Journal of Neuroimmunology 323 (2018) 36–42

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Increased costimulatory molecule expression of thymic and peripheral B cells and a sensitivity to IL-21 in myasthenia gravis

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ARTICLE INFO

Keywords:
Myasthenia gravis
B cells
Thymus
IL-21
Co-stimulation

ABSTRACT

B cells may contribute to the pathogenesis of myasthenia gravis with anti-acetylcholine antibodies (AChR + MG) by co-stimulation or selection of T cells.

In this study, we investigated costimulatory molecules on B cells in the blood and in the thymus as well as by TLR9 and IL-21 stimulations in AChR + MG patients with or without immunosuppressive treatment and controls.

CD80 and CD86 expression on B cells was increased in the peripheral blood and in the thymus of untreated patients. CD86 was further amplified by IL-21. A role for activated B cells, active thymic environment and IL-21 is implicated in MG.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease characterized by an autoantibody mediated immunological blockade of neuromuscular junction leading to increased muscle fatigability. A majority of the patients with MG have autoantibodies directed against nicotinic acetylcholine receptor (AChR). MG with anti-AChR antibodies (AChR + MG) is associated with thymic abnormalities such as thymic hyperplasia and thymoma (Drachman, 1994; Lefvert et al., 1981; Rødgaard et al., 1987).

B cells play an important role in the pathogenesis of AChR + MG through the production of anti-AChR antibodies. However, B cells are also capable of inducing antigen-specific T cell responses through antigen presentation and co-stimulation. Molecules such as MHC class II, CD80, CD86 and CD40 play a role in this B-T cell interaction. In multiple sclerosis (MS), B cells are shown to express higher levels of costimulatory molecules and can induce autoreactive T cell responses by antigen presentation (Fraussen et al., 2016). Increased costimulatory molecule expression by B cells is also reported as a marker for B cell activation in other autoimmune diseases such as systemic sclerosis (Sato et al., 2004), and systemic lupus erythematosus (SLE) (Rodríguez-Bayona et al., 2010). Furthermore, immunosuppression ameliorates the increased costimulatory molecule expression on B cells (Fraussen et al., 2016). There is only one previous study on MG reporting alteration of the costimulatory molecules on T, but not on B cells which did not control for the effect of immunosuppressive treatment (Teleshova et al., 2000).

Due to strong association with thymic abnormalities, the thymus is thought to play an important role in the pathogenesis of AChR + MG. The level of hyperplasia in thymic tissue is correlated with anti-AChR antibodies, suggesting thymic B cells could play a role in anti-AChR antibody production (Truffault et al., 2017). Additionally, antigen presentation by thymic B cells could be a mechanism for autosensitization to AChR and amplification of immune response in AChR + MG (Cron et al., 2017). In animal models, thymic B cells are found to have a more active phenotype compared to peripheral B cells with increased costimulatory molecule expression, suggesting increased antigen presentation capacity (Lu et al., 2015; Perera et al., 2013; Yamano et al., 2015). Furthermore, the presence of germinal centers and inflammation markers implicate thymus as an inflamed target organ in AChR + MG. In rheumatoid arthritis (RA) and psoriatic arthritis (PA), B cells in the synovium have an active phenotype with increased costimulatory molecule expression and antigen presentation capacity (Armas-González et al., 2015). In MS, cerebrospinal B cells are found to have a greater expression of CD80 and CD86 compared to peripheral blood (Fraussen et al., 2016). Similarly, the thymus in AChR + MG

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https://doi.org/10.1016/j.jneuroim.2018.07.006
Received 10 April 2018; Received in revised form 11 July 2018; Accepted 11 July 2018
0165-5728/

could harbor activated CD80 or CD86 expressing B cells with increased antigen presentation features.

A possible cause of increased costimulatory molecule expression by B cells in AChR+ MG patients could be a difference in exposure to signaling molecules. Both innate signals from Toll-like receptor (TLR) agonists or T cell dependent cytokines such as IL-21 could upregulate costimulatory molecule expression by B cells (Attridge et al., 2014; Geffroy-Luseau et al., 2011; Gerondakis et al., 2007; Meyer-Bahlburg and Rawlings, 2008; Zorro et al., 2009). TLR agonists are implicated in AChR+ MG by inducing AChR expression in thymic tissue as a possible trigger for autosensitization to AChR in MG patients (Cufi et al., 2014, 2013).

IL-21, a cytokine of T follicular helper (Tfh) cells, may also play a role in the pathogenesis of MG. In MG patients, serum levels of IL-21 and circulating Tfh cells were found to be elevated. Furthermore, Tfh cells of patients were reported to induce B cells to produce antibodies in a IL-21 signaling-dependent manner (Zhang et al., 2016). IL-21 could play a role in the recruitment of autoreactive B cells by Tfh in MG.

In this study, we aimed to elaborate on constitutive and induced costimulatory molecule expression of both thymic and peripheral blood B cells in AChR+ MG patients. Increased costimulatory capacity of B cells shown in other autoimmune diseases as well as ameliorating effect of immunosuppressive treatment were examined in this selected subgroup of the disease. Finally the probable role of TLR or Tfh mediated IL-21 on altered costimulatory molecule expression was studied by an in vitro stimulation system.

2. Material and methods

2.1. Patients and controls

Patients were recruited from the Neuromuscular Unit of Istanbul University, Istanbul Medical Faculty, Department of Neurology (Table 1). Two patient groups were included: immunosuppression negative (ISN) and immunosuppression positive (ISP). The ISN group included patients with a AChR+ MG diagnosis who, at time of blood sampling, were not using immunosuppressive drugs. The ISP group included patients with AChR+ MG diagnosis and were on immunosuppressive drugs (steroid alone or steroid plus azathioprine). The diagnosis of MG was based on clinical presentation, electrophysiological examination, and the presence of anti-AChR antibodies. Of the 45 AChR antibody positive patients, 33 had early-onset (< 50 years) and 12 had late-onset (> 50 years). In this group, 26 patients were thymectomized: 9 had thymoma and 17 had thymic hyperplasia. All of the patients had generalized MG except 3 patients, who had an ocular subtype. Myasthenia Gravis Foundation of America (MGFA) classification were used to assess patients clinical status at the time of blood sampling (Jaretzki Iii et al., 2000). According to MGFA, 3 patients had score I, 12 patients IIA, 21 patients IIB, 3 patients IIIA, 4 patients IIIB, 1 patient IVB and 1 patient had score V.

Table 1
The characteristic features of the AChR+ MG patient and HC groups. Median age (minimum, maximum) and sex distribution (M: men and W: women) are shown in ISN (immunosuppressive negative), ISP (immunosuppressive positive) and HC (Healthy controls) groups.

Group	N	Age	Sex
Peripheral blood			
AChR+ MG	45	41 (12–81)	16 M / 29 W
ISN	*22*	*42.5 (12–81)*	*9 M / 14 W*
ISP	*23*	*40 (17–74)*	*7 M / 15 W*
HC	21	35 (18–58)	6 M / 15 W
Thymus			
AChR+ MG	21	32 (12–62)	7 M / 14 W
ISN	*9*	*30 (12–52)*	*1 M / 8 W*
ISP	*12*	*38.5 (16–62)*	*6 M / 6 W*
NON MG	6	52 (14–77)	2 M / 4 W

Thymic samples were obtained from 21 patients with AChR+ MG who have undergone thymectomy. Blood samples of these patients were analyzed simultaneously. In the MG-thymus group, 14 patients had thymic hyperplasia and 7 patients had thymoma. Of the thymectomized patients, 9 were immunosuppression negative (ISN), while 12 patients were treated with immunosuppressive drugs (ISP). Furthermore, thymic tissues from 6 patients who had undergone thymectomy were included as thymic controls. Four of these patients had thymoma without MG, and the remaining 2 had moderate thymic hyperplasia without MG (disease excluded clinically and by negative antibody tests). None of these patients used immunosuppressive drugs.

The healthy control group (HC) included age- and sex-matched healthy volunteers from the staff. The study was approved by the Institutional Review Board, and a written informed consent was obtained from all patients and controls who participated in the study.

2.2. Phenotypic analysis of peripheral blood and thymic samples

Peripheral blood mononuclear cells (PBMC) were separated using Ficoll-density gradient centrifugation. Thymic samples were taken from thymectomy specimens under the guidance of the pathologist. Cell suspensions from the thymic samples were obtained by mechanical manipulation, filtered using a cell strainer (SPL Life Sciences) and separated using the/a density gradient method. B cells were analyzed in four distinct subpopulations based on their CD27 and IgD expression: $CD27^+IgD^-$ switched memory, $CD27^+IgD^+$transitional memory, $CD27^-IgD^+$ naive and $CD27^-IgD^-$ double negative (Fig. 1A). Both PBMC and thymic cell suspensions were then stained with anti-CD80-FITC, -CD27-PE, -CD86-APC, -IgD-PE.Cy.7, -CD40-APC (Bio legend), -CD19-Pe.Cy.5 (BD Biosciences) and -HLA-DR-PE (Beckman Coulter) antibodies by a 20 min incubation at room temperature. The samples were then analyzed by flow cytometry (Attune, Thermo Fischer Scientific). The gating was performed with florescence minus one controls as shown in Fig. 1B.

2.3. Cell culture and stimulation

The separated mononuclear cell suspensions from the blood were incubated in either complete medium (RPMI1640 with 2 mM L-glutamine, 100 IU/100 μg/ml penicillin/streptomycin (SIGMA) and 10% fetal bovine serum (Gibco)), with CpG ODN 2137 (5 μg/ml) (Coley), IL-21 (200 ng/ml) (Peprotech), or in a combination of both for 24 h in cell culture. After the incubation, the cells were washed and stained with the following conjugated antibodies, against CD80 (FITC), CD27 (PE), HLA-DR (PE), CD19 (PE.Cy.5), CD86 (APC) and CD20 (FITC), as above. The induced expression of the costimulatory molecules by IL-21 and TLR9 agonist were calculated by subtracting the frequency or the median fluorescence intensity (MFI) of positive cells for these markers in stimulated cells from the unstimulated cells.

2.4. Statistical analysis

Non-parametric two-tailed Mann-Whitney *U* test was used to compare patient subgroups and controls. $p < .05$ was considered as statistically significant. All statistical analyses were performed with the GraphPad Prism program.

3. Results

3.1. B cell subpopulations in the thymus and peripheral blood of AChR+ MG patients

To assess the proportions of B cell subpopulations in AChR+ MG, we measured the expression of CD27, IgD and CD19 on peripheral