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Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis ☆

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ABSTRACT

Growing evidence has demonstrated that dysfunction of follicular helper T (TFH) cells results in an abnormal positive selection of autoreactive B cells, which contributes to the development of autoimmune diseases. This study reveals that the frequency of circulating counterparts of TFH cells in myasthenia gravis (MG) patients is significantly higher compared to healthy controls. Interestingly, the frequencies of circulating TFH cells were positively correlated with the levels of serum anti-AChR Ab in MG patients. Our data suggest the presence of overactivation and expansion of circulating counterparts of TFH cells in MG patients, which may contribute to the immunopathogenesis of MG.

1. Introduction

Myasthenia gravis (MG) is an organ-specific immune disease that is characterized by autoantibodies such as the anti-Acetylcholine receptor antibody (anti-AChR Ab) and anti-Muscle-specific tyrosine kinase antibody (anti-MuSK Ab) (Conti-Fine et al., 2006; Meriggioli and Sanders, 2009). It has been established that specific IgG autoantibodies against corresponding proteins are involved in signaling at the neuromuscular junction (NMJ) and Th dependence in animals and humans (1998; Conti-Fine et al., 2006; Meriggioli and Sanders, 2009). In addition, several studies have demonstrated the relationship between thymic hyperplasia and anti-AChR Abs in MG patients (Berrih-Aknin et al., 1987; Melms et al., 1988; Conti-Fine et al., 2006; Meriggioli and Sanders, 2009); however, common treatments such as antibody removal, immunosuppressive therapy and thymectomy have been unable to achieve the desired therapeutic efficacy in MG patients (Meriggioli and Sanders, 2009). Thus, a more complex mechanism of dysfunction in the peripheral immune system may be involved in MG pathogenesis.

The interaction between T cells and B cells is a decisive point during antibody production (Vinuesa et al., 2009). Several studies have demonstrated that CD4+ T cells are involved in MG immunopathogenesis (Hohlfeld et al., 1988; Melms et al., 1988; Moiola et al., 1994a, 1994b; Meriggioli and Sanders, 2009), although it is still unclear which subtype of CD4+ T cells truly contributes to the disease. Recent studies have demonstrated that an additional effector CD4+ T cell subset, the follicular helper T (TFH) cell, is largely responsible for B cell help during an immune response (Schaerli et al., 2000; Fazilleau et al., 2009; Laurent et al., 2010; Crotty, 2011). Moreover, several studies have shown that the dysfunction of TFH cells results in an abnormal positive selection of autoreactive B cells, which contributes to the development of autoimmune diseases (King et al., 2008; Linterman et al., 2009; Vinuesa et al., 2009; Simpson et al., 2010). TFH cells express the chemokine receptor C-X-C chemokine receptor type 5 (CXCR5), which is critical for its function. Furthermore, TFH cells also express B-cell lymphoma 6 (Bcl-6), inducible costimulator (ICOS), programmed death-1 (PD-1) and IL-21, which are excellent markers for the identification of TFH cells (Laurent et al., 2010; Crotty, 2011).

TFH cells are normally located in the germinal center (GC) of secondary lymphoid tissue, which promotes B cells to produce antibodies (Vinuesa et al., 2005; Fazilleau et al., 2009; Crotty, 2011). Moreover, a large population of CXCR5+CD4+ T cells is present in human peripheral blood (Schaerli et al., 2000; Morita et al., 2011); however, the relationship between GC TFH cells and blood CXCR5+CD4+ T cells remains unclear and controversial. Most of the peripheral blood CXCR5+ CD4+ T cells expressing memory T cell surface markers (CD45RO+) are considered to be components of circulating memory T cells (Schaerli et al., 2000; Morita et al., 2011; Vinuesa and Cook, 2011). In comparison with TFH cells in the GC of secondary lymphoid tissue, blood CXCR5+CD4+ T cells express less active molecules (Schaerli et al., 2000).

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Recent studies have found that some blood CXCR5+CD4+ T cells highly express ICOS or PD-1. These cells are considered functional TFH cells in the peripheral blood and are defined as circulating TFH cells (Simpson et al., 2010). In MG patients, there is an increase in the number of CD4+ cells that express CXCR5, which also correlate with disease severity (Saito et al., 2005). Furthermore, the CXCR5 chemokine, CXC-chemokine ligand 13 (CXCL13), is preferentially produced by epithelial cells and overproduced by epithelial cells in the thymus of MG patients. Thus, CXCL13 may be responsible for GC formation in the MG thymus (Meraouna et al., 2006). In addition, the level of serum CXCL13 is high in MG patients and increases in more severe clinical stages (Shiao et al., 2010). These results suggest a dysregulation of blood CXCR5+CD4+ T cells in MG patients; however, little is known about other counterparts of TFH cells in the peripheral blood of MG patients.

In this study, we explored the frequency of the counterparts of TFH cells in the human peripheral blood of MG patients and examined the potential association between the frequency of circulating TFH cells and laboratory measurements. Here, we report on the expansion of circulating counterparts of TFH cells in MG pathogenesis.

2. Materials and methods

2.1. Patients

A total of 32 MG patients were recruited from the inpatient service, and an additional 31 healthy subjects (HC) were recruited from the outpatient service of the First affiliated Hospital of SUN YAT-SEN UNIVERSITY between Mar 2011 and Nov 2011. The diagnosis of MG was based on the patient's medical history, clinical manifestation, which was verified by an examination of muscular fatigability, a positive response to cholinesterase inhibitors, and/or a decreased response to repetitive motor nerve stimulation and/or positive single fiber electromyography. Subjects with other autoimmune diseases such as Graves' disease or systemic lupus erythematosus (SLE) and patients who received immunosuppressive therapy within the past 3 months, exhibited an acute inflammation within the preceding 4 weeks, or received a thymectomy prior to the study were excluded. The patients' clinical data are summarized in Table 1. MG patients were comparable with healthy control subjects in gender and age. This study complied with the guidelines of the Declaration of Helsinki and was approved by the Human Ethics Committee of SUN YAT-SEN UNIVERSITY, Guangzhou, China.

2.2. Sample processing

All venous blood samples were obtained after receiving the subject's informed consent. In addition, 3–4 ml of blood was collected directly into a drying tube and centrifuged at 3000 rpm for 10 min at 4 °C. Next, the serum was collected and stored in a −80 °C refrigerator until further analysis. Next, 8–10 ml of blood was collected directly into a heparin-containing tube, and the peripheral blood mononuclear cells (PBMCs) were isolated via density-gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Little Chalfont, UK) within 3 h.

Table 1
Clinical characteristics of the 32 MG patients and 31 healthy controls.

	MG patients	Healthy controls
N	32	31
Female, n%	18 (56.2%)	17 (54.8%)
Age	26.8 (12–54)	29.9 (20–56)
Age at onset (medians and range in years)	24.3 (2–52)	n.a
Disease duration (medians and range in months)	45.2 (1–300)	n.a
Anti-AChR positive[a]	28 (87%)	n.a

[a] The detection methods and the definition of positive values reference to Section 2.5.

2.3. Flow cytometry

We used CD4-Percp, CXCR5-Alexa 647, CD45RO-FITC, ICOS-PE, PD-1 PE-Cy7 and isotype-matched control IgG antibodies (BD Pharmingen™, USA). Human PBMCs (1×10^6/tube) were stained and incubated with antibody cocktails in the dark at room temperature for 20 min. After incubation, the cells were washed twice with PBS + 1% heat inactivated (HI) FCS, fixed with PBS + 1% paraformaldehyde, and stored in polystyrene tubes in the dark within 24 h at 4 °C until analysis. The cells were analyzed by flow cytometry using Canto (Becton Dickinson) and FlowJo software (v5.7.2). The flow cytometer settings and fluorescence compensation were standardized for each experiment using BD CompBeads and the BD FACSDiva Comp program (BD Immunocytometry Systems). The FSC and SSC gates were set to capture target cell populations including monocytes and lymphocytes and excluding any contaminating neutrophils and debris, before focusing on CD4+ T cells. Subsequently, each cell population was identified and analyzed using fluorescence surface markers. At least 50,000 events per sample were analyzed. Isotype controls were used to determine the positive cells. The gate for positive cells allowed 0.5% of the positive isotype controls in blood CD4+ cells, and all of the values were gated on the CD4+ cells. Circulating counterparts of the follicular helper T cells included CXCR5+CD4+, CD45RO+CXCR5+CD4+, $ICOS^{high}$CXCR5+CD4+ and PD-1^{high}CXCR5+CD4+ T cells.

2.4. Real-time PCR

The total PBMC RNA was isolated using the E.Z.N.A.® Total RNA Kit II (Omega Bio-Teck, Doraville, GA, USA). cDNA was produced using Taqman reverse transcription kits (TaKaRa Biotechnology, Dalian). Real-time polymerase chain reaction was performed on an ABI-Prism 7700 using SYBR Green II as a double stranded DNA specific dye according to the manufacturer's instructions (TaKaRa Biotechnology, Dalian). Each sample was tested in triplicate, and the average values were used for calculations. The primers used were 5′-CCTGTGAAAT CTGTGGCACCCG-3′ and 5′-CGCAGCTGGCTTTTGTGACGG-3′ for human Bcl-6, 5′-CCAAGGTCAAGATCGCCACA-3′ and 5′-TGCTGACTTTAGTTGGG CCTTC-3′ for human IL-21, 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′ for human GAPDH.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of serum CXCL13 and anti-AChR Ab in individual patients and healthy controls were determined by ELISA using the human CXCL13 ELISA Kit (R&D Systems) and Acetylcholine Receptor Autoantibody ELISA kit (RSR Ltd., Cardiff, United Kingdom), respectively, according to the manufacturer's instructions. Each sample was tested in duplicate using the average of the optical density (OD) values to calculate the concentrations. The serum anti-AchR Ab was defined as positive if the concentrations of serum anti-AChR Ab was higher than or equal to 0.45 nmol/L, which is in accordance with the manufacturer's instructions.

2.6. Statistical analysis

The data are expressed as the median and range unless otherwise specified. Differences between two groups were analyzed using an unpaired t-test with Welch's correction using SPSS version 17.0 software. Relationships between two variables were evaluated using the Spearman rank correlation test. A two-tailed P-value of 0.05