석사학위논문

육아종성다발혈관염 환자에서 단핵세포 및 거대세포의 염증반응의 저하에 관한 연구

Decreased Inflammatory Response of Monocytes and Macrophages in Patients with Granulomatosis with Polyangiitis

2014년7월

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이 논문을 박 진 균 석사학위논문으로 제출함

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ABSTRACT

Background: Granulomatosis with polyangiitis (GPA), formally known as Wegner's granulomatosis, is characterized by the necrotizing vasculitis involving medium and small vessels and granulomatous inflammation. Sinonasal airway of GPA patients is heavily colonized with bacteria despite extensive inflammatory infiltrates, indicating an impaired local immunity in patients with GPA. This study is aimed to investigate whether monocytes in patients with GPA are polarized towards alternative activation with a decreased immune response including tumor necrosis factor (TNF)-α production and whether the tissue infiltrating monocytes/macrophages in granulomatous GPA lesions express CD163, a marker of alternative macrophage activation.

Methods: CD16+ monocytes in peripheral blood mononuclear cells (PBMCs) from GPA patients (n=23) and health controls (n=14) were quantified by flow cytometry. Monocytes were stimulated with increasing concentrations of lipopolysaccharide (LPS), and TNFα production was measured at 4 and 24 hours using flow cytometry analysis and enzyme-linked immunosorbent assay. CD163 expression in lung biopsies of patients with GPA was detected by immunohistochemistry.

Results: Circulating CD16+ monocytes were more frequent in GPA patients compared to controls (4.7 ± 2.8% vs. 1.9% ± 1.2%, $P < 0.001$). Upon activation with LPS, TNFα production did not differ between CD16+ and CD16- monocytes. Activated monocytes from GPA patients produced

significantly less TNFα as compared with monocytes from healthy controls (2,903 ± 1,320 pg/mL vs. 8,335 ± 4,569 pg/mL, P<0.001). CD163 expression on surface of activated monocytes decreased with increased production of TNFα. Further, CD163+ macrophages were abundantly present in granulomatous lesions of GPA lungs.

Conclusions: Decreased TNFα production by circulating monocytes and CD163 overexpression by tissue monocytes/macrophages in granulomatous pulmonary lesions suggest that alternative activation of monocytes/macrophages might play an important role in GPA pathogenesis. Treatment to restore the normal monocyte differentiation might offer a new therapeutic target.

Key words: Granulomatosis with polyangiitis, monocytes, alternatively activated macrophages, CD163, inflammatory response.

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INTRODUCTION

Granulomatosis with polyangiitis (GPA), formerly known as Wegener's granulomatosis, is characterized by necrotizing vasculitis of medium and small sized vessels and granulomatous inflammation [1]. The granulomatous inflammation in GPA can involve any organ. It contains cells that differentiate from the monocyte lineage, i.e. macrophages, dendritic cells, epitheloid cell and multinucleated giant cells (MNGs) [2]. Monocytes within the peripheral blood mononuclear cells (PBMCs) in patients with GPA had a higher propensity to form MNGs compared to healthy controls and that this propensity was associated with the extent of systemic organ involvement, but not with disease activity or duration [3], suggesting the propensity of GPA monocytes to generate MNG as a key feature in GPA pathogenesis. Interestingly, this propensity is lost after monocytes are activated with inflammatory stimuli such as toll like receptor 2 and 4 agonists [4].

Activated monocytes are a major source of tumor necrosis factor alpha (TNFα), which promotes inflammation and aids in granuloma formation [5]. Specifically, circulating proinflammatory monocytes, which are phenotypically defined by the surface expression of CD16, have been reported to produce high amounts of TNFα and interleukin (IL)-1 upon activation [6]. These CD16+ monocytes are, indeed, accumulated in both infectious and systemic autoimmune diseases including sepsis and rheumatoid arthritis (RA)

[7]. Accordingly, treatment directed against TNFα has dramatically improved the prognosis of patients with RA and psoriatic arthritis [8, 9]. In contrast, such therapeutic benefit of anti-TNFα therapy was not observed in patients GPA. This lack of additional benefit of anti-TNFα therapy in GPA suggests that TNFα signaling may play a less important role in this disease development than expected, and that monocytes in GPA may contribute to disease progression by other mechanisms such as granulomatous inflammation [10].

The upper respiratory tract of GPA patients is heavily colonized with bacteria despite extensive local inflammation. This may indicate that local immunity in GPA is impaired despite the inflammatory state [11, 12]. Similar impaired immune response was observed in chronic sinusitis, where tissue infiltrating monocytes and macrophages express CD163 [13]. Expression of CD163, a scavenger molecule, defines a subset of monocytes/macrophages that are alternatively activated and they orchestrate resolution of inflammation and would repair [14, 15]. Taken together, monocytes in GPA might be already polarized towards alternative activation with a higher propensity for granulomatous inflammation at the expense of proinflammatory response with subsequent impaired immunity.

This study is aimed to investigate whether monocytes in GPA are alternatively activated with decreased inflammatory response including TNFα production and whether alternatively activated CD163+

monocytes/macrophages are abundant in granulomatous lesions of GPA patients.

Materials and Methods

Study population

GPA patients who received clinical care and healthy control subjects were enrolled in the study (Table 1). Informed consent was obtained under the auspices of an Institutional Review Board-approved research protocol. Paraffin-embedded lung tissues from GPA patients and controls were obtained from the Pathology archive.

Cell isolation and activation

PBMCs were isolated from heparinized peripheral venous blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare, NJ, USA). Monocytes were isolated using CD14 Microbeads as per manufacturer's instructions (MACS, Miltenyi Biotec).

PBMCs or CD14+ cells (1 x 10^6 cells per well) were activated in RPMI, supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, and 1% streptomycin, in the presence of 10, or 100 ng/mL lipopolysaccharide (LPS) (InvivoGen, catalog # tlrl-eblps, San Diego, CA, USA). Cells were incubated in 5% carbon dioxide at 37°C for 4 hours or 24 hours, respectively. Supernatants were collected for enzyme-linked immunosorbent assay (ELISA).

Flow cytometry analysis

Activated PBMCs were incubated with anti-human CD14 PerCP (BD

Biosciences, San Jose, CA, USA) and anti-human CD3 antibodies (BD Biosciences, San Jose, CA, USA) for 30 minutes at 4℃. Intracellular staining of TNFα was performed using anti-human TNFα antibodies (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction (GolgiStop, Pharmigen, San Diego, CA, USA). Cells were analyzed on a FACSAria III (BD, San Jose, CA, USA). Flow data were analyzed by FlowJo software version 8.8 (Treestar, Ashland, OR, USA).

Enzyme-linked immunosorbent assay (ELISA) for TNFα

TNFα levels were measured using a commercially available ELISA kit (R&D Systems). The optical density was determined using a microplate reader (Biotrack, Pharmacia) set at 450 nm. TNFα concentrations were extrapolated from a standard curve and expressed in pg/mL. All samples were assayed in duplicate.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were de-paraffinized with xlyenes and rehydrated. After antigen retrieval and blocking, tissues were incubated with antibodies directed against CD68 (DAKO) and CD163 (MAB1652, Abnova, Walnut, CA, USA). The staining was visualized with 3,3-diaminobenzidine (DAKO). Nuclei were counterstained with Mayer's hematoxylin.

Statistical Analysis

Student t-test and Mann Whitney tests were used for continuous variables as appropriate. Correlations between data were performed using Spearman correlation. P-values <0.05 were considered significant. Statistical analyses were computed in GraphPad Prism (La Jolla, CA, USA).

RESULTS

CD16+ monocytes are more frequent in GPA

First, the percentage of CD16+ monocytes in PBMCs of patients with GPA (n=23) and healthy subjects (n=14) was determined. PBMCs in GPA showed a significantly higher percentage of CD16+ monocytes than healthy controls (4.7 ± 2.8% vs. 1.9% ± 1.2%, P <0.001) (Figure 1). However, there was no association between CD16+ monocyte levels and GPA disease activity (Spearman r=-0.105, P=0.65) or disease duration (Spearman r=0.288, P=0.21) (Figure 2). Of note, patients in the cohort had a relatively low disease activity with Birmingham Vasculitis Activity Score (BVAS) ranging from 0 to 5 (Table 1).

Monocytes in GPA produce less TNFα

As M-CSF, a cytokine crucial for the generation of alternatively activated macrophages with anti-inflammatory property [16, 17], is known to induce the expression of CD16 on monocytes, it is possible that CD16+ monocytes in GPA are not as proinflammatory as observed in other inflammatory conditions. As such, the inflammatory response of CD16+ monocytes in GPA was examined. Of note, monocytes from GPA patients and controls expressed toll-like receptor (TLR) 4 on their surface to comparable degree (Figure 3). Upon activation with LPS, the percentage of TNFα producing cells did not differ in the CD16- and CD16+ monocyte fractions of both GPA patients and controls

(in controls, 52.2 ± 1.42% vs. 59.0 ± 3.9%, P=0.06; in GPA 44.1 ± 16.3% vs. 52.4 ± 10.5%, P=0.20) (Figure 4A).

However, monocytes from patients with GPA (n=3) produced significantly less TNFα after 4 hours of stimulation with LPS than healthy controls (n=3) (for 10 ng/mL LPS, 7,254 ± 1,004 MFI vs. 4,015 ± 2,322 MFI, P=0.08; for 100 ng/mL LPS, 9,562 ± 1,219 MFI vs. 4,855 ± 2,322 MFI, P= 0.04) (Figure 4B). To quantify levels of secreted TNFα, 2 x 10^5 isolated monocytes from healthy controls (n=3) and GPA patients (n=3) were stimulated with LPS for 24 hours. Monocytes from healthy controls produced TNFα in a dose-dependent manner, whereas the secretion of TNFα in GPA plateaued with 10 ng/mL LPS. At 100 ng/mL LPS, monocytes of healthy controls produced significantly more TNFα compared to those from GPA patients (8,335 ± 4,569 pg/mL vs. 2,903 ± 1,320 pg/mL, P <0.01) (Figure 4C).

CD163+ cells are abundant in GPA lung granuloma

Cheadle et al previously demonstrated that the transcriptome of active GPA patients differs from healthy controls [18]. Interestingly, CD163 was one of the most up-regulated transcripts in active GPA patients. To investigate if the inflammatory lesions in GPA reflect these peripheral transcriptional changes, we stained lung tissues (n=4) for expression of CD163. In healthy lung, alveolar macrophages expressed little to no CD163 (Figure 4A). By contrast, monocytes/macrophages in the GPA alveolar space adjacent to the inflammatory lesions expressed high amounts of CD163 (Figure 4B). As the

TNFα levels could not be examined in lung tissue due to technical limitation, the association between expression levels of CD163 and TNFα production was examined after *in vitro* activation. Surface expression of CD 163 inversely correlated with amount of TNFα production (Figure 6).

DISCUSSION

The present study demonstrates that circulating monocytes in GPA patients produce less TNFα upon stimulation as compared to healthy controls, and that lesional monocytes/macrophages in granulomatous inflammation strongly express CD163, a marker of the alternatively activated macrophages with anti-inflammatory and wound-healing properties [15].

Circulating proinflammatory monocytes, characterized by high TNFα production and the surface expression of CD16, have been reported to be enriched in infectious and autoimmune diseases [7, 19]. In this study, CD16+ monocytes were found to be enriched in patients with GPA but this was not associated with disease activity or duration (Figure 1 and 2). Similar observation was made that the levels of activated monocytes that express CD11b and CD64 were elevated regardless of GPA disease activity [20]. This raises a question whether CD16 expression is truly a specific marker of the proinflammatory state or a general marker of activation and differentiation of monocytes in GPA pathogenesis. Indeed, Chui *et al* showed that cytokines including M-CSF, a crucial cytokine in alternative activation of monocytes, can

induce monocytes to express CD16 and that those CD16+ monocytes can differentiate into osteoclasts, i.e. specialized multinucleated giant cells (MNGs) with bone resorptive capacity [16]. In the present study, CD16+ and CD16- monocytes in GPA and controls did not differ in TNFα production (Figure 4A), indicating that those cells might not be more pro-inflammatory than CD16- monocytes in GPA. Although the functional role of CD16+ monocytes in GPA pathogenesis remains to be defined, it is tempting to speculate that CD16+ monocytes contribute to granulomatous inflammation by increased MNG formation in addition to regulating other immune cells [7, 21].

The decreased TNFα production of monocytes in GPA as compared with healthy controls is surprising and contrasts the previous findings in systemic lupus erythematosus (SLE), a chronic systemic autoimmune disease; Sule *et al* demonstrated that monocytes in patient with SLE produced more TNFα after activation with apoptotic cells and the production was independent of disease activity of SLE [22]. One possible explanation for the decreased TNFα production in GPA may be the phenomenon known as LPS tolerance in which activated monocytes produce less proinflammatory cytokines after repetitive LPS stimulations [23]. It is possible that circulating monocytes are already activated in GPA, expressing higher level of CD16, and therefore are

less responsive to *in vitro* LPS stimulation. Of note, all monocytes from GPA patients and healthy controls expressed TLR4, the receptor for LPS, on their surface. Another tempting explanation comes from the previous observation that circulating GPA monocytes had a higher propensity to form MNGs compared to control monocytes and this MNG formation did not correlate with disease activity or duration, but with the extent of systemic manifestation [3]. Therefore, monocytes in GPA might be alternatively activated and exhibit an increased propensity to form granulomatous inflammation at the expense of inflammatory response including TNFα production. Indeed, cytokines such as IL-4 and M-CSF can promote both MNG formation and alternative activation of monocytes [15].

The abundance of CD163 expressing cells indirectly suggests that monocytes/macrophages in the granulomatous inflammation of GPA lung produce less TNFα *in situ,* as TNFα production inversely correlates with CD163 expression (Figure 6). In GPA, monocytes may preferentially differentiate into MNGs, contributing to the granulomatous inflammation. Similarly, a strong CD163 expression was observed in cells forming granuloma in sarcoidosis, a systemic granulomatous disease of unclear etiology [24]. Possibly, impaired cellular immunity with decreased TNFα production may contribute to

the higher rate of bacterial colonization of sinonasal inflammation in GPA as well [11, 12].

This study has several limitations. The functional studies were performed with monocytes from a relatively small number of GPA patients who were receiving immunosuppressive therapy. As such, the decreased TNFα production could result from effects of the immunosuppressive treatment on monocyte function. Further studies are needed to investigate whether monocytes from untreated GPA patients with quiescent and high disease activity exhibit the decreased TNFα production. In addition, the TNFα production by tissue infiltrating monocytes/macrophages in vivo could not be directly examined due to limited accessibility to fresh GPA lung samples.

In conclusion, GPA monocytes produce less TNFα upon activation and express CD163 as a possible result of alternative monocyte activation. Understanding the mechanisms regulating monocyte polarization and differentiation might provide new insights in GPA pathogenesis and novel therapeutic opportunities.

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Table 1. Characteristics of the patients with granulomatosis with polyangiitis.

	Age	Sex	Disease duration (years)	BVAS	Type	ANCA type	Medication
1	27	F	5	4	Systemic	PR3	AZA/Pred
2	28	F	6	5	Systemic	PR3	AZA/Pred
3	60	F	6	0	Limited	PR3	AZA/Pred
4	46	F	3	1	Limited	Negative	AZA/Pred
5	42	F	3	0	Systemic	Negative	AZA
6	31	M	1	0	Systemic	PR3	AZA/Pred
7	52	M	13	1	Systemic	PR3	CTX/Pred
8	74	M	7	2	Systemic	MPO	RTX/Pred
9	32	F	12	2	Systemic	PR3	None
10	79	F	2	0	Systemic	MPO	AZA/Pred
11	74	M	1	4	Systemic	PR3	MTX/Pred
12	72	F	6	1	Systemic	PR3	AZA/Pred
13	59	F	2	5	Systemic	Negative	Pred
14	48	F	2	2	Limited	PR3	None
15	47	F	3	2	Limited	PR3	CTX/Pred
16	75	M	34	0	Systemic	PR3	AZA/Pred
17	64	M	3	1	Systemic	PR3	AZA
18	31	F	5	1	Limited	PR3	AZA/Pred
19	36	F	2	1	Limited	PR3	AZA/Pred
20	72	M	2	0	Systemic	Negative	None

21	71	M	3	0	Systemic	Negative	AZA
22	51	F	5	2	Limited	PR3	AZA
23	52	F	6	2	Systemic	MPO	RTX/MMP/Pred

ANCA, anti-neutrophil cytoplasmic antibody; AZA, azathioprine; BVAS, Birmingham vasculitis activity score; CTX, cyclophosphamide; F, female; M, male; MMP, mycophenolate mofetil. MPO, myeloperoxidase; PR3, proteinase 3; Pred, prednisolone; RTX, Rituximab.

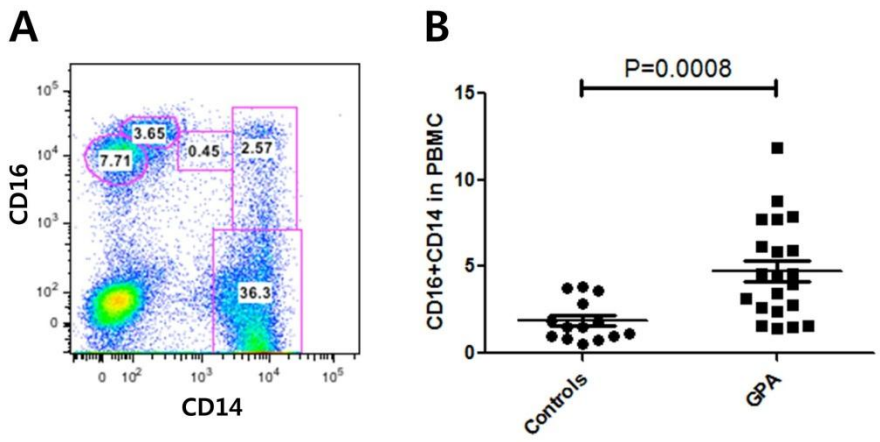

Figure 1. Increased frequencies of CD16+ monocytes in patients with GPA. PMBCs were stained with anti-human CD14 and CD16 antibodies. There were CD14+ monocytes which expressed CD16 cells (A). Percentages of CD16+ CD14 monocytes in PBMCs were determined from 22 GPA patients and 14 healthy controls. CD16+ monocytes were significantly enriched in patients GPA as compared to healthy controls (4.8 ± 2.8% vs. 1.0 ± 1.2%, P <0.001) (B).

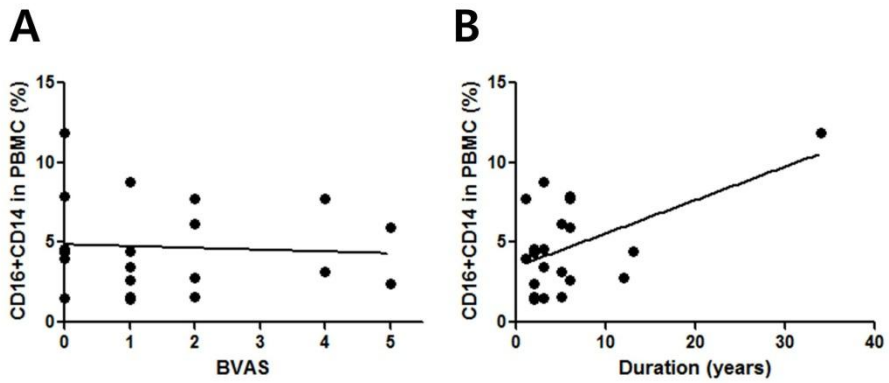

Figure 2. Frequency of CD16+ monocytes are independent of disease activity in GPA. The levels of CD16+ monocytes in PBMCs did not correlate with disease activity, expressed as Birmingham vasculitis activity score (BVAS) (A), or with disease duration (B) in patients with GPA.

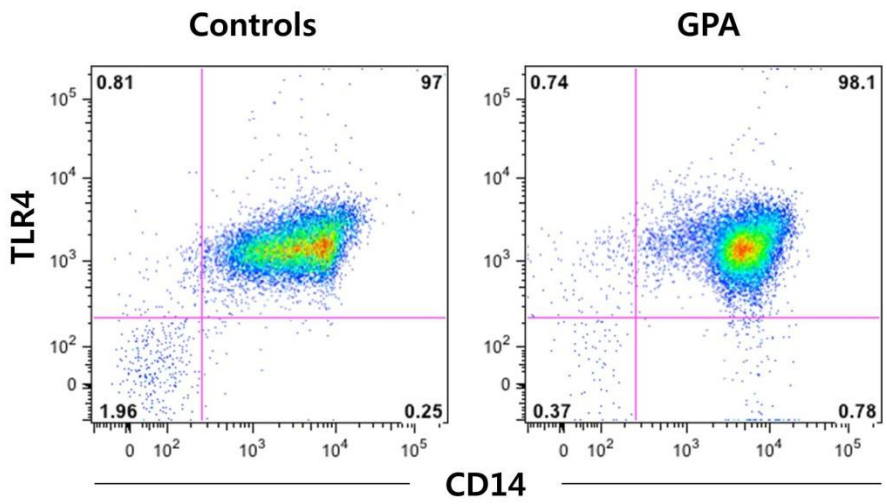

Figure 3. Toll-like receptor (TLR)-4 expression on monocytes. Isolated monocytes were stained with anti- CD14 and TLR4 antibodies. All CD14+ monocytes expressed TLR4 on their surface.

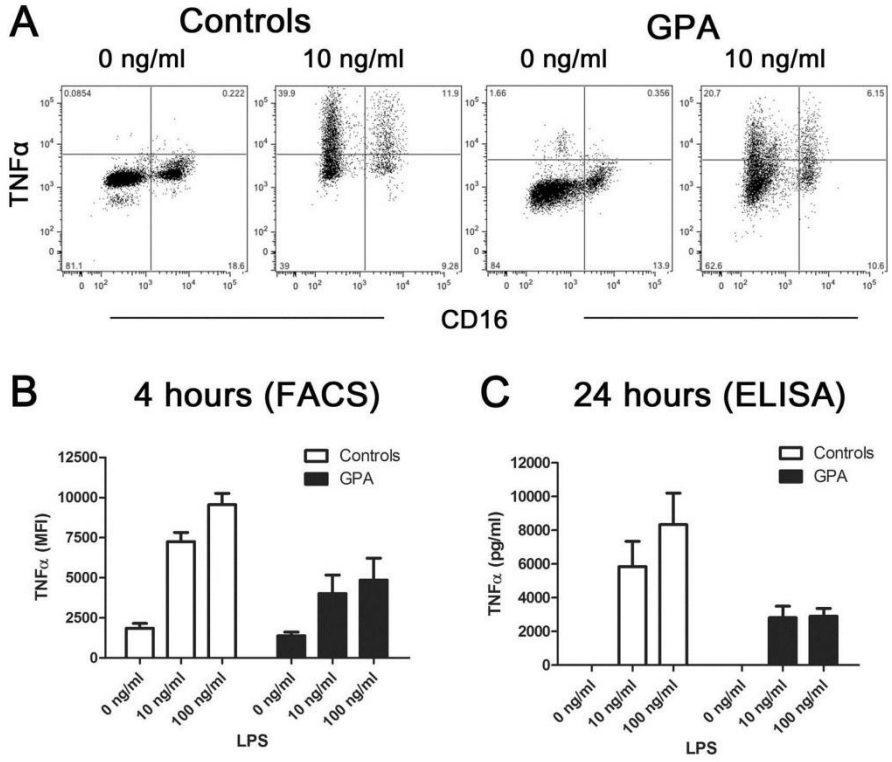

Figure 4. GPA monocytes produce less TNFα upon activation. PBMCs from healthy (n=3) and GPA (n=3) were stimulated with LPS for 4 hours. Both CD16+ and CD16- CD14+ monocytes from healthy controls and GPA produced TNFα (A, gated on CD14 positive cells). After 4 hour stimulation with 10 or 100 ng/mL LPS, CD14+ monocytes from GPA produced significantly less TNFα (B). Isolated CD14+ monocytes from patients with GPA (n=3) and controls (n=3) were activated with LPS for 24 hours and the secreted TNFα were measured using ELISA. GPA monocytes secreted less TNFα compared to control monocytes (C). FACS, fluorescence activated cell sorting; ELISA, enzyme-linked immunosorbent assay.

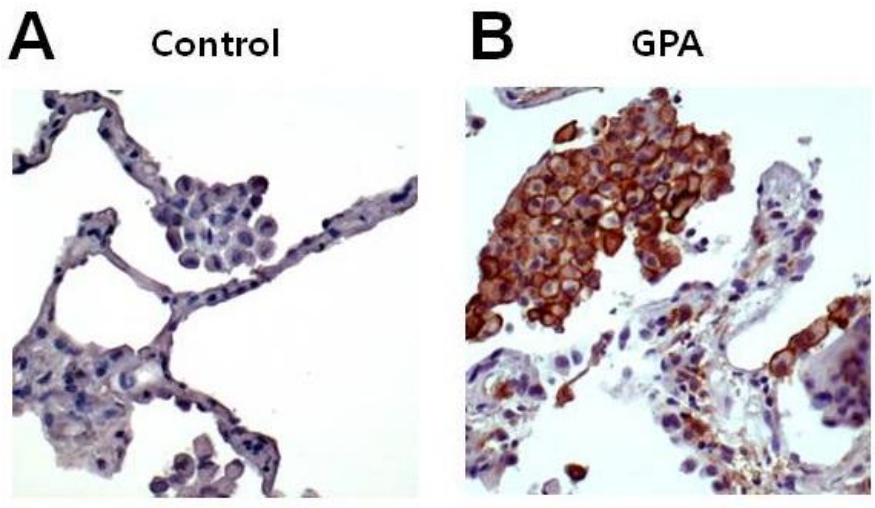

Figure 5. CD163+ monocytes/macrophages are abundant in GPA lung lesions. In healthy lung tissue, alveolar macrophages did express little to no CD163 (A). In contrast, the monocytes-macrophages in granulomatous inflammation expressed strongly CD163 on the cell surface in GPA.

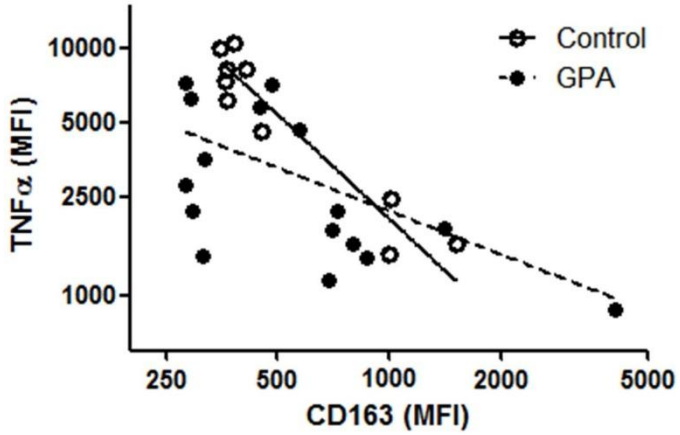

Figure 6. Inverse correlation between CD163 expression and TNFα production. Monocytes from GPA and controls were activated with LPS in increasing concentrations and the levels of TNFα production was correlated with surface expression CD163 using flow cytometry analysis. After activation, monocytes from healthy controls (n=3) and GPA patients (n=3) decreased CD163 expression on surface that correlated inversely with the amount of produced TNFα. MFI, mean fluorescence intensity.

국문 초록

육아종성다발혈관염 환자에서 단핵세포 및 거대세포의 염증반응의 저하에 관한 연구

서론: 육아종성다발혈관염은 중간크기 및 미세 혈관의 괴사성 혈관염과 육아종성 염증을 특징으로 갖는 원인 미상의 자가면역질환이다. 이 질환은 상기도의 지속적 염증에도 불구하고 세균 집락이 일반인에 비해 매우 높은 소견을 보이는데, 이는 면역력 저하로 인한 세균 제거 능력의 저하를 시사한다. 본 연구는 육아종성다발혈관염 환자에서 단핵세포(monocytes)의 대체적 활성화(alternative activation)로 인한 염증반응저하를 조사하고, 육아종성염증 내에 CD163를 발현하는 대체 활성 대식세포(alternatively activated macrophage)의 존재를 규명하고자 하였다.

방법: 환자군(n=23) 및 대조군(n=12)에서 말초혈액에 존재하는 CD16양성 단핵세포를 유세포분석을 통해 정량하였다. 이후 환자군 및 대조군으로부터 분리한 단핵세포를 지질다당류(lipopolysaccharide, LPS)로 자극하고, 4시간 및 24시간 경과 시점에 알파 종양괴사인자(tumor necrosis factor alpha, TNFα)생성 등의 염증반응을 유세포분석 및 효소면역측정법으로 비교분석 하였다.

또한 환자군 및 대조군의 폐조직에서 CD163을 발현하는 대체 활성 대식세포의 존재를 면역조직화학염색을 통해 분석하였다.

결과: 환자군에서 CD16양성 단핵세포의 양이 대조군에 비해 유의하게 많았다(4.7 ± 2.8 % vs. 1.9 % ± 1.2 %, P < 0.001). LPS 자극 후 TNFα 발현은 CD16양성 및 CD16음성 단핵세포 사이에 차이가 없었다. 환자군의 단핵세포는 대조군의 단핵세포보다 TNFα를 적게 생산하였다 (2,903 ± 1,320 pg/mL vs. 8,335 ± 4,569 pg/mL, P<0.001). 자극된 단핵세포에서 CD163 발현은 TNFα 생산과 반비례하며 CD163을 발현하는 대체 활성 대식세포는 환자군의 폐조직에서 더 많이 관찰되었다.

결론: 육아종성다발혈관염 환자에서 관찰되는 단핵세포의 TNFα 생산저하와, 폐 육아종 조직의 단핵세포/대식세포에서 관찰되는 CD163의 과발현 등은 이들 세포들의 대체적 활성화가 질환의 병태생리에 중요한 역할을 할 것임을 시사하였다. 향후 단핵세포의 정상적 분화로 회복시키는 새로운 치료법이 기대된다.

주요어: 육아종성다발혈관염, 단핵세포, 대체 활성 대식세포, CD163, 염증반응.

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