

Is CD19 an Immunological Diagnostic Marker for Acute Appendicitis?

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ABSTRACT

Background: The appendix is considered as part of the gut-associated lymphoid tissue; however, lymphocyte subsets in this tissue are not fully defined. **Objective:** To investigate and compare the function and phenotype of lymphocyte subsets in peripheral blood and appendix of patients with normal and inflamed appendix tissues. **Methods:** Peripheral blood samples and appendiceal mononuclear cells were obtained from 81 patients (mean age; 23 ± 10.5 years), clinically suspected of having appendicitis. The phenotypic characteristics of lymphocyte subsets in peripheral blood (before and 48-72 hrs after appendectomy) and in appendix tissue were analyzed by three color-flow cytometry. The proliferative response of mononuclear cells was assessed by MTT method. **Results:** The frequency of CD19+DR+, HLA-DR+ and CD19⁺ cells in the appendix tissue were significantly higher than that of the peripheral blood in all the groups ($p < 0.001$). The percentage of CD19⁺ cells and HLA-DR⁺CD19⁺ cells significantly decreased after appendectomy in the peripheral blood of the patients with acute appendicitis ($p = 0.047$ and $p = 0.03$, respectively). CD19 and HLA-DR plus CD19 had better diagnostic efficiency compared with T cell markers (area under the ROC curve [AUC]= 0.76 and 0.73, respectively). **Conclusion:** These results indicate a significant difference in CD19+ and HLA-DR+ lymphocytes between peripheral blood and the appendix tissue.

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Keywords: Acute Appendicitis, CD19, HLA-DR, Lymphocyte Subsets, Proliferation

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INTRODUCTION

Acute appendicitis is one of the most common causes of abdominal surgical emergencies with prevalence of approximately 6-10% during lifetime. Early diagnosis and intervention is important for successful management of acute appendicitis. Acute appendicitis is commonly diagnosed with clinical and physical examination findings (1,2). However, this diagnosis especially in children is problematic based on the patient's difficulty in communicating symptoms and the large overlap in presentation with other common diseases. The difficulty of diagnosis, as expressed by the 15% to 40% of unnecessary appendectomies, has motivated some authors to search for better diagnostic tools (3).

Acute appendicitis is an inflammation of the appendix tissue starting with obstruction of the appendiceal lumen and impaired blood flow, leukocytes infiltration and elevated of white blood cell (WBC) count and C-reactive protein (4-6). Recent studies reported that several serum biomarkers such as proinflammatory cytokines, granulocyte colony stimulating factor, calprotectin, leucine-rich alpha glycoprotein-1, D-lactate and matrix metalloproteinases elevate in patients with acute appendicitis and may be useful in the diagnosis and staging of appendicitis (7-9).

During acute appendicitis the WBC and neutrophil count increases in peripheral blood and lymphocytes are recruited to the inflamed appendix (10). The process of homing and migration of lymphocytes to the inflamed appendix may affect phenotype and function of lymphocytes in peripheral blood. Few researchers have attempted to characterize the phenotype and function of lymphocytes in acute appendicitis (11-13). However, the immunological function of appendix as part of the gut associated lymphoid tissue has not been clearly known. In the present study we analyzed the subsets and function of lymphocytes in peripheral blood and appendix tissues of patients with normal and inflamed appendix.

MATERIALS AND METHODS

Patients. Eighty-one patients who were clinically suspected of having appendicitis were included in this study. Inclusion criteria for clinically acute appendicitis were based on the clinical judgment of the referring surgeons and emergency care physicians. The criteria used to establish the diagnosis of acute appendicitis were CT scan and peri-appendiceal inflammatory changes such as leucocytosis and C-reactive protein. An appendix larger than 6 mm in transverse diameter was considered abnormal. Surgical appendectomy was performed by one surgeon, according to the hospital protocol. Forty patients had histologically confirmed acute appendicitis, 16 patients had gangrenous appendicitis. Twenty-five patients had no demonstrable histopathology of appendicitis and were classified as normal appendix. The demographic characteristics of the patients are presented in Table 1. The study was approved by the ethics committee of Arak University of Medical Sciences (AUMSEC-89-88-2).

Blood and Tissues Samples. Peripheral blood samples were collected from patients before and 48-72 hours after appendectomy. Appendix tissue was divided to two parts by longitudinal section. One section of the appendix was examined for routine histology and the rest of appendix was floated in phosphate buffered saline (PBS, pH=7.2)

containing 200 U/ml penicillin and 200 µg/ml streptomycin and transferred to immunological laboratory.

Table 1. Demographic and clinical characteristics of appendectomy patients.

Type of Appendix	Male/Female	Age(years) ± SD	WBC (×10 ³ cells/mL)	PMN (%)	Lymp (%)
Normal	10/15	20.16 ± 9.5	14 ± 3.56	78.2 ± 6.42	15.3 ± 8.6
Acute	21/19	22.8 ± 10.3	12 ± 3.25	79.4 ± 9	15.7 ± 8
Gangrenous	10/6	27.1 ± 12.5	15.2 ± 3.8	79.7 ± 6.7	16.4 ± 5.7
		NS*	NS	NS	NS

*NS: P value Not significant

Preparation of Single-Cell Suspensions. A single-cell suspension from each appendix was prepared by blunt dissection in sterile petri dishes containing PBS (200 U/ml penicillin and 200 µg/ml streptomycin).

Mononuclear Cell Separation from Blood and Appendix Tissue. The diluted blood and the appendiceal cells were isolated using Ficoll-Isopaque. Mononuclear cells were isolated by centrifugation (600 ×g for 20 minutes) with 1.077 g/mL Lymphoprep (Nyegaard, Oslo, Norway) and washed twice in PBS. The viabilities of the isolated mononuclear cells were determined to be more than 95% by trypan blue staining. The cells were then resuspended in RPMI-1640, 5 mM HEPES, 100 U/mL of penicillin and 100 µg/mL of streptomycin supplemented with 10% FBS (All obtained from Gibco, Life Technologies, Inc., Gaithersburg, Md.) and used for phenotype and function assay as described below.

Phenotypic analysis. Phenotypic analyses of mononuclear cells were performed by two- and/or three- color direct staining and flow cytometry. Conjugated monoclonal antibodies (MoAbs) were used for the phenotyping as follows: anti-CD3 FITC, anti-CD19 PerCP, anti-CD45RA PE, anti-CD45RO FITC, anti-CD3 PerCP-Cy5.5, anti-CD5 FITC, anti-gamma delta TCR PE, anti-alpha beta TCR FITC, anti-CD4 FITC, anti-CD8 PE anti- HLA-DR PE. FITC, PE or PerCP-conjugated mouse IgG1 or IgG2a were used as isotope controls. The monoclonal antibodies were purchased from eBioscience (San Diego, CA) or BD Biosciences (San Jose, CA). Mononuclear cells (1-2×10⁶ cells/100µL PBS) were incubated with appropriate concentration of FITC, PE and/or PerCP-conjugated MoAbs for 30-45 min on 4°C. After 3 washing with PBS, the cells were resuspended in 2 ml PBS containing 1% BSA. The cells were then analyzed using Becton Dickinson FACSCalibur flow cytometer by the CellQuest program (BD Biosciences). The lymphocyte population was gated by its forward and side scatter characteristics. 10,000 events in gated population were acquired for each sample. To illustrate how the flow cytometric data were obtained, representative dot plots from blood and appendix cells of an acute appendicitis patient are shown in Figure 1.

A: Peripheral blood

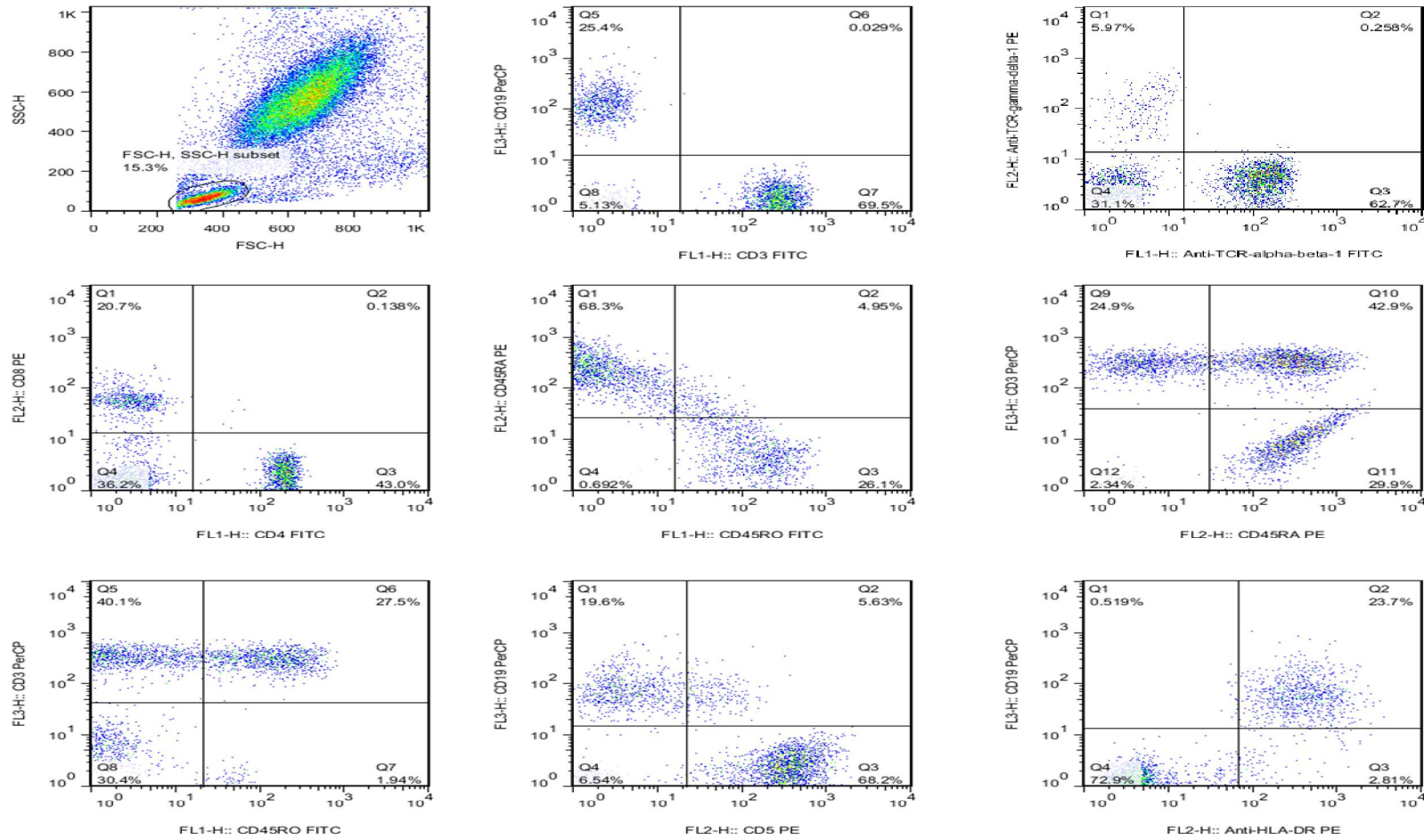
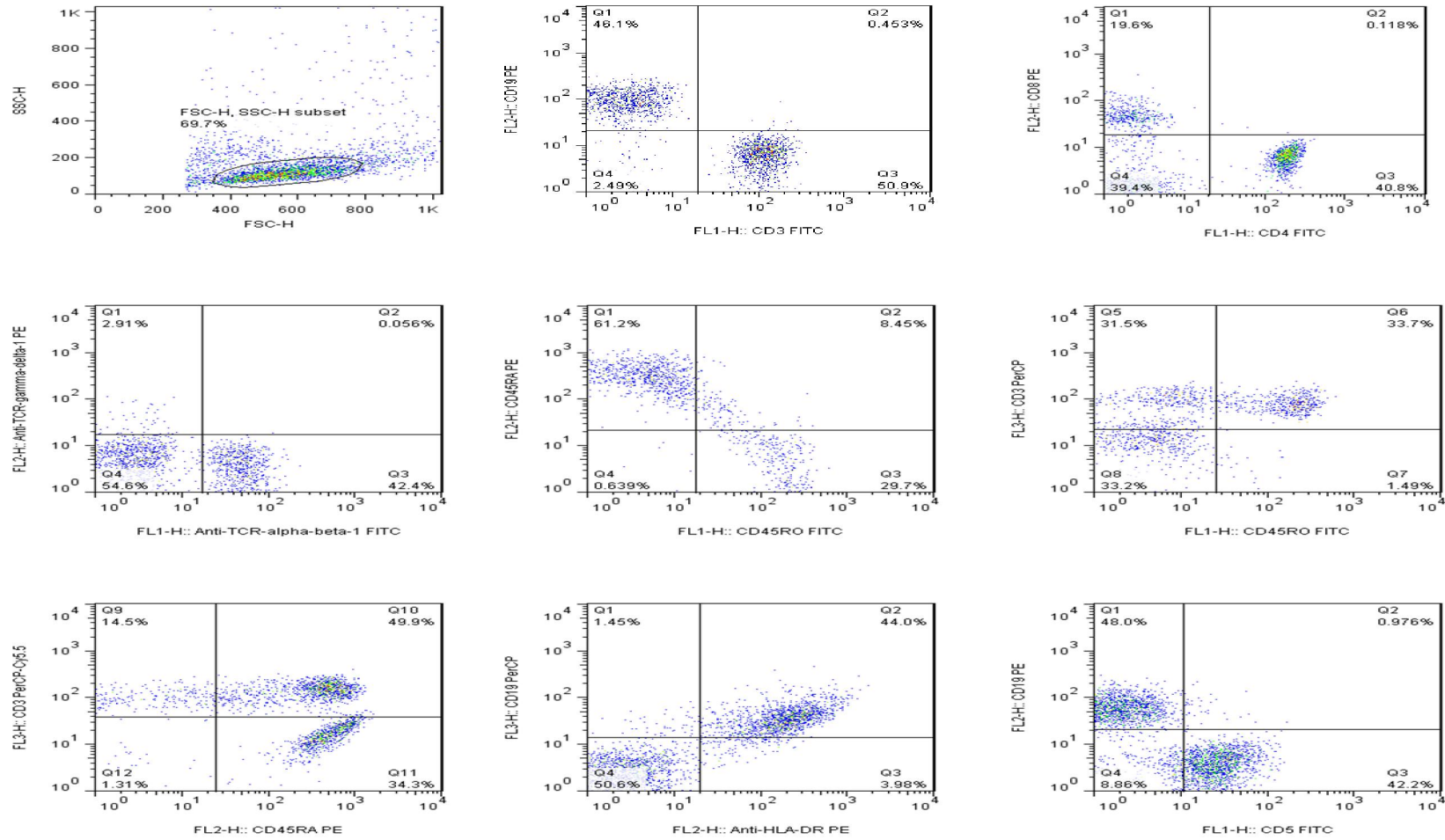


Figure 1. Flow cytometry analysis of lymphocytes derived from peripheral blood (A) and appendix tissue (B) of a patient with acute appendicitis.

B-cells in acute appendicitis

B: Appendix tissue



Proliferation of PBMCs. Proliferation was checked by the MTT assay. A total of 3×10^3 cells in 200 μ l RPMI-1640 supplemented with 10% FBS were incubated in 96-well round-bottomed plates (Nunc, Denmark) in the presence, or absence 1 μ g/mL phytohemagglutinine (PHA) (Sigma) or 1 μ g/mL Con-A (Sigma) in a 5% CO₂ incubator at 37°C for 72h. Twenty microliters of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl; Sigma-Aldrich, St. Louis, MO) were added to the cells, followed by incubation for 4 h in a 5% CO₂ incubator at 37°C. After centrifugation, the medium was removed, and 200 μ l of DMSO was added to each well. The optical density (OD) values of stimulated and non-stimulated cells were measured at 540 nm using a microtiter plate reader (Stat Fax2100, USA). All experiments were performed in triplicates. Proliferation responses for the MTT assay were expressed in terms of the mean stimulation index (SI) and obtained by dividing the OD values of stimulated cells by the respective OD values of the non-stimulated ones.

Statistical Analysis. For statistic evaluation, One-way ANOVA with *post hoc* contrast was used to assess the statistical significance of differences between the groups. An independent sample *t*-test (2-tailed) was used to evaluate differences between variables in the groups. Paired-Sample *t*-test was used for comparing variables before and after appendectomy. Receiver operator characteristic (ROC) curves were drawn to define the diagnostic accuracy as determined by the area under the ROC curve (AUC) of the CD lymphocyte markers. Data were presented as mean \pm standard deviation. *p* values less than 0.05 were considered significant.

RESULTS

General Findings. Mean of age was similar in the three groups. Also, there was no significant difference between the three groups based on the mean frequency of WBC and percentage of lymphocytes and polymorphonuclear cells (Table 1).

Frequency of T Cell and B Cell Subsets in Peripheral Blood and Appendix Tissue. Overall, percentage of CD3⁺ cells in peripheral blood of appendectomy patients was approximately 58-62%, higher than that of the appendix tissue (34-46%), (Table 2). There was no significant difference in the ratio of CD3⁺CD4⁺ cells/CD3⁺CD4⁺ cells, and alpha-beta TCR/gamma-delta TCR T cells between peripheral blood and appendix tissue. The frequency of CD19⁺ cells (*p*=0.001), CD5⁺CD19⁺ cells (*p*=0.023), HLA-DR⁺ cells (*p*=0.001) and HLA-DR⁺CD19⁺ cells (*p*=0.001) in appendix tissue were significantly higher than that of peripheral blood sample in all groups (Table 2).

Frequency of T Cell and B Cell Subsets in Appendectomized Patients. In peripheral blood, the percentage of CD3⁺ cells and T cell subsets (CD3⁺CD4⁺, CD3⁺CD8⁺, CD3⁺ alpha-beta-TCR, CD3⁺ gamma-delta-TCR, CD3⁺RA⁺, CD3⁺RO⁺) were not significantly different between groups (Table 2). The frequency of CD19⁺ cells and HLA-DR⁺ CD19⁺ cells in patients with acute (21.36 ± 9.04 and 19.17 ± 7.98) and gangrenous (20 ± 7.52 and 20.8 ± 6.61) appendicitis were significantly higher than that from patients with normal appendix (14.46 ± 6.08 and 12.46 ± 5.10 , *p*=0.012).

In appendix tissue, there was no significant difference in percentage of T cell subpopulations among three groups. But the percentage of CD19⁺ cells and HLA-DR⁺CD19⁺ cells in patients with acute appendicitis were significantly higher than that from other groups (*p*=0.04, Table 2).

Table 2. The percentage of lymphocytes subsets in appendix tissue and peripheral blood samples from patients who underwent appendectomy.

Markers	Blood			Appendix		
	Normal %	Acute %	Gangrenous %	Normal %	Acute %	Gangrenous %
CD3+	62.07 ± 11.97	60.10 ± 10.81	58.71 ± 16.22	40.65 ± 11.68	46.50 ± 13.85	34.25 ± 7.81
CD3+CD4+	65.92 ± 7.35	56.21 ± 9.90	57.71 ± 9.12	63.76 ± 10.01	56.53 ± 12.67	57.06 ± 11.58
CD3+CD8+	31.69 ± 11.54	40.78 ± 5.81	35.85 ± 13.08	28.75 ± 4.77	38.68 ± 9.42	39.75 ± 1.84
CD3+alpha-beta- TCR	96.66 ± 12.42	94.34 ± 14.43	97.71 ± 14.45	94.72 ± 11.48	89.05 ± 12.78	90.62 ± 13.53
CD3+ gamma- delta-TCR	5.91 ± 2.81	6.71 ± 5.22	2.28 ± 1.32	4.05 ± 7.27	6.21 ± 6.88	4.93 ± 3.52
CD3+CD45RA+	45.13 ± 10.36	39.23 ± 8.46	39.83 ± 14.51	43.19 ± 8.92	27.52 ± 9.89	22.12 ± 10.47
CD3+CD45RO+	46.69 ± 6.39	52.41 ± 5.60	54.95 ± 10.7	35.84 ± 7.34	53.34 ± 8.00	53.75 ± 7.68
CD45RA+CD45RO+	4.16 ± 2.66	3.81 ± 2.38	4.83 ± 2.51	5.36 ± 2.05	6.48 ± 2.91	6.67 ± 3.21
CD19+	14.46 ± 6.08	21.36 ± 9.04	20.00 ± 7.52	37.40 ± 16.42	43.00 ± 14.87	30.62 ± 13.60
CD19+CD5+	2.52 ± 0.28	3.51 ± 1.10	3 ± 0.37	6.83 ± 1.88	7.85 ± 2.78	8.18 ± 1.12
*HLA-DR+	19.90 ± 6.83	24.28 ± 10.55	17.66 ± 7.47	41.45 ± 15.33	46.94 ± 15.19	35.62 ± 7.19
*HLA-DR+ CD19+	12.46 ± 5.10	19.17 ± 7.98	20.80 ± 6.61	27.50 ± 15.51	34.43 ± 16.22	29.62 ± 7.96

*; The percentages are presented from all events in lymphocyte gate

Table 3. The percentage of lymphocytes subsets in peripheral blood samples of patients with normal appendix (Normal) and patients with acute appendicitis before and 48-72 hrs after appendectomy.

Markers	Normal (n=10)			Acute appendicitis (n=18)		
	Before %	After %	P Value	Before %	After %	P Value
CD3+	64.8 ± 9.6	68.3 ± 9.8	N.S	59.3 ± 12.5	63.3 ± 11.4	N.S
CD3+CD4+	65.5 ± 5.4	61 ± 5.3	N.S	54.2 ± 8.9	68.4 ± 9.2	N.S
CD3+CD8+	30.1 ± 8.6	33 ± 7.6	N.S	41.2 ± 7.08	30 ± 7.2	N.S
CD3+alpha-beta- TCR	95.5 ± 10	97 ± 14.7	N.S	91.58 ± 16.8	95.6 ± 9.5	N.S
CD3+ gamma- delta-TCR	5 ± 2.13	4 ± 1.7	N.S	8.4 ± 10.5	7.4 ± 3.75	N.S
CD3+CD45RA+	45.8 ± 10.36	44.23 ± 8.43	N.S	40 ± 10	38 ± 9.4	N.S
CD3+CD45RO+	46.2 ± 6.39	50.41 ± 5.60	N.S	53.1 ± 7	54 ± 10.5	N.S
CD45RA+CD45RO+	4.3 ± 2.8	4 ± 2.2.8	N.S	3.8 ± 2.9	3.48 ± 2.7	N.S
CD19+	15 ± 5.8	14.7 ± 3.4	N.S	22.2 ± 6.38	15.01 ± 7.1	0.047
CD19+CD5+	2.1 ± 0.6	2.51 ± 0.2	N.S	4.23 ± 1.2	5.85 ± 0.78	N.S
*HLA-DR+	20.2 ± 5.5	12.8 ± 5.6	0.012	27.54 ± 14.67	17 ± 19.24	0.02
*HLA-DR+ CD19+	12.07 ± 4.7	13.3 ± 5.3	N.S	20 ± 9.5	14.3 ± 3.1	0.03

*; The percentages are presented from all events in lymphocyte gate

Lymphocytes Variations in Peripheral Blood before and after Operation. Changes of lymphocyte populations were analyzed in peripheral blood of patients with the normal and the inflamed appendix before and 48-72 hrs after appendectomy (Table 3). The results showed that there was no significant difference in T-cell subsets in peripheral blood of patients with acute appendicitis or normal group at before and after appendectomy. Also, there was no difference in the frequency of CD19⁺ cells, CD5⁺CD19⁺ cells and HLA-DR⁺CD19⁺ cells before and after operation in normal group, although the absolute count of HLA-DR⁺ cells was different (p=0.012, Table 3). The percentage of CD19⁺ cells and HLA-DR⁺CD19⁺ cells significantly decreased after appendectomy in patients with acute appendicitis (p=0.047 and p=0.03, respectively).

Value of CD Markers in Predicting Appendicitis. Data of 76 patients (52 appendicitis patients and 24 patients with normal appendix) was analyzed for CD markers of peripheral blood lymphocyte subset. Sensitivity and specificity for the lymphocyte CD markers were calculated as the area under the receiver operating characteristic curve (AUC-ROC). These AUC-ROC values with 95% confidence intervals are shown in Table 4. CD19⁺ cells (AUC=0.76, Figure 2A) and HLA-DR⁺CD19⁺ cells (AUC=0.73, Figure 2B) had better diagnostic efficiency in compared with other CD markers (Table 4).

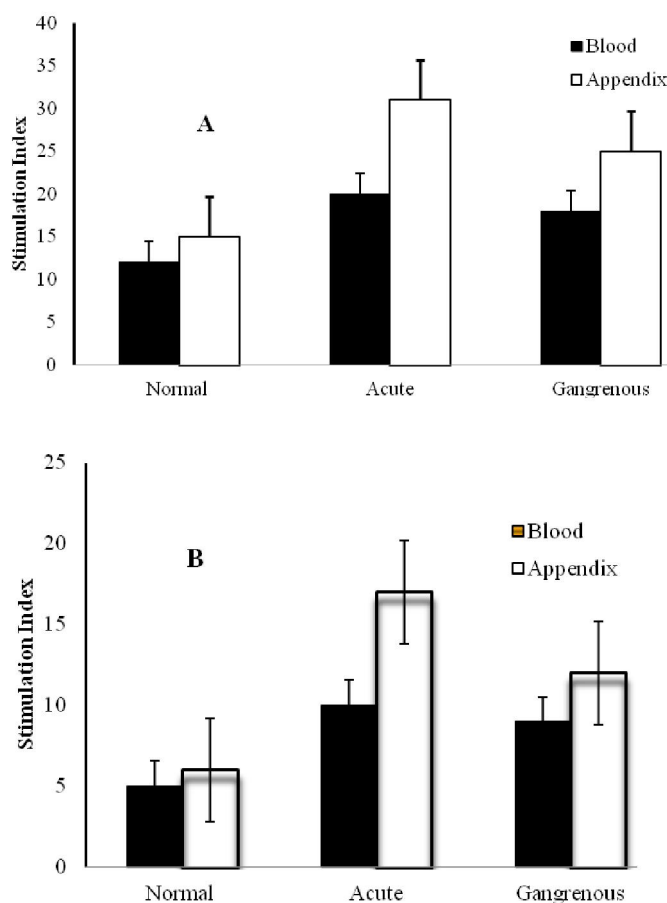


Figure 2. Mean stimulation index of peripheral blood and appendiceal lymphocytes induced by 1 µg/mL PHA (A) or 1 µg/mL Con-A (B) in appendectomy patients.

Lymphocytes Proliferation. We analyzed lymphocytes proliferation in peripheral blood and appendix of appendectomy patients by MTT assay. Figures 3A and 3B show the proliferative responses of the peripheral blood and appendiceal lymphocytes to PHA and Con-A, respectively. In all patient groups, there is no significant difference in proliferation responses induced by PHA or Con-A between peripheral blood lymphocytes and appendiceal lymphocytes in patients with normal appendix. In patients with acute and gangrenous appendicitis, appendiceal lymphocytes had significantly higher proliferative responses to PHA or Con-A compared with peripheral blood lymphocytes.

Table 4. Area under the Curve (AUC) values with 95% Confidence Intervals for the lymphocyte CD markers.

CD markers	AUC	95% Confidence Intervals	Specificity (%)	Sensitivity (%)	Cut off
Alpha-Beta TCR	0.58	0.38-0.76	60	72	60
Gamma- delta TCR	0.54	0.34-0.73	100	22	8
CD3+CD4+	0.55	0.35-0.70	70	50	32
CD3+CD8+	0.68	0.48-0.87	50	89	25
CD3+CD45RA+	0.7	0.53-0.80	70	67	30
CD3+CD45RO+	0.62	0.42-0.80	50	83	30
CD19+	0.76	0.57-0.9	80	72	14
HLA-DR+CD19+	0.73	0.59-0.85	82	71	15

DISCUSSION

The lymphoid tissue of the appendix is considered as part of the gut associated lymphoid tissue (GALT). GALT houses more lymphocytes than all secondary lymphoid tissues combined. Bacterial colonization in the appendix results in considerable changes in mucosal immune architecture, initiating proliferation and phenotypic differentiation of specific lymphocyte lineages (14). Compared to the peripheral blood, the appendix from three groups (acute appendicitis, gangrenous appendicitis and normal appendix) contained a significantly higher percentage of CD19⁺ CD5⁻ cells (B-2 B cells) and CD19⁺ CD5⁺ cells (B-1 B cells). These finding is in agreement with Somekh et al. that CD19 cells and B1 B cells were higher in the appendix against the peripheral blood (11). Here, we demonstrate that the percentage of CD19⁺CD5⁻ cells in patients with acute appendicitis was significantly higher than that from other groups but decreases after appendectomy, whereas the percentage of CD19⁺CD5⁺ cells remains relatively constant.

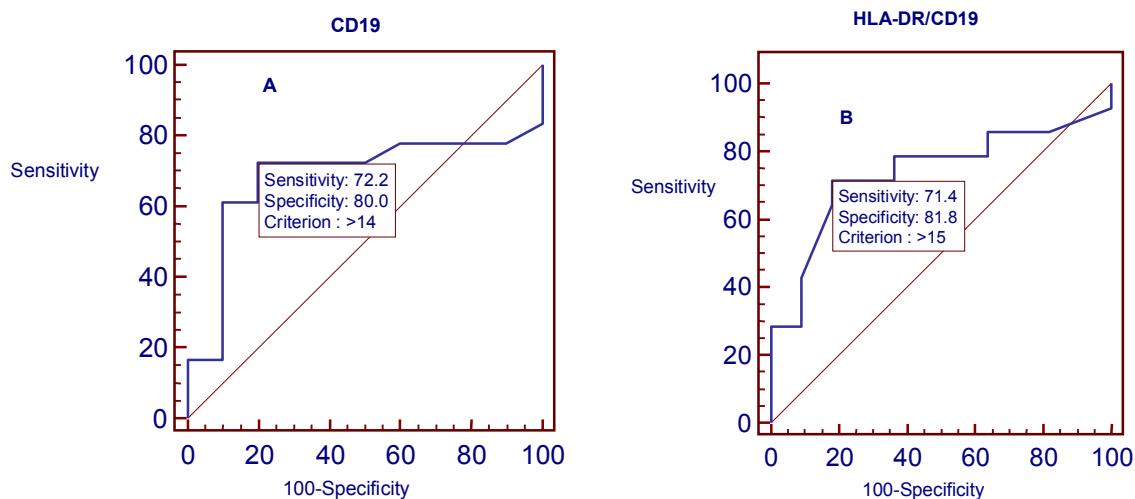


Figure 3. Receiver-operator characteristics for CD19 (A) and HLA-DR/ CD19 markers in peripheral blood.

Protective humoral immunity against invading pathogens is contributed by distinct subset of B cells, conventional or B-2 B cells ($CD5^-$) and B-1 B cells ($CD5^+$) (15). A major function of B-1 B cells is the production of polyspecific natural antibodies in steady-state in the apparent absence of antigenic or pathogenic stimulations (16). This provides the first line of humoral antibody-mediated immune defense before antigen-induced antibodies leads to increased susceptibility to infections with both viral and bacterial pathogens (17). B-1 B cells constitute roughly 1-7% of peripheral mononuclear cells in normal human adults (18). These cells have been reported to constitute 10-25% of the B cell population in adult secondary lymphoid tissues such as spleen, tonsils and lymph nodes. In conclusion, these results permit us to conclude that the phenotype of the appendiceal immunological competent cell population is unique in its composition of B cells. Appendix tissue contains higher number of B-1 and B-2 B cells compared to the peripheral blood and that these cells play a role in the primary immune response to acute infection/inflammation in the appendix. Our results show that the frequency of $HLA-DR^+CD19^+$ cells in appendix tissue of patients with acute appendicitis was significantly higher than that of other groups. This indicates that the increase in $HLA-DR^+$ cells may be an inflammation to gut antigens. In a gut-associated lymphoid tissue, such as appendix, food and bacterial antigens continuously prime lymphocytes. Activated lymphocytes play an important role in bowel inflammatory diseases (19). We also found that patients with appendicitis had no impaired lymphocyte proliferative response to PHA and Con-A.

Acute appendicitis is the most common cause of abdominal surgical emergencies all over the world, but its accurate diagnosis remains difficult. After obtaining a history and physical examination, the treating clinician(s) may decide to send the patient home without further testing, perform diagnostic imaging using ultrasound or computed axial tomography (CT scan), admit for observation, or proceed to the operating room (2). The costs of unnecessary surgery are balanced by an increased risk of perforation if the diagnosis is missed on initial evaluation. Perforated appendicitis results in greater morbidity (20). The routine laboratory tests are not accurate enough to diagnose or exclude appendicitis. Recently, many studies have highlighted the diagnostic value of serum biomarkers in acute appendicitis (21-23). Acute appendicitis is characterized by

peripheral blood lymphopenia and leukocyte infiltration to appendix tissue. The process of homing of lymphocytes from peripheral blood to tissue and vice versa may be affected the distribution of lymphocyte subsets. Our results on the Sensitivity and specificity of the lymphocyte CD show that the percentages of T lymphocyte subsets were not significantly different between peripheral blood and appendix tissue in all groups. There was no significant change in T-cell subsets in peripheral blood of patients with acute appendicitis or normal group at before and after appendectomy. Using a cut off value of CD19⁺ > 14% in our patient, CD19 marker appeared as a diagnostic tool with would a sensitivity of 72% and specificity of 80%. Value of the AUC-ROC for CD19 marker was 0.76, indicating a good discrimination power of the test. This marker can be a candidate tool to discriminate between normal and inflamed appendix tissue.

In conclusion, these results show that appendix tissue has a special lymphocyte profile Also, lymphocytes phenotypic changes (CD19⁺ cells) in peripheral blood of appendicitis patients may be used as a diagnostic marker for appendicitis. However, there is limited information on phenotype and function of lymphocytes in inflamed appendix tissue. More studies should be done to investigate the diagnostic value of lymphocytes subtypes as blood marker disease for determination of appendicitis

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