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Innate immunity in myasthenia gravis thymus: Pathogenic effects of Toll-like receptor 4 signaling on autoimmunity

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ABSTRACT

The thymus is the main site of immune sensitization to AChR in myasthenia gravis (MG). In our previous studies we demonstrated that Toll-like receptor (TLR) 4 is over-expressed in MG thymuses, suggesting its involvement in altering the thymic microenvironment and favoring autosensitization and autoimmunity maintenance processes, via an effect on local chemokine/cytokine network. Here, we investigated whether TLR4 signaling may favor abnormal cell recruitment in MG thymus via CCL17 and CCL22, two chemokines known to dictate immune cell trafficking in inflamed organs by binding CCR4. We also investigated whether TLR4 activation may contribute to immunodysregulation, via the production of Th17-related cytokines, known to alter effector T cell (Teff)/regulatory T cell (Treg) balance. We found that CCL17, CCL22 and CCR4 were expressed at higher levels in MG compared to normal thymuses. The two chemokines were mainly detected around medullary Hassall's corpuscles (HCs), co-localizing with $TLR4^+$ thymic epithelial cells (TECs) and $CCR4^+$ dendritic cells (DCs), that were present in higher number in MG thymuses compared to controls. TLR4 stimulation in MG TECs increased CCL17 and CCL22 expression and induced the production of Th17-related cytokines. Then, to study the effect of TLR4-stimulated TECs on immune cell interactions and Teff activation, we generated an in-vitro imaging model by co-culturing $CD4^+$ Th1/Th17 AChR-specific T cells, naïve $CD4^+CD25^+$ Tregs, DCs and TECs from Lewis rats. We observed that TLR4 stimulation led to a more pronounced Teff activatory status, suggesting that TLR4 signaling in MG thymic milieu may affect cell-to-cell interactions, favoring autoreactive T-cell activation. Altogether our findings suggest a role for TLR4 signaling in driving DC recruitment in MG thymus via CCL17 and CCL22, and in generating an inflammatory response that might compromise Treg function, favoring autoreactive T-cell pathogenic responses.

1. Introduction

Myasthenia gravis (MG) is a T-cell dependent antibody-mediated disease that affects neuromuscular junction (NMJ), causing weakness and fatigability of ocular and skeletal muscles. In most cases (~80%), the autoimmune attack is caused by autoantibodies against the acetylcholine receptor (AChR) of the post-synaptic NMJ [1].

Several lines of evidence indicate that the thymus is the main site of the autosensitization process in AChR-positive MG (AChR-MG) [2]. Hyperplastic MG thymus is characterized by the presence of B cells either infiltrating the thymic medulla (diffused hyperplasia or thymitis) or organized into ectopic germinal centers (GCs) forming follicles (follicular hyperplasia) [3]. AChR-specific T cells and autoantibody-producing plasma cells have been found in hyperplastic MG thymus [3], suggesting an ongoing intra-thymic autoimmune response to AChR.

Accumulating data show that innate immunity activation via Toll-like receptors (TLRs) and chronic inflammation characterize MG thymus [4–6]. TLR4 signaling is activated by both Gram-

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negative bacteria and viral components [7], and is one of the TLR family members suspected to play a role in MG [4]. In our previous studies, we demonstrated TLR4 over-expression and the presence of poliovirus-infected TLR4$^+$ macrophages in the thymus of MG patients [4,8], suggesting dysregulated TLR4-mediated innate immune responses. Activation of the immune system and increased expression of AChR-α subunit in thymic epithelial cells (TECs) [5,6] are possible TLR-mediated inflammatory mechanisms leading to intra-thymic AChR autosensitization. Published data showed that regulatory T cells (Tregs) from MG thymuses are functionally defective [9] and therefore unable to control the T-cell-mediated autoimmune response to AChR. Whether dysregulated innate immune response, through TLR4 or other TLRs, contributes to self-tolerance disruption in MG thymus via an effect on Treg-mediated immunoregulation is not completely understood.

Chemokines and their receptors are key regulators of lymphocyte trafficking; they are able to recruit T cells and antigen presenting cells (APCs) into sites of inflammation and orchestrate APC, T-, and B-cell encounters in these sites [10]. The interactions among these cells, as well as the balance among different T-cell subtypes (i.e. Th1, Th2, Th17 and Tregs), may determine the outcome of the immune reactions; in this regard, the molecular settings regulating immune cell migration and interaction is of great importance. The Thymus and Activation-Regulated Chemokine CCL17 (TARC) and the Macrophage-Derived Chemokine CCL22 (MDC) are known to dictate trafficking of Th cells, Tregs and dendritic cells (DCs) to inflamed areas [11]. CCL17 and CCL22 are mainly produced by DCs and can be up-regulated by several stimulating factors, such as lipopolysaccharide (LPS), the prototypical TLR4 agonist [12,13]. The receptor for CCL17 and CCL22 is the C–C chemokine receptor type 4 (CCR4) [14,15], a G protein-coupled receptor known to be expressed in functionally distinct subsets of T cells, including activated Th2 cells and Tregs, and DCs [16,17].

CCL22 and CCR4 are normally expressed in the thymus and participate in thymopoiesis by regulating the migration of maturing thymocytes through this organ [18]. However, their role in the intra-thymic MG pathogenesis has been never explored.

Aim of this study was to asses the pathogenic effects of TLR4 signaling in MG thymus, by investigating whether this signaling may lead to altered CCL17 and CCL22 expression, and to immunodysregulation, via the production of Th17-related cytokines, known to impair Treg function and favor autoimmunity [19]. The TLR4 effect on chemokine expression was also studied in thymic epithelial cell cultures derived from experimental autoimmune myasthenia gravis (EAMG) animals [20]. Finally, to better address the effect of the inflamed pathologic thymic milieu on the cellular interactions and functions, we set-up an in vitro model by co-culturing rat DCs, TECs, Tregs and AChR-specific Th1/Th17 effector cells (Teffs).

Our overall findings suggest that TLR4 signaling may participate in the intra-thymic pathogenesis of MG, by promoting the recruitment of CCR4$^+$ DCs in the inflamed MG thymus, where the ongoing TLR4-mediated innate immune responses may favor autoantigen presentation and alter Teff/Treg balance.

2. Materials and methods

2.1. MG patients and thymuses

The study was carried out in 12 thymuses with follicular hyperplasia (MG-FH), 8 thymuses with diffused hyperplasia (MG-DH), and 8 involuted thymuses obtained from early-onset (EOMG; ≤50 years of age) AChR-positive MG patients (22 females and 6 males), who underwent thymectomy as therapeutic treatment. Fifteen patients were treated with corticosteroids before thymectomy, and 13 were untreated or treated only with cholinesterase inhibitors. Patients' clinical characteristics are summarized in Table 1. Nine non-pathologic thymuses from cardiopathic patients (mean age: 31.9 ± 17.0) were also examined as controls. Written informed consent was obtained from patients and controls for the use of thymus for research purposes. The study was approved by the Ethic Committee of the Neurological Institute 'Carlo Besta'. For each thymus, some fragments were fixed in 10% formalin and others were snap-frozen and stored at −80 °C.

2.2. Animals and EAMG induction

Female Lewis rats (6–8 week old, $n = 20$) were purchased from Charles Rivers Laboratories International, maintained and bred at the animal house of the Institute "Carlo Besta". Animal studies were approved by the Institute (codes: IMP-03-11 and IMP-04-11), and performed in accordance with the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC). Animals were sacrificed after deep anesthesia by exposure to carbon dioxide. EAMG was induced via immunization with purified *Torpedo californica* electroplax tissue (Aquatic Research Consultants) AChR (TAChR; 50 μg/rat) in Complete Freund's Adjuvant (CFA; Beckton Dickinson-BD-Difco) or with the rat immunodominant AChR epitope, R97-116 (200 μg/rat), in CFA [21]. At chronic disease stage, 2 animals/group were sacrificed to obtain thymic cell suspensions for primary TEC cultures. Control primary TEC cultures were obtained from age- and sex-matched PBS/CFA-immunized ($n = 2$) and naïve ($n = 2$) animals. EGFP$^{+/+}$ and EGFP$^{+/-}$ transgenic Lewis rats [22] were used for T-cell primary cell cultures and bone marrow-derived DCs.

2.3. Immunohistochemistry (IHC) on thymic sections

Six-μm thick serial sections from snap-frozen thymic tissues ($n = 4$ for each thymic pathology and control) were immunostained with primary antibodies specific for: CCL17 (Santa Cruz); CCL22 (Peprotech); CCR4 (BD Pharmingen); TLR4 (Santa Cruz); cytokeratin (CK; Dako); desmin (Dako); DC-LAMP (Immunotech); FoxP3 (Thermo Scientific) and CD20 (Dako); non-immune IgG staining was used as isotype control. Secondary labeling was performed with HRP Anti-Mouse or Anti-Rabbit antibodies (Dako) followed by incubation with 3,3′ diaminobenzidine (DAB; Dako) and hematoxylin counterstaining. Images were digitally acquired with the ScanScope system (Aperio). DC-LAMP$^+$ cells were counted in normal ($n = 3$) and MG ($n = 9$) thymic sections and their number was normalized to medullary area size (mm^2).

Table 1
Clinical features of MG Patients included in the study.

Thymic pathology	Follicular hyperplasia ($n = 12$)	Diffuse hyperplasia ($n = 8$)	Involuted thymus ($n = 8$)
Sex (F:M)	9:3	8:0	5:3
Early-onset (≤50 years)	12/12	8/8	8/8
Age at surgery (years, mean ± SD)	28.6 ± 8.4	29.6 ± 11.1	28.5 ± 10.9
Ab AChR-positive	12/12	8/8	8/8
Immunosuppressive therapy	5/12	4/8	6/8