Stimulated/Unstimulated	Patients(N=6)	Controls(N=6)	P value
Unstimulated (Mean ± SE)	0.00 ±0.00	2.25 ±1.46	0.071
Stimulated (Mean ± SE)	143.00±162.38	87.00 ± 36.79	0.033*

* *P*<0.05

Table 5: Statistical analysis of IL-17 by Elispot

Stimulated/Unstimulated	Patients(N=6)	Controls(N=6)	P value
Unstimulated (Mean ± SE)	246.25 ±42.75	111.38 ± 17.90	0.05*
Stimulated (Mean ± SE)	488.50 ± 61.84	187.96 ±19.06	0.01**

Table 5: Statistical analysis of IL-17 by Elispot
**P*<0.05, ** *P*<0.01

Table 6: Correlation analysis of IL-10 in patients and controls

IL-10	Patients (N=6)	Pearson's Correlation significance (P)	Controls (N=6)	Pearson's Correlation significance (P)
Eosinophils	8.67 ± 2.16	0.76	3.17 ± 1.47	0.048
Neutrophils	54.5 ± 5.09	0.59	60.83 ± 5.04	0.13
IgE	1470.83 ± 1243.10	0.79	94.13 ± 36.78	0.88

Table 7: Correlation analysis of IL-17 in patients and controls

IL17	Patients (N=6)	Pearson's Correlation significance (P)	Controls (N=6)	Pearson's Correlation significance (P)
Eosinophils	8.67 ± 2.16	0.374	3.17 ± 1.47	0.596
Neutrophils	54.5 ± 5.09	0.253	60.83 ± 5.04	0.938
IgE	1470.83 ± 1243.10	0.246	94.13 ± 36.78	0.996

IL-10	Stimulated	Un-stimulated
Patient		
Control		
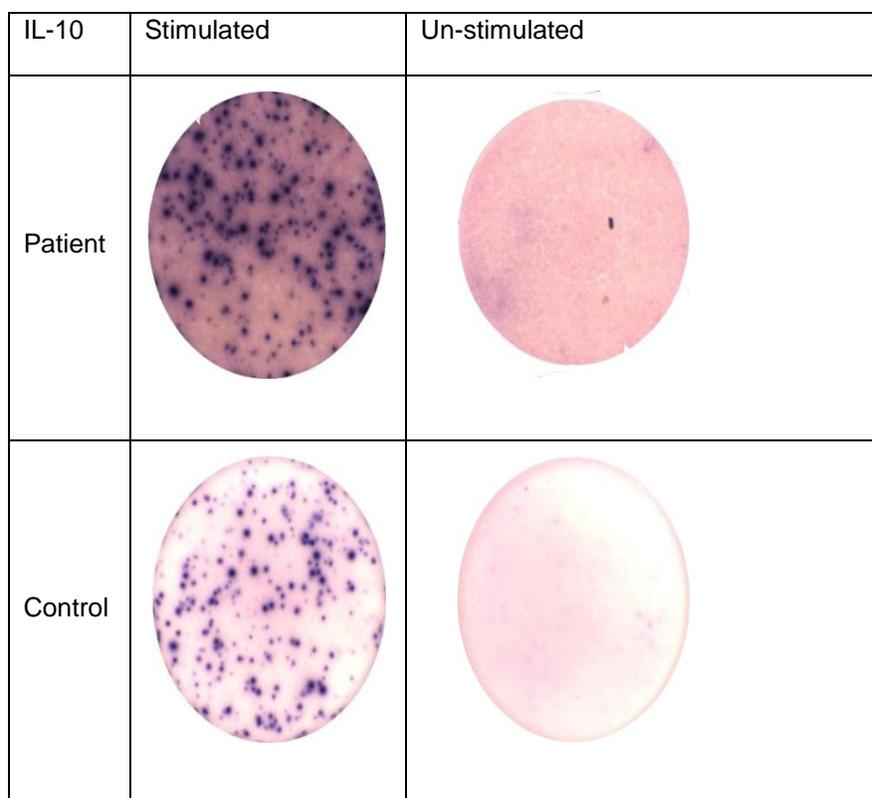
Figure 1: Elispots for IL-10 cytokine in asthmatic patient and control

| IL17	Stimulated	Un-stimulated
Patient		
Control		
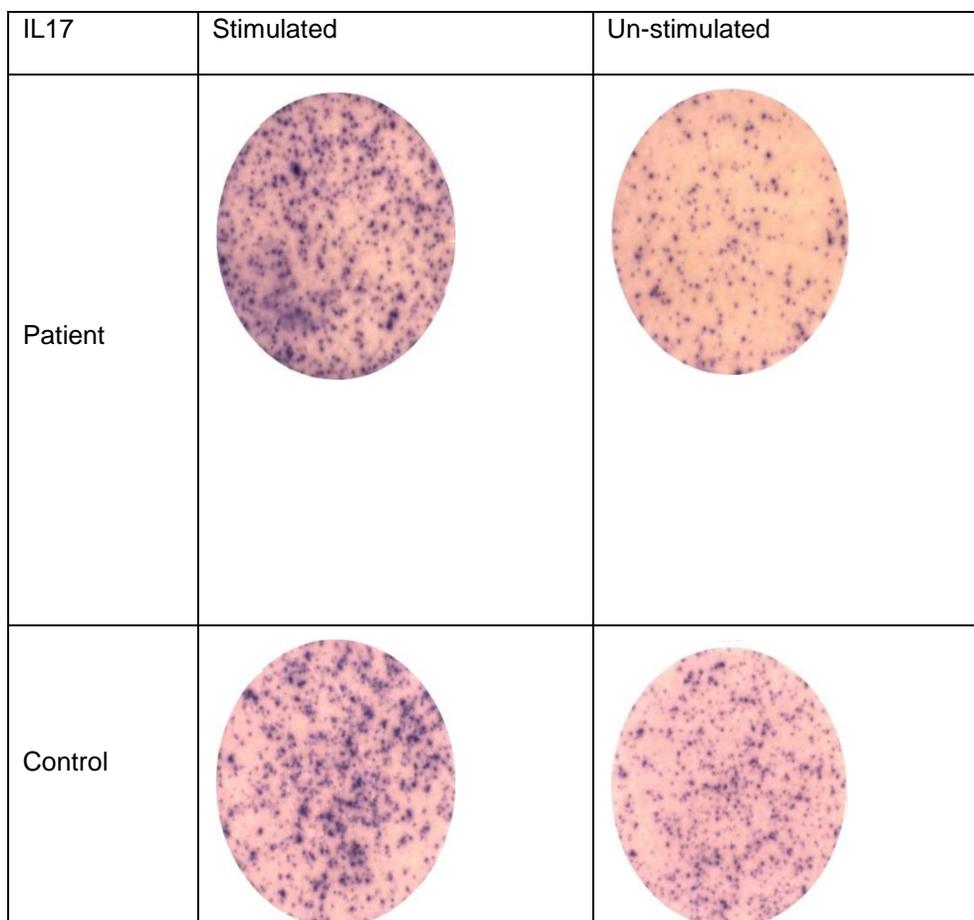
Figure 2: Elispots for IL-17 cytokine in asthmatic patient and control

Table 2 gives the analysis of differential count in asthmatic and control subjects. Percentage of different cells in the peripheral blood was performed by differential count and we observed significant increase in eosinophil percentage in asthmatic than in controls. Similarly, the percentage of monocytes in patients' blood was also found to be significant.

Table 3 shows the results of total IgE in serum performed by Elisa. Total IgE in the patients being 1070±314I U/ml while that in healthy controls being 102 ±35 IU/ml. This is found to be highly significant showing allergic conditions.

IL-10 and IL-17 cytokines were studied by Elispot assay. Table 4 and 5 give the results of the two cytokines taken for our study. These subjects were divided into stimulated and unstimulated using mitogen PHA for our stimulation. We were able to get results in 6 patients and 6 healthy controls. There was significant difference in IL-10 cytokines by elispot only when the cells were stimulated by mitogen. While when the cells were stimulated or without stimulation there was significant difference in IL-17 cytokine by elispot. We tried to correlate our results with the percentage of eosinophils, neutrophils and total IgE in patients and controls as shown in Table 6, 7 for these six patients. We did not get significant correlation of the cytokines with respect to eosinophils, neutrophils and total IgE.

Figures 1 and 2 show the diagrammatic representation of the elispot results for IL-10 and IL-17. There is clear difference in the number of spots in stimulated and unstimulated cells in patients and controls.

The spot size corresponds to the amount of IL-10/IL-17 secreted by individual cells. Thus, the mean distribution of spot size was used as a means to semi-quantitatively estimate
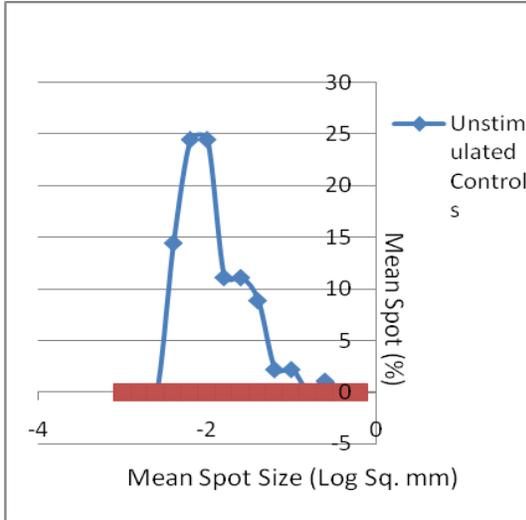
Figure 3: Mean spot distribution for IL-10 in patients and controls

and compare the secretions per cell in patients and controls. It was seen that cells showed spots in the range of -2.2 (Log Sq. mm) and -0.8 (Log Sq. mm) for IL-10 (Table 6 for IL-10) and -2.8 to -1.6 (Log Sq. mm) for IL-17 (Table 7 for IL-17). When the spot sizes were compared with the percentage mean spots it was seen that cells with larger spot sizes were found more in patients than controls for both for IL-10 (Figures 3 and 4 for IL-10) as well as IL-17 (Figures 5 and 6 for IL-17). Thus, patient cells secreted more IL-10 and IL-17 than controls.
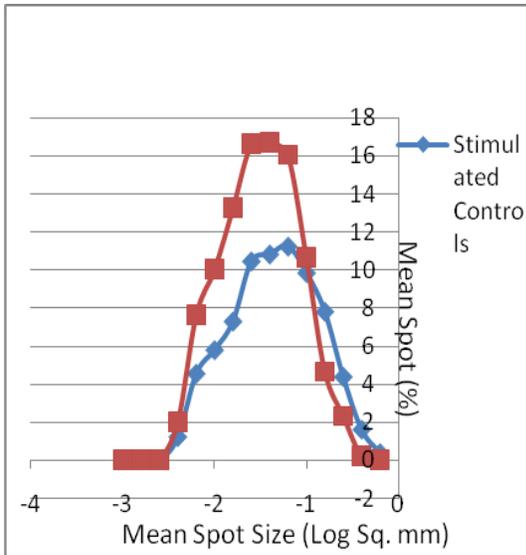
Figure 4: Mean spot distribution for IL-10 in patients and controls
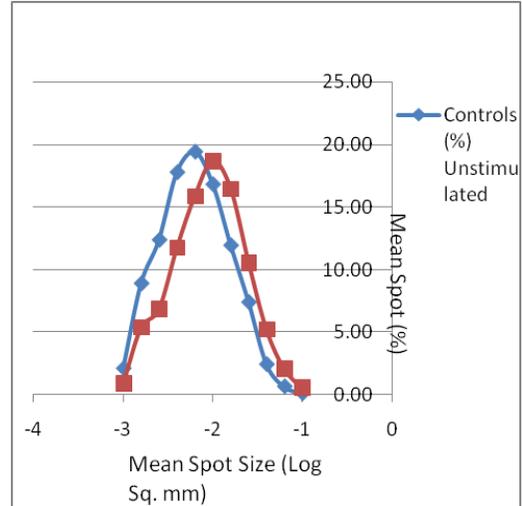
Figure 5: Mean spot distribution for IL-17 in patients and controls
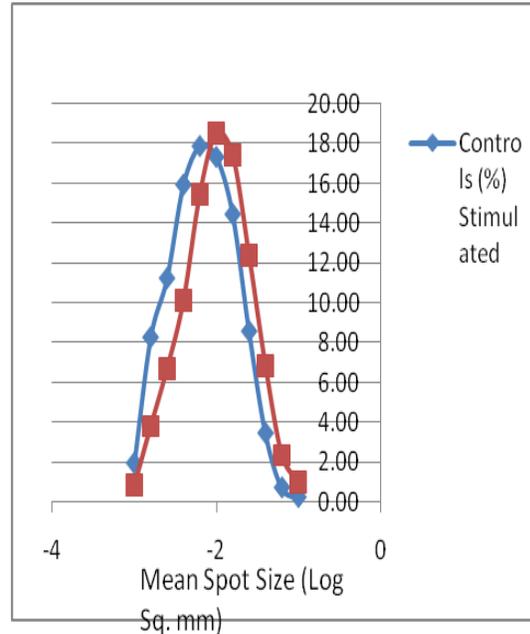
Figure 6: Mean spot distribution for IL-17 in patients and controls

DISCUSSION

Studies conducted in our laboratory reveal that there was significant difference in IL-10 cytokines by elispot only when the cells were stimulated by mitogen. But when the cells were stimulated or without stimulation there was significant difference in IL-17 cytokine by elispot on comparison with controls.

Thus we can confirm that there is spontaneous production of IL-17 cytokines in asthmatic patients, while increase in IL-10 cytokines is only after the trigger of and by the allergens that may act as mitogens. As IL-10 acts as a negative regulator of immune response, we can say that there are low levels of IL-10 cytokine production while IL-17 seems to be increased even without the action of stimulant. Thus we may conclude that IL-17 plays an important role in asthmatic patients.

It is seen that during an immune response low frequency antigen specific naïve T cells clonally expand.[23-25] The overall clonal size when reached, defines the effector cell mass and therefore the magnitude of the acquired immune response.[23-25] During initial encounter with the antigen, naïve T cells differentiate into memory cells with specific cytokine producing or killing effector function that defines the quality of the immune response.[23-25]

The Elispot assay takes the advantage of the fact that memory T-cell population do not secrete effector cytokine until they encounter cognate antigen.[26] Appropriated cognate is required to detect these T-cells. Since T-cells recognize processed antigen, the mode of antigen administration and the type of antigen presenting cell defines what antigen is presented to the cell.[26,27] Soluble antigens added to cell culture are preferentially presented on major histo-compatibilty complex (MHC) II molecules generating the ligand for activating CD4 cells.[27]

Elispot has been used successfully to delineate some of the cellular re-activities involved in contact allergy. Studies conducted on allergic patients reveal that nickel allergy involves T cells with a production of both T cell type 1(IFN –γ) and type 2 (IL-4) cytokines. The type 2 cytokines appear to be most consistently expressed and elispot tests on specific detection of IL-4 or IL-13 can potentially be used as a diagnostic alternative to current patch tests.[27]

CONCLUSION

Findings from this study revealed that in allergy research, Elispot assay are important in assessing antigen-specific T-cell responses. This method is sensitive enough to detect low frequency T-cells in peripheral blood, unlike ELISA. Thus ELISPOT assays for specific cytokines may provide a method that could be used to monitor antigen-specific T cell responses in peripheral blood.[21,28,29]

Abbreviations: 1. Th2: T helper type 2 cells
2. ELISA: Enzyme-linked immunosorbent assay.
3. ELISPOT: Enzyme-linked immunospot assay
4. IL: Interleukin
5. PBMNC-Peripheral blood mononuclear cells.
6. OPD-Out patient department.
7. IgE- Immunoglobulin E.

ACKNOWLEDGEMENT

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