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Evaluation of the frequency of invariant natural killer T (iNKT) cells in nasal polyps

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ABSTRACT

Nasal polyps (NP) are associated with inflamed mucosa of unknown etiology. The role of T cells in nasal polyposis is unclear. Invariant natural killer T cells (iNKT) can promote Th2 responses and have been implicated in some types of asthma. As there are shared inflammatory pathways involved in asthma and NPs, we evaluated the frequency of iNKT in 17 patients with NPs, but without asthma. A median of 6% polyp cells were T lymphocytes, of which iNKT were 0 to 2.38% (mean 0.674%). In the matched group (n = 10), iNKT in NPs was significantly higher than PBMCs (1.057% vs 0.155%, $P < 0.05$). Relative expression of Vα24 to TCR-beta genes in polyps (n = 14) was higher than blood in matched samples (n = 4). The presence of greater proportions of iNKT in NPs than in blood suggests that iNKT may play a role in the pathogenesis of nasal polyposis.

1. Introduction

Nasal polyposis (NP) is a chronic inflammatory state of the nasal cavity and sinuses [1]. The prevalence of NP ranges between 2 and 5% and is usually accompanied by some comorbid conditions such as chronic rhinosinusitis (CRS), allergic rhinitis (AR) and asthma, which in total decrease the patients' quality of life [2]. The etiology and pathogenesis of NPs are still not fully understood [1,3], but some evidence supports the role of the immune system in this disorder. It has been suggested that an ineffective local Th1 response may increase the level of Th2 cytokines, which contribute to chronic infection and accumulation of eosinophils and consequently further polyp formation [4–6]. In this regard, the presence of inflammatory cells such as Eosinophils, Th2 [7], Th17 and regulatory T cells [8] in polyp tissue has been reported.

Invariant natural killer T (iNKT) cells are a rare subset of lymphocytes that express a highly specific and invariant T-cell receptor and recognize conserved glycolipids presented by the non-polymorphic MHC-like molecule CD1d [9-11]. These cells are activated early in different immune reactions and are able to secrete a large amount of Th1 and/or Th2 cytokines and act as a bridge between the innate and adaptive immune responses [5,10–13]. Many studies have shown potential roles of iNKT cells in Th2 related immune responses such as IgE synthesis [14], cow's milk allergy [15], eosinophilic esophagitis [16,17], atopic dermatitis [18], intestinal polyposis (18) and particularly some subsets of asthma [5,10,13, 19]. Frequent lines of evidence correlate asthma with NP [20–23]. Both diseases show inflammation as well as remodeling and sharing similar histopathologic features such as infiltration of eosinophils [24] and T cell subsets [25,5]. From an epidemiological point of view, asthma is more frequent in patients suffering from nasal polyps as almost 45% of NP patients have asthmatic symptoms [26] but conversely, only 7% of asthmatic patients have nasal polyps [27]. Finally, recent results with a mouse model suggest NKT cells specifically making Th2 cytokines can contribute to intestinal

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Table 1
Primer sets for RT-PCR analysis.

Targets	Forward	Reverse	Size
TCRB	CAGCGAGCCCTACTCAAATTAG	GACCTGTGGAAGAGAGAACATT	104bp
TRAV24	AAGCATCTGACGACCTTCTTG	AACAGGACCTCTCCCAGTATC	192bp

polyposis (18).

Taking into consideration the importance of iNKT cells in asthma and Th2-mediated immune responses in general and the relations between asthma and nasal polyposis in particular, we aimed to investigate the frequency of iNKT cells in patients with nasal polyposis, but without asthma, in the hope of shedding light on the pathogenesis of nasal polyposis.

2. Methods

2.1. Patients and samples

Seventeen patients with chronic rhinosinusitis who were candidates for polypectomy were enrolled in this study. The study was approved by the Ethics Committee of the Mashhad University of Medical Sciences and all participants received written informed consent. Demographic data, history of asthma and other allergic or immunologic disorders were collected by a questionnaire. Patients with asthma, any serious health problem or those using immunosuppressive drugs or drugs affecting skin prick test result were excluded from this study.

2.2. Skin prick test (SPT) and total IgE

To evaluate the allergic sensitization, skin prick test was performed with a panel of 20 common allergenic extracts including grass, weeds, trees, mites, moulds, cat and cockroach (Holishter-Ster, USA). Albumin and total IgE in serum and polyp homogenate were measured in duplicate by clinical chemistry analyzer (Roche, USA) and commercial ELISA kit (Radim, Italy). The level of albumin was used for normalization of polyp tissue's total IgE.

2.3. Sample collection and processing

Before polypectomy, 5 ml of whole blood samples were collected and peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation. A part of fresh PBMCs was used for flow cytometry and the rest for RNA isolation and RT-PCR.

During polypectomy, resected polyp samples were washed with normal saline and divided into several parts for histopathology evaluation and RNA extraction. Tissue slides were stained with Hematoxylin and Eosin, and light microscopic evaluation of polyp type and cellularity performed coded by an expert pathologist.

2.4. Flow cytometry

To perform flow cytometry, single cell suspension from fresh tissue samples were made using medimachine (BD Bioscience, USA) and filtered through 50 and 30-μm nylon mesh cell strainers sequentially. PBMCs and polyp cell suspensions with viability < 90% were not analyzed. Phenotypic analysis of samples was performed by FACSCalibur machine (Becton Dickinson). Anti-CD3 (PerCP), anti-Vα24-Jα18 (clone 6B11) (PE) and anti-CD4 (FITC) antibodies and matched isotype controls all from BD (CA, USA) were used for flow cytometry. To reduce nonspecific binding, the cells were incubated with 10% human AB serum in RPMI 1640 for 30 min and then routine staining protocol was performed. Because of the low frequency of iNKT cells in PBMC, at least 2×10^5 cells were analyzed, but the number of cells in tissue was limited and the maximum of accessible cells was counted.

2.5. Reverse-transcription polymerase chain reaction (RT-PCR)

RNA extraction from tissue and PBMCs was done by a commercial kit (RNeasy mini kit, Qiagen, Germany). All samples were treated with DNase-I (Qiagen, Germany) and then cDNA synthesis was performed using cDNA Reverse Transcription kit (Thermofisher, USA). Qualitative expression of T cell receptor beta constant 2 region (TRBC2) and Vα24 genes as the representative of CD3 + T lymphocytes and iNKT cells was assessed by an RT-PCR method using specific primers for both genes (Table 1). PCR products were electrophoresed on 3% agarose gel and relative expression of V-alpha-24 was normalized against TCRβ using ImageJ software (ImageJ, NIH USA, 2008).

3. Data analysis

Flow cytometry data were analyzed using FCS Express 3. The lymphocyte population (gated based on the forward versus side scatter) and CD3-positive population (gated based on the side scatter and CD3) were used for analysis of frequency and phenotype of CD4 ± T cells and iNKT cells.

4. Statistical analysis

Data were analyzed by SPSS software package version 11 (Chicago, USA). When appropriate, two-tail Mann-Whitney test or Student's *t*-test was used. Spearman's nonparametric correlation test was used for correlation analysis. P values < 0.05 were considered significant.

5. Results

Seventeen patients with nasal polyps enrolled in this study (mean age was 36.7 ± 12.6 Range 17–60 yrs., M/F; 9/8). 62.5%of patients reported allergy and had at least one positive reaction in the skin prick test. The mean polyp IgE was significantly higher than serum IgE (202.6 ± 198 vs. 121.6 ± 173, P = 0.002) (Fig. 1) and there was a significant positive correlation between them (Spearman's r = 0.782, P = 0.002). In the microscopic evaluation of polyp's slides, the percentage of lymphocytes (50.0% vs 11.6%, P = 0.036) and neutrophils

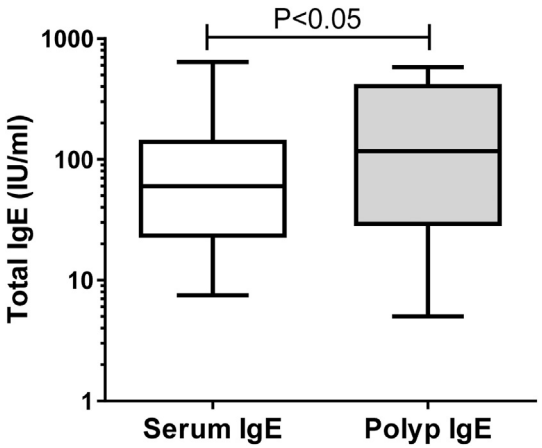

Fig. 1. Comparison of the total IgE in serum and polyp samples.

Table 2
Descriptive Statistics of different cell types in PBMCs and polyp tissue.

	N	Minimum%	Maximum%	Mean%	Std. Deviation
Age	17	17	60	36.65	12.60
B-iNKT	10	0.028	0.39	0.155	0.12
B-CD4+	16	49	65	56.5	5.75
B-CD3+	16	32	87	60.28	16.47
B-iNKT,CD4+	15	38	63	47.25	10.10
P-iNKT	17	0	2.38	0.674	0.689
P-CD4+	13	16	52	35.83	11.47
P-CD3+	13	2.11	32	6.67	7.39
P-iNKT,CD4+	13	20	80	48	19.29
P-Lymphocyte*	17	3	80	23.50	25.99
P-Neutrophil*	17	0	35	9.50	10.06
P-Eosinophil*	17	3	95	54.25	34.54
Serum-IgE (IU/ml)*	16	7.50	640	121.60	173.10
Polyp-IgE (IU/ml)*	16	5	580	202.64	198.07

P: polyp B: blood.
* Histopathology evaluation of Polyp tissues.

(20.0% vs 5.8%, P = 0.034) were significantly higher in non-allergic polyps than allergic ones while eosinophils were significantly higher in allergic forms (21.3% vs 72.7%, P = 0.01).

5.1. Flow cytometry

In polyp single cell suspension, the average number of T lymphocytes was almost 6% of total counted cells. Among CD3+ T gate, the mean percentage of iNKT cells was 0.674% (range: 0% to 2.38%) and 48% of iNKT were positive for CD4+ (range: 20%–80%). In the peripheral blood samples, the average number of CD3+, CD4+, iNKT cells, and iNKT CD4+ cells, were 60.2%, 56.5%, 0.155% and 47.2%, respectively (Table 2).

The percentage of iNKT cells was significantly higher in polyp tissues than matched PBMCs (1.057% vs 0.155%, P < 0.05), while CD4+ cells were significantly higher in PBMCs than polyps (56.50% vs 35.83%, P = 0.001) (Fig. 2A). There was no significant difference between the number of CD4+ iNKT cells in polyp tissues and blood samples. There was a strong positive correlation between the percentage of CD3+ lymphocytes and iNKT cells in polyps (Spearman's r = 0.805, P = 0.016).

In contrast, there was a weak but significant inverse correlation between the polyp's iNKT cell proportions and those of eosinophils (Spearman's r = 0.558, P = 0.036).

5.1.1. RT-PCR data of Vα24 and TCR constant genes

Expression of TCRβ Constant region 2 (as a measure of total T cells) and Vα24 genes were evaluated by RT-PCR in 14 polyp tissues and 4 PBMCs samples.

TCRβ and Vα24 bands were present in all polyp samples, but the relative expression of Vα24 to TCRβ showed high variation among samples and in 7 samples was high. (Figs. 3 & 4). Comparative expression of these genes in 4 polyps and their corresponding PBMCs samples showed that expression of Vα24 in polyp tissues is higher than corresponding PBMCs. (Fig. 5).

6. Discussion

Nasal polyps (NPs) are benign lesions originating from the nasal mucosa or sinuses possibly because of chronic inflammation and cell infiltration [2,28]. In the current study, we confirmed the presence of iNKT cells and expression of their corresponding TCR Vα24 gene in polyp tissue in varying degrees, but significantly higher than in the matched peripheral blood. To the best of our knowledge, there is only one report by Yamamoto, which evaluated the relative expression of Vα24Jα18 to CD3 genes in patients suffering from chronic sinusitis

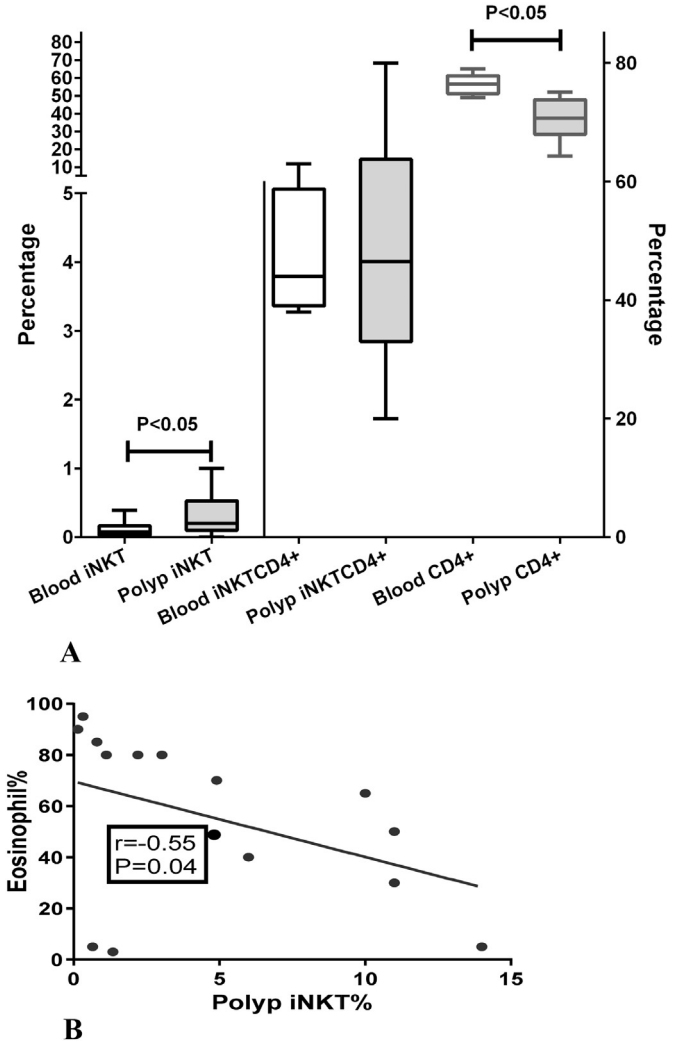

Fig. 2. A. Comparison of the percentage of different cell subsets in the peripheral blood and polyp tissue, B. The correlation between iNKT and eosinophilia in polyp tissue.

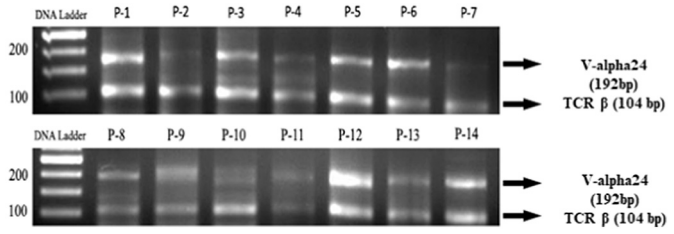

Fig. 3. Image of gel electrophoresis for PCR products, TCRβ constant region and V-alpha-24 gene bands in polyp tissue. (P: polyp sample).

with asthma. [5]. In Yamamoto's study, iNKT cell RNA was detected at various levels in all patients' peripheral blood, but just in polyps of asthmatic patients. In spite of the difference in detection methods and study population, their study showed high variation in iNKT cells in polyp samples [5]. In contrast to our study, Yamamoto detected iNKT in five out of six patients with eosinophilic polyps, but in just one case of neutrophilic polyps, while we found a negative correlation between iNKT and eosinophils in polyps. This discrepancy can be explained by a different methodology, as we study the correlation of the two cell types, but Yamamoto did not evaluate the correlation and detected iNKT cells by PCR. Several studies evaluate different subsets of T lymphocytes in the pathogenesis of nasal polyposis. Two studies reported that Th17

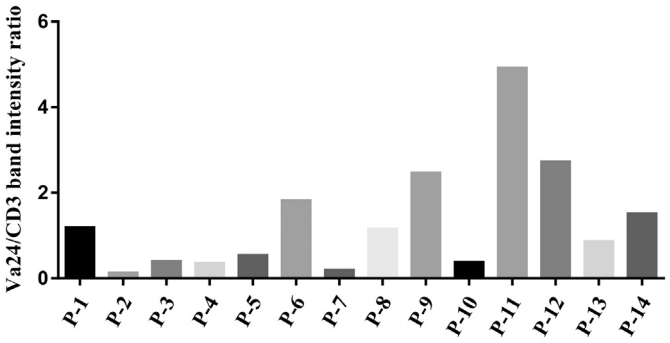

Fig. 4. Density ratio of Vα24 gene's bands to TCRβ constant gene in gel electrophoresis of RT-PCR products-nasal polyposis.

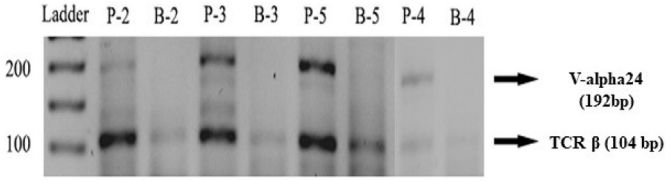

Fig. 5. Gel electrophoresis of PCR products TCR constant gene and Vα24 gene in polyp tissue and their corresponding blood samples in 4 cases; (P: Polyp sample, B: Blood sample).

frequency is increased in the peripheral blood of patients with NP, but Treg frequency is decreased in the peripheral blood of these patients. The Th17/T regulatory (Treg) ratio is important in the systemic and local immune responses and the impaired Th17/Treg balance seems to be critical for the development of NP [29].

Similar to Tregs, iNKT are regulatory cells which can promote Th2 responses [30,31]. In spite of the lack of data about iNKT cells in nasal polyposis, several studies supported the importance of iNKT cells in the pathogenesis of some subsets of asthma [9,32], which has many similarities to nasal polyposis. Akbari et al. reported that a high percentage of $CD4^+CD3^+$ cells in Bronchoalveolar lavage (BAL) fluid of patients with moderate-to-severe persistent asthma were iNKT cells which produced type 2 helper cytokines [13]. Similarly, in two other studies, $V\alpha24^+$ presumptive iNKT cells were increased in sputum of asthmatic patients [33,34]. Xiao et al. identified a role of IL-21 in nasal polyposis, which is a supportive finding for the role of iNKT cells in nasal polyposis, as iNKT cells can produce IL-21 and in an autocrine loop, IL-21 enhances survival and proliferation of NKT cells [6]. However, other studies reported lower levels of iNKT cells in other asthmatic patients [35,36]. Considering the correlation between asthma and nasal polyps and to prevent any bias in our results due to asthma, asthmatic patients were excluded from the current study. Nasal polyps are histologically characterized by the accumulation of eosinophils [4]. iNKT cells can produce high levels of Th2 cytokines which promote proliferation, differentiation, and accumulation of eosinophils [37,38]. In this regard, several reports suggest the contribution of iNKT cells in IgE production and eosinophilic inflammation such as eosinophilic esophagitis (EoE) and airway hyper-responsiveness [13,39,40]. We expected a positive correlation between iNKT cells and eosinophils in NP tissue, but interestingly there was a weak but significant inverse correlation between eosinophils and iNKT cells in polyp tissue. Consistent with this finding, two studies have shown that alpha-galactosylceramide, a potent activator of iNKT cells, can suppress the eosinophilic inflammation [41,42]. Other studies also reported that allergic responses can establish without iNKT cells [43,44]. Conflicting reports also exist about the role of iNKT cells in Th2 responses and asthma. [10,43–46]. Heterogeneity of polyp samples and a variation in eosinophil infiltration can also be a reason for this finding. In this regard, the correlation for T-lymphocyte with eosinophils, which had less variation in frequency, was strong and showed that our finding is not a technical bias. The lower frequency of CD4+ cells in our Polyp samples is supported by previous studies that report a lower number of CD4+ cells in nasal mucosa and polyps of adults compared to children [47,48]. On the other, hand nasal polyp T cells may not completely originate from the same source as stably circulating peripheral blood T cells, and their proliferation is in part attributed to external inducers such as allergens, bacteria and other agents [49]. In this study, the mean percentage of iNKT in blood was fairly high, but it is in concordance with our previous report about the frequency of blood iNKT in Iranians [12]. But the main finding of this study was that the abundance of iNKT cells in the majority of polyp samples was substantially higher a frequency than in matched peripheral blood.

iNKT cells are hard to study, as they are rare in humans and collecting enough cells for accurate analysis from tissues such as a polyp is difficult. In addition, there are several methods of detection that may give significantly different results [50]. To overcome these limitations, we collected and counted as many cells as were available from polyp tissues using the bright PE-conjugated anti Vα24-Jα18 antibody, which in combination with anti-CD3 antibody has high specificity and sensitivity for iNKT cell detection [51–53]. Strict gating strategy, using isotype controls as well as Fc Receptor blocking buffers also decrease the possibility of non-specific staining. Furthermore, for validation of the flow results, Vα24 and TCR constant genes were used as representatives of iNKT and T cells detected by RT-PCR methods in polyp tissues and available matched PBMCs. Relative expression of Vα24 to TCR constant region in polyp sample confirms the presence of iNKT cells in varying degrees. Comparison of the expression of Vα24 in polyp tissue and peripheral blood also supports the higher percentage of iNKT cells in tissue than blood. Considering all the above-mentioned precautions, it is unlikely that this finding has a technical bias and it seems that iNKT cells can have a role in the pathogenesis of nasal polyposis.

7. Conclusion

The present study showed that iNKT cells are more abundant in the nasal polyps than the peripheral blood of non-asthmatics and may likely play a role in the pathogenesis of polyposis through interaction with other cells. Further studies with more samples need to reveal the precise mechanism of action for these regulatory cells.

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