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ORIGINAL INVESTIGATION / ÖZGÜN ARAŞTIRMA

Toll-Like Receptor 2 Expression and Peripheral Blood CD4+/CD8+ T Cell Ratio in COPD

KOAH'da Periferik Kan CD4+/CD8+ T Hücre Oranları ve Toll Like Reseptor-2 Ekspresyonu

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Abstract

OBJECTIVES: We aimed to evaluate toll-like receptor 2 (TLR-2) expression on monocytes and peripheral blood CD4+/CD8+ T cell ratio, as well as the relationship of these cells with pulmonary functions in chronic obstructive pulmonary disease (COPD) patients.

MATERIAL AND METHODS: Forty COPD patients and 40 healthy volunteers were included. Participants were analysed in four groups according to their smoking status. Peripheral blood CD4+ and CD8+ T cells and monocyte TLR-2 expression were measured by flow cytometry in the whole study population.

RESULTS: No significant difference was observed in TLR-2 expression, number of CD4+ and CD8+ T cells, and CD4+/CD8+ T cell ratio between the study groups. CD4+/CD8+ T cell ratio and FEV_1/FVC were found to have a mild positive correlation (r=0.295, p=0.022). A mild negative correlation was observed between smoking intensity and CD4+/CD8+ T cell ratio (r=-0.274, p=0.034).

CONCLUSION: We demonstrated a mild correlation between pulmonary functions and peripheral blood CD4+/CD8+ T cell ratio. However, we did not find a significant difference in TLR-2 expression of CD14+ monocytes in patients with airway obstruction.

KEY WORDS: COPD, inflammation, innate immunity, CD4+/CD8+ T lymphocytes, TLR

Özet

AMAÇ: Bu çalışmada kronik obstrüktif akciğer hastalığı (KOAH) bulunan hastalarda monositer Toll-Like Reseptör-2 (TLR-2) ekspresyonu ve periferik kan CD4+/CD8+ T hücre oranlarının yanı sıra bu hücrelerin solunum fonksiyonlarıyla ilişkisinin araştırılması amaçlanmıştır.

GEREÇ VE YÖNTEMLER: Kırk KOAH'lı hasta ve 40 sağlıklı gönüllü çalışmaya dahil edildi. Çalışmaya alınan katılımcılar sigara içme durumlarına göre 4 grupta analiz edildi. Periferik kan CD4+/CD8+ T hücreleri ve monositer TLR-2 ekspresyonu "flow-sitometri" yöntemiyle çalışıldı.

BULGULAR: Çalışma grupları arasında TLR-2 ekspresyonu, CD4+, CD8+ T hücreleri ve CD4+/CD8+ oranları arasında istatistiksel anlamlı fark saptanmadı. CD4+/CD8+ oranları ve FEV_1/FVC arasında hafif düzeyde pozitif ilişki mevcuttu (r=0,295, p=0,022). Sigara içme yoğunluğu ve CD4+/CD8+ oranları ise hafif derecede ters ilişkiliydi (r=-0,274, p=0,034).

SONUÇ: Solunum fonksiyonları ve periferik kan CD4+/CD8+ oranları arasında hafif düzeyli bir ilişki saptamış olsak da, hava yolu obstrüksiyonu olan hastalarda CD 14+ monositlerdeki TLR-2 ekspresyonunda bir farklılık gözleyemedik.

ANAHTAR SÖZCÜKLER: KOAH, inflamasyon, doğal bağışıklık, CD4+/CD8+ T lenfositler, TLR

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD), defined as a preventable and treatable condition characterised by poorly reversible airflow limitation, is a global health problem [1]. Airflow limitation is usually progressive and associated with an abnormal response of the lungs to noxious particles or gases that eventually results in chronic airway inflammation [1]. A wide range of inflammatory cells such as macrophages, neutrophils, T and B lymphocytes, eosinophils, and epithelial cells are involved in the inflammatory process of COPD [2]. However, the mainstay of inflammation in COPD depends on macrophages, neutrophils, and CD8+ T lymphocytes [1].

Alveolar macrophages are located between the air and lung tissue and have an important role as first-line defence for both innate and acquired immunity [3]. Increased numbers of alveolar macrophages in the airways and parenchyma of COPD patients have been reported [4]. The role of the innate immune response is to discriminate between self cells and pathogens by receptors known as toll-like receptors (TLRs), which are expressed on the surface of the macrophages [5].

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TLRs are a family of transmembrane receptors that recognise specific pathogen-associated molecular patterns (PAMPs) and signal to initiate immune responses [6]. Eleven different TLRs have been defined to date [7]. TLRs also induce adaptive immune responses due to cytokine production after stimulation of the innate immune system [8]. TLR-2 recognises a wide range of components like lipoproteins, glycopeptides, peptidoglycan, and lipoarabinomannan [9]. It has been shown that human alveolar macrophages and type II alveolar epithelial cells express TLR-2 mRNA; endothelial cells also express TLRs [10-12]. The TLRs on airway monocytes and neutrophils may contribute to disease development and progression by inducing matrix metalloproteinase release [13]. Besides, an alteration in pathogen recognition of monocytes and alveolar macrophages may be important in COPD in terms of bacterial colonisation and airway inflammation [14].

T lymphocytes constitute the majority of the peripheral blood lymphocytes. They have T cell receptors (TCRs) on their surface, which easily distinguish them from other blood cell types. Alpha beta TCR molecules further differentiate T lymphocytes as CD4+ and CD8+ T cells [15]. CD4+ T lymphocytes are important in cellular and humoral immune responses, while CD8+ cells are cytotoxic to intracellular pathogens [15]. Increased numbers of CD8+ T cells have been established in small airways of COPD patients [16]. Pulmonary CD8+ T cell numbers and lung function impairment have been reported to be correlated. The CD4+/CD8+ T cell ratio in COPD patients has been demonstrated to be decreased [17]. Although COPD has been defined as a systemic disease, circulating T cells of COPD patients have less been investigated with respect to pulmonary T cells. There are studies reporting differences in the CD4+/CD8+ T cell ratio of COPD patients [18,19].

The aim of the present study was to analyse the features of peripheral inflammatory cell types such as T lymphocytes and monocytes, which have been reported to be involved in the chronic inflammatory process of COPD, since any predictive inflammatory marker for future development of COPD will lead to early diagnosis of airway obstruction. Therefore, we investigated the difference in CD4+ and CD8+ T lymphocyte numbers, the CD4+/CD8+ T cell ratio, and variations in TLR-2 expression of CD14+ monocytes of COPD patients and healthy controls with regard to the smoking status of the subjects. We also assessed the relationship between pulmonary function test results and the inflammatory cells that were evaluated.

MATERIAL AND METHODS

Between April 2008 and October 2009, 20 current smoker and 20 nonsmoker (who were ex smokers that quitted at least 20 years ago) consecutive COPD patients who were referred to our pulmonary diseases outpatient clinic and 20 current smoker and 20 nonsmoker healthy controls among family members of hospitalised patients were recruited in the study. Inclusion criterion for COPD patients was being in a stable state (no exacerbations in the last 6 weeks). COPD was defined as a history of smoking more than 20 pack-years and a FEV_1/forced vital capacity (FVC) ratio of less than 70% 20 minutes after salbutamol administration [20]. Inclusion criterion for the control group was the absence of COPD confirmed by history, physical evaluation, and/or spirometry where necessary. Exclusion criteria for both COPD patients and controls were: having other inflammatory diseases (inflammatory bowel disease, rheumatological diseases, vasculits); having acute infections; and having respiratory diseases other than COPD. The study was approved by the local human-research review board (February 14 2008/0030) and all participants provided written informed consent.

Demographic features and medical history including smoking status of the study population were recorded. Nonsmokers were defined as never smokers or ex smokers. Those with a history of less than 10 years of smoking or who quit smoking at least 20 years ago were accepted as ex smokers. COPD patients (groups 1 and 2) and smoker controls (group 3) underwent pulmonary function tests. Venous blood samples were obtained from all participants.

Pulmonary Function Tests

Pulmonary function tests were performed with a Jaeger Master Screen Pneumo V452I (Germany) device by a single technician. The best test among three consecutive tests was accepted. FEV_1, FVC, and FEV_1/FVC were measured according to ATS criteria [20]. COPD staging was done according to GOLD 2009 [1].

Assessment of CD4+ and CD8+ T Lymphocyte Subgroups and TLR-2 Expression

Peripheral blood samples were obtained and collected into tubes that contained ethylene diamine tetra acetic acid (EDTA) preservative. Peripheral blood leukocytes, CD4+ and CD8+ T lymphocyte subgroups, and TLR-2 receptors on CD14+ monocytes were assessed daily by a flow cytometric method. CD4+ and CD8+ T lymphocyte subgroups were assessed by commercial antibodies (IO Test CD4-FITC/CD8-PE, Immunotech SAS, Beckman Coulter Company, Marsille Cedex, France). Isotypic controls were assessed as negative controls (CD4/CD8 combined isotype controls IgG1 FITC/PE/PC5, Immunotech SAS, Beckman Coulter Company, Marsille Cedex, France).

TLR-2 expression on CD14+ monocytes was investigated by antibodies (BD Pharmingen Alexa Flour 488 Mouse Anti-Human CD 282, BD Pharmingen PE Mouse Anti-Human CD 14 BD Biosciences, USA). Isotopic controls (BD Pharmingen Alexa Flour 488 Mouse IgG1 κ Isotype Control and BD Pharmingen PE Mouse IgG2a κ Isotype Control BD Biosciences, USA) were used as negative controls.

Erythrocyte lysis was performed on analyser (Coulter TQ-Prep Beckman Coulter Inc., Fullerton, CA, USA) using original reagents (Immunoprep Reagent System (Beckman Coulter Inc., Fullerton, CA, USA). Antibody- and control-added blood specimens were assessed on a flow cytometry analyser (Cytomics FC 500, Beckman Coulter Inc., Fullerton, CA, USA). Data were analysed by original software (CXP Cytometer 2.2 Beckman Coulter Inc., Fullerton, CA, USA).

Statistical Analysis

Data were analysed by the Statistical Package for the Social Sciences (SPSS) 10.0 program package. Groups were compared by unpaired t tests and one-way analysis of variance (ANOVA) and covariance analysis for continuous variables.

Table 1. Demographic features and functional parameters of the study population

	Group 1 Nonsmoker COPD (n=20)	Group 2 Smoker COPD (n=20)	Group 3 Smoker control (n=20)	Group 4 Nonsmoker control (n=20)	p value**
Age (years)*	64.6±7.4	58.6±12.0	29.7±7.2	47.4±6.3	<0.001
Gender (n) (female/male)	2/18	0/20	9/11	10/10	<0.001†
Smoking					
(package/year)	0	52.2±24.7	11.9±10.4	0	<0.001††
Steroid therapy (n)	8	7			
FEV_1 (%)*	52.1±17.7	52.6±19.3	100.2±8.1	-	<0.001
FEV_1 (L)*	1.5±0.6	1.7±0.8	3.7±1.1	-	<0.001
FVC (%)*	67.7±17.3	70.6±21.7	103.5±10.4	-	<0.001
FEV_1/FVC*	58.1±12.5	56.9±11.0	82.2±6.3	-	<0.001

COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity
*Values are expressed as mean±standard deviation (SD). p<0.05 was considered significant
†Chi square test
††Student's t test
**One-way analysis of variance (ANOVA) with least significant difference (LSD) as a post hoc comparison test
Smoker control < nonsmoker control < smoker COPD < nonsmoker COPD for age
Nonsmoker COPD = smoker COPD < smoker control for other comparisons

Table 2. Proportions of peripheral blood toll-like receptor 2 expression on CD14+ monocytes, CD4+ and CD8+ T cell rates, and CD4+/CD8+ T cell ratios in the study population

	Group 1 Nonsmoker COPD (n=20)	Group 2 Smoker COPD (n=20)	Group 3 Smoker control (n=20)	Group 4 Nonsmoker control (n=20)	p value**
TLR-2 expression (%)	56.41±24.49	64.47±22.93	64.18±23.47	51.82±24.79	0.269
CD4+ (%)	33.72±10.9	33.31±14.15	38.64±12.79	38.83±15.59	0.396
CD8+ (%)	29.21±12.08	25.76±9.85	25.23±7.89	24.88±9.06	0.486
CD4+/CD8+	1.41±0.74	1.33±0.47	1.58±0.56	1.65±0.64	0.310

COPD: chronic obstructive pulmonary disease; TLR-2: toll-like receptor 2
*Values are expressed as mean±standard deviation (SD). p<0.05 was considered significant
**Covariance analysis, adjusted for age and sex

Table 3. CD4+ and CD8+ T cell distribution and CD4+/CD8+ T cell ratio according to age in the study population

	<49 (n=36)	50–64 (n=27)	>65 (n=17)	p value
CD4+ (%)	37.43±13.89	38.11±13.26	30.21±11.86	0.122
CD8+ (%)	25.11±8.45	25.61±8.98	29.75±13.07	0.253
CD4+/CD8+	1.56±0.61	1.59±0.61	1.18±0.56	0.064

Values are expressed as mean±standard deviation (SD). p<0.05 was considered significant

The group making the difference was analysed by post hoc comparison (least significant difference). Normality assumption was checked by Kolmogorov Smirnov test. Chi-square analysis was used for categorical data. The relationship between continuous variables was analysed using Pearson's correlation test. A p value of <0.05 was considered statistically significant.

RESULTS

Demographic and functional characteristics of the study population are depicted in Table 1. Patients were staged according to GOLD criteria [1] and 7.5% of the patients were in stage 1, 55% were in stage 2, 25% were in stage 3, and 12.5% were in stage 4.

TLR-2 expression rates on CD14+ monocytes of the study groups are shown in Table 2. We did not find a statistically significant difference among the study groups (p=0.269). However, smokers (64.33±22.91) had numerically higher TLR expression rates than nonsmokers (54.12±22.44). CD4+ and CD8+ T cell rates as well as CD4+/CD8+ T cell ratios were also not significantly different among the four groups (Table 2). Additional covariance analysis was performed in order to eliminate the effect of age and gender on the results, but a significant difference was still not detected (p>0.05).

CD4+ T cell rate (p=0.002) and CD4+/CD8+ T cell ratio (p=0.0001) were found to be different among the groups when analysed according to gender. However, there was no difference between the groups for CD4+ and CD8+ T cell rates and CD4+/CD8+ T cell ratio when analysed according to age (Table 3).

CD4+ and CD8+ T cell rates and CD4+/CD8+ T cell ratio were compared with pulmonary function tests. CD4+ T cells were found to be positively correlated with FEV_1 (r=0.311, p=0.016) and FEV_1/FVC (r= 0.293, p=0.023). CD4+/CD8+ T cell ratio was also found to be correlated with FEV_1/FVC (r=0.295, p=0.022) (Figure 1). Smoking history was found to be negatively correlated with CD4+/CD8+ T cell ratio.

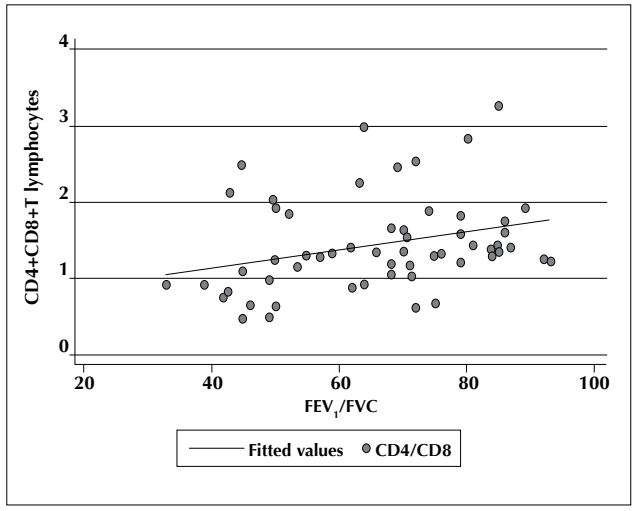

Figure 1. Correlation of CD4+/CD8+ T cell ratio with FEV_1/FVC in the patients and smoker controls

DISCUSSION

COPD is a chronic disease with important an economic and social burden as a consequence of high morbidity and mortality related to the disease [1]. However, despite great attempts in diagnosis and treatment of COPD, most of the patients are underdiagnosed [1]. FEV_1 has been used as a diagnostic and prognostic tool for many years; nevertheless, it is incapable of evaluating the disease's multidimensional nature. Currently, accurate and feasible biomarkers other than FEV_1 are necessary for the diagnosis and follow-up of COPD. Also, understanding the pathophysiological mechanisms in COPD may help us to develop new therapeutic modalities.

Chronic inflammation is the major underlying pathology in COPD. Adaptive immunity has been intensively investigated; however, innate immunity has recently been recognised as a new therapeutic target in many of the chronic inflammatory diseases. TLRs are a family of interleukin receptors and play an important role in both innate and adaptive immunity [21]. Innate immunity activates adaptive immunity by TLRs, which have a critical role in antigen presentation [22]. Monocyte-dependent TLR-induced inflammatory reactions can evoke the activation of airway smooth muscle [23,24] and neutrophils by which antimicrobial signalling is mediated [25,26]. TLRs not only play a role in infective inflammatory responses and but also in non-infective processes. It has been reported that TLRs are involved in the pathophysiology of acute respiratory distress syndrome (ARDS), asthma, and COPD [27]. In COPD, TLR-mediated interactions between monocytes and smooth muscle cells lead to matrix metalloproteinase release that plays an important role in parenchymal injury [13]. On the other hand, TLRs are critical in protective immunity as their participation in the defence against viral and bacterial infections of the airways is indispensable. TLR-2 expression, which has been reported to be involved in the recognition of *P. aeruginosa,* was found to be depleted on circulating neutrophils in cystic fibrosis patients [28,29].

Droemann et al. [30] showed decreased TLR-2 expression on alveolar macrophages of COPD patients and smokers. Similarly, Pons et al. [31] found reduced anti-inflammatory capacity of macrophages in COPD patients with respect to smokers without airflow limitation. In another study that investigated TLR-2 and TLR-4 expression on CD14+ monocytes of COPD patients and healthy controls, TLR-2 expression in COPD patients (52.9±20.5%) was found to be lower than that in the healthy controls, independent of smoking status [32]. A positive correlation between TLR-2 expression and pulmonary functions in terms of FEV_1% and FEV_1/FVC was also reported. However, in another study, Pons et al. [33] reported upregulated TLR-2 expression on peripheral blood monocytes of COPD patients either in the stable state or exacerbation. There was no difference between smoker and nonsmoker healthy controls, while TLR-2 expression was significantly higher in smokers with COPD than in the smoker controls. Furthermore, systemic steroids were found to cause dose-dependent reduction in TLR-2 expression. Another recent study reported no difference in TLR-4 and TLR-9 expression in lung tissue and peripheral blood of COPD patients, while lung CD8+ T cells was found to express increased TLR-4 and TLR-9 [34]. Similarly, we did not find any statistically significant difference in CD14+ monocyte TLR-2 expression among our study groups (p=0.269). However, although statistically insignificant, the proportion of TLR-2 expression was higher in the COPD group compared with healthy controls both for smokers and nonsmokers and the lowest TLR-2 expression was found in healthy nonsmokers. This may be due to the smoking-induced upregulation of TLR-2 receptors as well as the biological variations and small number of subjects in the groups.

As discussed above, the results of studies on TLR-2 expression of the monocytes in COPD patients are controversial. This may be related to steroid treatment and/or bacterial colonisation in some COPD patients. Detailed and well-conducted population-based studies are required to demonstrate the relationship between pulmonary functions and TLR-2 expression in patients with airflow limitation.

Local inflammation in COPD patients is characterised by increased numbers of neutrophils, macrophages, and particularly CD8+ T lymphocytes [16]. In very severe COPD, CD4+ T lymphocytes have also been shown to be increased [35]. Circulating lymphocyte populations in COPD patients and smokers have not been investigated much. A relative decrease in peripheral blood CD4+ T lymphocytes and increase in CD8+ T lymphocytes has been reported in heavy smokers as well as nonsmoker COPD patients [36,37]. It has been hypothesised that T lymphocyte variations might be important in the pathogenesis of airflow limitation. Glader et al. [38] investigated the correlation between lung functions and peripheral blood CD4+ and CD8+ T cells and proportions of lymphocyte populations in COPD patients and matched smokers. The number of CD4+ T cells was found to be higher in smokers than in both never smokers and COPD

patients. CD4+ T-cells with CD69 expression and lung function were found to be correlated in smokers regardless of airflow limitation. The authors concluded that increased peripheral CD69+ CD4+ T cells might be protective against airway obstruction in smokers [38]. Kulawik et al. [39] found that CD8+ T lymphocyte proportions were higher in COPD patients with respect to healthy smokers, although this difference was statistically insignificant. However, they demonstrated that CD4/CD8 rates were significantly lower in COPD patients than in healthy smokers ($p<0.05$). FEV_1/FVC showed a negative correlation with CD8+ T cells and a positive correlation with CD4/CD8 rates [39]. Similarly, another study showed that CD8+ T cells were significantly increased in patients with COPD than in healthy smokers [40].

Kim et al. [18] did not find significant differences in circulating T lymphocyte subsets among healthy smokers, nonsmokers, and COPD groups. However, when they grouped the study population according to physiological indices, they found that the normal diffusing capacity of the lung for carbon monoxide per unit of alveolar volume (DLCO/VA) subgroup had a significantly higher proportion of CD8+ T lymphocytes and a lower CD4+/CD8+ T cell ratio than the healthy smokers or the low DLCO/VA subgroup [18]. In our study, we also showed no statistically significant difference in CD4+ T cells, CD8+ T cells, and CD4+/CD8+ T cell ratio between COPD patients and healthy controls regardless of smoking status. However, our results demonstrated that FEV_1 and FEV_1/FVC were greater in patients with increased CD4+ T cells ($r=0.311$, $p=0.016$ and $r=0.293$, $p=0.023$, respectively). The correlation coefficients were similar to those of Glader and Kluwik's study [38,39]. Although a statistically significant relationship for CD8+ lymphocytes and pulmonary functions was not established in our data, a mild negative correlation was observed between CD8+ T cells and FEV_1 and FEV_1/FVC. Smoking history was also found to be negatively correlated with CD4+/CD8+ T cell ratio. We interpreted these results as the reflection of smoking burden and airway obstruction to peripheral CD4+/CD8+ T cell ratios. However, the results of the studies investigating peripheral T lymphocyte subtype proportions in COPD patients are controversial. Therefore, well-conducted and comprehensive studies are needed to highlight this issue.

We did not demonstrate a difference in TLR-2 expression of peripheral blood monocytes or in CD4+ and CD8+ T cell distribution. We suggest that TLR-2 expression is not a feasible marker of airway inflammation. However, the TLR family is an interesting area of research and therapies targeting these receptors might be popular in the near future. The positive correlation of pulmonary functions and CD4+/CD8+ T lymphocyte ratio is promising in the evaluation of the pathophysiology of airflow limitation. Thus, we believe that CD4+/CD8+ T cell ratio may be used as one of the biomarkers for the pathogenesis of COPD.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Celal Bayar University Faculty of Medicine.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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