Contents lists available at ScienceDirect

Journal of Autoimmunity

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

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

Chiara Cordiglieri^a, Roberta Marolda^a, Sara Franzì^a, Cristina Cappelletti^a, Carmelo Giardina^b, Teresio Motta^b, Fulvio Baggi^a, Pia Bernasconi^a, Renato Mantegazza^a, Paola Cavalcante^{a,*}

^a Neurology IV Unit, Neurological Institute 'Carlo Besta', Via Celoria 11, 20133 Milan, Italy

^b Department of Pathological Anatomy, Azienda Ospedaliera Bolognini Seriate, Via Paterno 21, 24068 Seriate Bergamo, Italy

ARTICLE INFO

Article history:

Received 18 July 2013

Accepted 15 December 2013

Keywords:

Myasthenia gravis

Thymus

TLR4

Chemokines

Dendritic cells

Regulatory T-cells

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 auto-sensitization 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.

© 2013 Elsevier Ltd. All rights reserved.

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 auto-sensitization 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-

* Corresponding author. Neurology IV Unit – Neuromuscular Diseases and Neuroimmunology, Neurological Institute 'Carlo Besta', Via Celoria 11, 20133 Milan, Italy. Tel.: +39 02 2394 4511/4651; fax: +39 02 7063 3874.

E-mail addresses: chiara.cordiglieri@istituto-besta.it (C. Cordiglieri), roberta.marolda@istituto-besta.it (R. Marolda), sara.franzi@istituto-besta.it (S. Franzì), cristina.cappelletti@istituto-besta.it (C. Cappelletti), anatoniapat.seriate@bolognini.bg.it (C. Giardina), teresio.motta@bolognini.bg.it (T. Motta), baggi@istituto-besta.it (F. Baggi), pbernasconi@istituto-besta.it (P. Bernasconi), rmantegazza@istituto-besta.it (R. Mantegazza), pcavalcante@istituto-besta.it (P. Cavalcante).

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 auto-sensitization. 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 assess 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 $\mu\text{g}/\text{rat}$) in Complete Freund's Adjuvant (CFA; Beckton Dickinson-BD-Difco) or with the rat immunodominant AChR epitope, R97-116 (200 $\mu\text{g}/\text{rat}$), 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 $\pm$ SD)	28.6 $\pm$ 8.4	29.6 $\pm$ 11.1	28.5 $\pm$ 10.9
Ab AChR-positive	12/12	8/8	8/8
Immunosuppressive therapy	5/12	4/8	6/8

2.4. Double immunofluorescence (IF) on thymic sections

Frozen 6- μ m thymic sections were immunostained with combinations of antibodies against CCL17, CCL22, CCR4 (BD Pharmingen or Abnova), peripheral-node addressing (PNAd; BD), CK, DC-LAMP, TLR4 (Santa Cruz) and FoxP3, followed by secondary labeling with Alexa Fluor 488-(Molecular Probes, Life Technologies), Cy3- and Cy2-(Jackson Immunoresearch Lab.) conjugated goat anti-mouse and anti-rabbit IgGs, and 4',6-diamidino phenylindole-dihydrochloride (DAPI; Life Technologies) staining. Images were captured via confocal laser-scanning microscopy (EZ-C1 scan-head equipped with Eclipse TE2000-E microscope; Nikon). Single- and double-positive cells were counted on 6 adjacent field areas per section at $\times 60$ magnification using Image J software v1.43u.

2.5. Laser-capture microdissection (LCM)

Normal ($n = 3$) and MG ($n = 9$) thymuses were subjected to LCM of medullary areas containing Hassall's corpuscles (HCs) using a laser microdissector CellCut (MMI) equipped with an Eclipse

TE2000-S microscope (Nikon). For each thymus, three 15- μ m thick serial sections were mounted on the same LCM membrane slide, stained with hematoxylin and fixed in RNase-free 75%–100% ethanol. 500 μ m-diameter medullary areas around each HC were selected, cut and pooled in a single cap and resuspended in lysis buffer. Sections devoid of microdissected areas were separately collected.

2.6. Reverse transcription and real-time PCR

Total RNA was extracted from frozen thymic fragments and cells using TRIzol reagent (Life Technologies), and from LCM samples with RNeasy Micro kit (Qiagen). DNase I-treated RNA was retro-transcribed using Superscript VILO cDNA synthesis kit or SSIII reagent (Life Technologies) and amplified in duplicates using pre-designed functionally tested assays (Life Technologies). Human GAPDH and rat β -actin were used as endogenous controls (Life Technologies). GAPDH was stably expressed in thymic (Ct mean $\pm$ SD: 23.25 ± 0.49 for normal thymuses; 22.85 ± 0.48 for MG thymuses) and TEC samples (Ct mean $\pm$ SD: 19.91 ± 0.48). β -actin

Fig. 1. Preferential localization of CCL17- CCL22- and CCR4-expressing cells in thymic medullary areas around Hassall's corpuscles (HCs). A. and B. Immunohistochemical analysis of CCL17, CCL22 and CCR4 in normal (A) and follicular hyperplastic MG (B) thymuses. C. Characterization of the MG thymic microenvironment associated with HCs for the presence of TLR4⁺ cells, CK⁺ TECs, desmin⁺ myoid cells, DC-LAMP⁺ mature DCs, FoxP3⁺ Tregs, and CD20⁺ B cells. D. Double immunofluorescence staining of follicular hyperplastic MG thymuses showing co-localization of CCL17 (green) with PNAd⁺ high endothelial venules (HEVs) (red) and presence of CCR4⁺ cells (green) in close proximity to HEVs (red), that were also present near HCs. Magnification bars: 100 μ m in A-C; 50 μ m in D. E. Real-time PCR analysis to assess CCL17, CCL22 and CCR4 transcriptional levels in laser-capture microdissected thymic medullary areas (500 μ m of diameter), containing HCs plus areas surrounding HCs from sections of Normal, MG-FH, MG-DH and MG-Involuted thymuses. Transcriptional levels of the three genes were normalized towards glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. The dashed line corresponds to CCL17, CCL22 and CCR4 mRNA levels in whole sections devoid of HC-associated medullary areas normalized to 1. Graphs show means ($\pm$ SEM) of results obtained with three different donors per group.

was stably expressed in rat TECs (Ct mean $\pm$ SD: 19.22 ± 0.23) and co-cultures (Ct mean $\pm$ SD: 17.67 ± 0.35).

2.7. Human and rat TEC isolation and TLR4 stimulation in vitro

Human TEC primary cultures were established from freshly isolated hyperplastic thymuses of MG patients treated with cholinesterase inhibitors ($n = 2$) or corticosteroids ($n = 2$) prior to thymectomy. Finely cut thymic fragments were seeded into 60 mm collagen-coated dishes (BD) in culture medium (MEM with D-valine, Cambrex Bioscience) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM Hepes (all from Euroclone) and 10 ng/ml epidermal growth factor (EGF; Life Technologies). After 10–15 day culture, the confluent monolayers were detached and subcultured. Rat primary TECs were established from thymic tissues of naïve Lewis rats ($n = 2$), PBS/CFA- ($n = 2$), R97-116/CFA- ($n = 2$) or TACHR/CFA-immunized ($n = 2$) Lewis rats. Cell suspensions were cultured for 10–15 days into collagen type IV (Sigma)-coated 75 mm² flasks in RPMI complete medium (10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin) plus 10 ng/ml EGF (Peprotech). TEC purity (>85%) was checked by anti-CK (Novocastra) IF staining followed by Alexa-488- or Cy2-conjugated IgGs. For TLR4 stimulation experiments, TECs (1×10^5 cells/cm²) were seeded in 5% FBS-containing culture medium for 24 h and treated with 1 µg/ml LPS (Sigma) for 3–48 h. In some experiments, human TECs were stimulated with LPS in the presence of 20 µg/ml of a cyanobacterial LPS antagonist (CyP) [23]. For IF stainings, 5×10^3 cells/well were plated on 4-well culture chamber slides (Nunc, Thermo Scientific), stimulated with LPS and immunostained with anti-TLR4 (Abcam) and anti-CK antibodies followed by Alexa-594 and -488 conjugated IgGs (Molecular Probes) and DAPI nuclei labeling. Images were acquired via confocal microscopy (Nikon).

2.8. Analysis of human TEC protein secretion

Supernatants were collected from human TECs at basal condition and after 24 and 48 h of LPS stimulation. Fifteen Th17-related proteins were measured by the Bio-Plex Pro Human Th17 Cytokine Assay kit (Bio-Rad Laboratories), following manufacturer's instructions.

2.9. R97-116 specific effector rat Teff cell lines

EGFP^{+/+} transgenic and wild type (wt) Lewis rats were immunized with 200 µg of peptide R97-116 in CFA as previously described [21]. Ten days post-immunization (p.i.) lymph node (LN) cell suspensions were cultured in RPMI medium, containing 1% Napyrivate, 1% non-essential aa, 1% L-glutamine, 1% penicillin-streptomycin (Euroclone), 50 µM 2-mercaptoethanol (Sigma), 2% rat serum and stimulated with R97-116 (5 µg/ml) as described [24]. T cells were maintained and expanded as reported [25]. For live imaging, EGFP^{+/+} R97-116-specific T cells were loaded with CFSE and wt R97-116-specific T cells were loaded with Fluo-4 dye to visualize intracellular calcium fluxes (both dyes from Molecular Probes).

2.10. Rat DC cultures

Single-cell suspensions of myeloid precursor cells (MPCs) were derived from bone marrows (BM) of naïve wt and EGFP^{+/+} Lewis rats and cultured in complete RPMI medium in the presence of GM-CSF and IL-4 (both 20 ng/ml; Peprotech) for 10 days to differentiate into immature DCs (iDCs) [24]. iDCs were then seeded either in live imaging glass-inserted plates (3×10^5 EGFP^{+/+} DCs/dish) or transwell plates (1×10^5 wt DCs/well) for specific assays.

2.11. Rodent TEC/DC transwell assay

Naïve wt TECs (1×10^5) were added in the lower chambers of 12-well transwell plates (Corning Costar, 3 µm pore size) and incubated with LPS (1 µg/ml) for 12 and 24 h. After medium change, differentiated wt iDCs were added in the upper chambers (1×10^5). After 24 h incubation, TECs and DCs were collected from bottom and lower chambers for RNA sampling and qPCR analysis.

2.12. Rat Treg cultures

Naïve natural occurring CD127⁺CD4⁺CD25⁺ rat Tregs were obtained from pool of LNs extracted from naïve wt Lewis rats. LN cell suspensions were processed using 3-step negative-positive MACS separation procedure (Miltenyi). Anti-CD127-Biotin, anti-CD4-FITC and anti-CD25-PE antibodies (all eBioscience) and conjugate-specific magnetic beads (Miltenyi) were sequentially used. Tregs were finally labeled with CMRA dye (Molecular probes) and used in live imaging co-culture experiments (8×10^5 cells/dish).

2.13. Live imaging for Teff cell motility

Time-lapse videomicroscopy was performed using a live-imaging Nikon set-up equipped with temperature/CO₂ control unit (OKOLab). Naïve wt TECs (3×10^5), EGFP^{+/+} DCs (3×10^5), R97-116-specific CFSE-labeled EGFP^{+/+} Teffs (8×10^5) and CMRA-labeled wt naïve Tregs (8×10^5) were co-cultured on glass-inserted imaging collagen-coated dishes. Differential interference contrast (DIC), green and red channels images were acquired on a 512×512 pixel² field of view, with 1.31 µm/pixel conversion. Recordings were performed in 30 s time intervals as previously reported [26] using an inverted microscope (with 20×, 0.5 NA, objective) and a Q-imaging Fast Camera (Roper scientific), and processed by NIS Elements AR software v3.1 (Nikon). Image J software was used to evaluate cell trajectories and velocities. Cells were defined as stationary if they moved less than 10 µm/10 min, motile if they moved more than 10 µm/10 min [26,27].

2.14. Cytosolic Ca²⁺ measurements of Teff cells

Intracellular Ca²⁺ concentration ([Ca²⁺]_i) was determined in Fluo4-loaded R97-116-specific T cells (8×10^5 cells/dish) co-cultured with DCs, TECs and CMRA-Tregs in the presence of appropriate stimuli (R97-116 10 µg/ml, LPS 1 µg/ml) onto glass-inserted imaging collagen-coated dishes. Calcium imaging was obtained via quick run-mode acquisition for 1 h using the live-imaging Nikon set-up previously cited. One DIC image was acquired at the beginning and at the end of each imaging for morphological cell analysis. Fluo-4 positive cells were counted and analyzed as percentage of total Teffs in each frame. [Ca²⁺]_i calibration curve was performed using CD3/CD28 stimulation and ionomycin-induced calcium fluxes.

2.15. Statistical analysis

Data distribution was tested via Kolmogorov test: normally distributed data were analyzed via Student's *t* test or one-/two-way ANOVA, followed by Bonferroni post-hoc test; non-parametric data were analyzed by Mann–Whitney *U* test. *P* < 0.05 was considered statistically significant. GraphPad Prism v5.0 (GraphPad Prism) was used for data elaboration and statistical analysis.

Fig. 2. CCL17 and CCL22 co-localized with TECs and mature DCs over-expressing TLR4 in MG thymus. Double immunofluorescence staining of thymic sections from A. normal and B. MG thymuses for CK⁺ TECs (green) and TLR4 (red), and for DC-LAMP⁺ mature DCs (green) and TLR4 (red). Double immunofluorescence staining of thymic sections from C. normal and D. MG thymuses for: i) CK⁺ TECs (green) and CCL17 (red); ii) DC-LAMP⁺ mature DCs (green) and CCL17 (red); iii) CK⁺ TECs (green) and CCL22 (red); iv) DC-LAMP⁺ mature DCs (green) and CCL22 (red). E. Double immunofluorescence staining of thymic sections from hyperplastic MG thymuses for TLR4 (green) and CCL17 (red), and for TLR4 (green) and CCL22 (red). The arrows indicate double positive cells. Insets in D. and E. panels show double positive cells stained with DAPI (blue). Magnification bars in the main panels (A to E): 50 μ m; magnification bars in the insets (D and E): 10 μ m. HC: Hassall's corpuscles.

3. Results

3.1. CCL17, CCL22 and CCR4 are enriched in medullary areas around HCs in MG thymus

Despite a similar distribution of CCL17⁺ and CCL22⁺ cells in control and pathologic thymuses, being mainly observed in medullary areas containing HCs, an increased expression of CCL17 and CCL22 in HCs and in cells surrounding HCs was observed in MG thymuses (Fig. 1A and B; Supplementary Fig. 1). CCL17 was also expressed in PNA⁺ HEVs, highly present in MG thymic medulla (Fig. 1D), suggesting that CCL17 might drive peripheral cell entrance via HEVs. An increased number of CCR4⁺ cells was found in MG thymuses, mainly concentrated around HCs, (Fig. 1A, B and D; Supplementary Fig. 1) and/or in close proximity to PNA⁺ HEV (Fig. 1D).

CCL17- and CCL22-enriched thymic areas surrounding MG HCs showed a strong TLR4 positivity and presence of TECs, myoid cells, mature DCs, Tregs and B cells (Fig. 1C; Supplementary Fig. 1), suggesting that fine cellular interactions in these restricted medullary areas could play a role in thymic MG etiology.

Preferential CCL17, CCL22 and particularly CCR4 expression by HCs and surrounding cells was confirmed at mRNA levels on microdissected HC-associated areas, compared with whole thymic sections devoid of HCs (Fig. 1E).

3.2. CCL17 and CCL22 co-localize with TLR4⁺ TECs and mature DCs in MG thymus

To verify whether CCL17 and CCL22 expression in MG thymuses was linked to the expression of TLR4, we analyzed the two chemokines in TECs and DCs, known to over-express TLR4 in MG thymuses [4] (Fig. 2A and B). Indeed, CCL17 and CCL22 co-localized with CK⁺ TECs and DC-LAMP⁺ mature DCs more prominently in MG thymuses, and especially in microenvironment around HCs (Fig. 2C and D), and with TLR4 (Fig. 2E). This suggested an expression of the two chemokines by TLR4⁺ TECs and/or DCs in MG thymus.

3.3. Increased number of CCR4-bearing DCs in MG thymus

We wondered whether inflamed MG thymus was characterized by altered proportions of CCR4⁺ DCs and CCR4⁺ Tregs. We found an increased proportion of mature highly ramified DC-LAMP⁺ DCs, but not of FoxP3⁺ Tregs, in MG compared to normal thymuses (Fig. 3A, B, C and F). DC-LAMP⁺ DC increase was not merely due to expansion of medullary area in MG thymuses (Fig. 3D). Interestingly, the percentage of DC-LAMP⁺ DCs expressing CCR4, but not of CCR4⁺ FoxP3⁺ Tregs, was higher in MG compared to normal thymuses (Fig. 3A, B, E and G).

3.4. CCL17, CCL22 and CCR4 mRNA expression is increased in MG thymuses over-expressing TLR4

Besides confirming up-regulated levels of TLR4 mRNA [4], qPCR analysis showed that CCL17 and CCL22 mRNA levels were significantly increased in both follicular and diffuse hyperplastic MG thymuses compared with controls, whereas CCR4 mRNA was increased in follicular hyperplastic MG thymuses only (Fig. 4A). Immunosuppressive treatment does not seem to affect thymic TLR4, CCL22 and CCR4 expression since the mRNA levels did not differ between untreated and corticosteroid-treated patients (Fig. 4B), whereas CCL17 transcriptional levels were significantly reduced in thymuses from corticosteroid-treated MG patients, thus suggesting that immunosuppressive treatment could normalize CCL17 levels in MG thymuses (Fig. 4B).

3.5. TLR4 stimulation by LPS increases CCL17/CCL22 expression and Th17-related cytokine production in MG TECs

Since CCL17 and CCL22 were found to co-localize with TECs in MG thymuses, we wondered whether TLR4 signaling could modulate their expression. We first demonstrated that LPS stimulation increased TLR4 expression in MG TECs, and IL-6 as control for

a proper TLR4 activation (Supplementary Fig. 2). Then, we found that LPS stimulation increased CCL17 and CCL22 mRNA levels in MG TECs, with a peak at 6 h of stimulation (Fig. 4C). This effect was blocked by the specific LPS-antagonist CyP [23] (Fig. 4C), thus confirming LPS binding to TLR4.

To better understand the pathogenic role of TLR4 signaling in MG TECs, we investigated whether LPS stimulation induced secretion of Th17-related cytokines. We analyzed TEC culture supernatants at basal condition and after LPS stimulation (24 and 48 h). The data confirmed high levels of IL-6 at basal and stimulated conditions (Table 2), accompanied by a significant increase of IL-1 β production after 24 h of LPS treatment. Moreover, we observed a slight but significant increase of IL-4 and IFN- γ after 48 h of LPS treatment. Despite IL-17 family cytokines were secreted at low levels by TECs, we found a significant increase of IL-17F in MG TECs after 48 h of LPS stimulation, suggesting that IL-17F could participate in the pro-inflammatory cytokine network that characterizes MG thymus.

3.6. Th-17-related genes are up-regulated in MG thymus

To provide insights into the possible involvement of Th17-related cytokines in the thymic MG pathogenesis, we verified whether the expression of IL-6, IL-17A, IL-17F, IL17C and TGF- β 1

Fig. 3. Increased number of CCR4⁺ DCs, but not of CCR4⁺ Tregs, in MG thymuses. A. Sections from normal and follicular hyperplastic MG thymuses co-labeled for DC-LAMP⁺ mature DCs (green) and CCR4 (red). B. Sections from normal and follicular hyperplastic MG thymuses co-labeled for CCR4 (green) and FoxP3⁺ Tregs (red). The arrows indicate double positive cells. Insets in A. and B. right panels show double positive cells stained with DAPI (blue). Magnification bars in the main panels: 50 μ m; magnification bars in the insets: 10 μ m. HC: Hassall's corpuscles. C. DC-LAMP⁺ and E. CCR4⁺ DC-LAMP⁺ DCs, and F. FoxP3⁺ Tregs and G. CCR4⁺ FoxP3⁺ Tregs were counted in 6 adjacent fields per section in 3 normal and 9 MG thymuses (3 for each pathological group). Bars in C. and F. graphs correspond to the mean percentage ($\pm$ SEM) of DC-LAMP⁺ (C) or FoxP3⁺ (F) cells among total (DAPI⁺) cells per field in each sample group. Bars in the E. and G. graphs correspond to the mean percentage ($\pm$ SEM) of CCR4⁺ cells among DC-LAMP⁺ DCs (E) or among FoxP3⁺ cells (G) per field in each sample group. Values in D. correspond to the mean number ($\pm$ SEM) of DC-LAMP⁺ cells per thymic medullary area (mm²) obtained in each sample group. *P* values were assessed by student *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table 2
Effect of TLR4 stimulation by LPS on secretion of Th17-related cytokines in MG TECs.

Gene symbol	Time of LPS treatment		
	0 h	24 h	48 h
IL-1 β	152.40 $\pm$ 31.64	270.5 $\pm$ 27.19*	216.20 $\pm$ 56.84
IL-4	20.62 $\pm$ 3.98	25.30 $\pm$ 4.90	39.32 $\pm$ 11.07**
IL-6	22210.00 $\pm$ 871.86	24128.57 $\pm$ 124.39*	23932.00 $\pm$ 478.00
IL-17F	147.70 $\pm$ 53.42	109.6 $\pm$ 54.28	422.20 $\pm$ 25.29*
IFN- γ	29.98 $\pm$ 5.98	25.50 $\pm$ 6.58	72.87 $\pm$ 8.07*
IL-10	21.41 $\pm$ 6.48	18.46 $\pm$ 2.67	21.31 $\pm$ 6.51
IL-17A	50.35 $\pm$ 34.76	60.51 $\pm$ 21.96	39.50 $\pm$ 17.53
IL-17C/IL-21	23.23 $\pm$ 3.42	26.27 $\pm$ 7.32	15.00 $\pm$ 5.03
IL-22	187.30 $\pm$ 69.71	159.7 $\pm$ 44.22	115.40 $\pm$ 25.73
IL-23	36.78 $\pm$ 5.04	26.39 $\pm$ 16.36	29.84 $\pm$ 14.85
IL-17E/IL-25	17.77 $\pm$ 3.93	20.19 $\pm$ 9.47	13.03 $\pm$ 4.10
IL-31	21.57 $\pm$ 5.52	18.04 $\pm$ 9.75	14.81 $\pm$ 1.68
IL-33	21.54 $\pm$ 7.58	25.80 $\pm$ 7.20	19.83 $\pm$ 1.50
sCD40L	53.66 $\pm$ 24.13	42.37 $\pm$ 26.39	31.43 $\pm$ 5.52
TNF- α	23.07 $\pm$ 1.08	32.86 $\pm$ 12.09	19.79 $\pm$ 4.11

Values given in the table represent the mean concentration (pg/ml) $\pm$ SD of each cytokine in supernatants of TECs from three different MG patients at basal condition (0 h) and after 24 and 48 h of LPS stimulation. Statistical differences between untreated and treated samples were assessed by ANOVA followed by Bonferroni post hoc tests. * P < 0.05, ** P < 0.01.

TECs and with direct LPS stimulation of Ag-DCs. Interestingly, untreated TECs induced a slight, but significant, Ag-DC activation (Fig. 7B). To evaluate whether Ag-DC activation was due to soluble factors from LPS-stimulated and untreated TECs, an indirect co-

culture transwell set-up was established (Supplementary Fig. 4B). After 24 h of transwell co-culture, a significant increase in activation (CCR7, IL-6, IFN- γ) and immunomodulation (TGF- β) gene expression was observed when DC were exposed to soluble factors released from LPS-treated TECs (Fig. 7C). Untreated TEC medium induced down-regulation of Ag-DC endogenous IFN- γ expression. Moreover, we observed that LPS-stimulated TECs induced a significant up-regulation of CCL17 and CCL22 expression in Ag-DCs, accompanied by TLR4 and CCR4 down-regulation (Fig. 7D), likely due to a post-activation response [28]. In contrast, direct Ag-DC LPS stimulation led to increased CCL17, CCL22, TLR4, and CCR4 increased expression, together with TGF- β suppression (not shown), suggesting that the effect of LPS-activated TECs on Ag-DCs differs from direct LPS stimulation of Ag-DCs.

3.9. Live-imaging of cellular interactions among TECs, DCs, Teff and Treg cells

To address the cellular interactions that might occur in the inflamed thymic milieu, we performed in-vitro live imaging experiments, using multiple co-culture systems employing R97-116 specific Th1/Th17 effector cells (Teffs), naïve CD127⁺CD4⁺CD25⁺ T cells (Tregs), Ag-DCs, and untreated or LPS-treated TECs, all from Lewis rats (Supplementary Fig. 4C). When co-cultured with Ag-DCs, R97-116 Teffs showed a random movement [29,30] (Fig. 8A; Supplementary Video 1), and then acquired a stationary phenotype when encountering the proper R97-116-MHC complex, showing a

Fig. 5. Analysis of Th17-related cytokine gene expression in normal and MG thymuses. Real-time PCR analysis of A. IL-6, B. IL-17A, C. IL-17F, D. IL-17C, E. TGF- β 1 and F. FoxP3 transcriptional levels in Normal ($n = 9$) and 28 MG thymuses (MG-FH, $n = 12$; MG-DH, $n = 8$; MG-Involuted, $n = 8$). mRNA levels were expressed as relative values ($2^{-\Delta\Delta C_t} \times 100$) normalized towards GAPDH. Values shown in the graphs are means $\pm$ SEM of duplicate determinations. P values were assessed by the Mann–Whitney U test. * P < 0.05; ** P < 0.01.

Fig. 6. TLR4 stimulation by LPS increases the expression of CCL17, CCL22 and CCR4 in rat EAMG TECs. **A.** Gene expression evaluated via real-time PCR of TLR4, CCR4, CCL17 and CCL22 in naïve (white bars), PBS/CFA-(light gray bars), R97-116/CFA-(dark gray bars) and TACHR/CFA-immunized (EAMG; black bars) rat derived TECs: relative values ($2^{-\Delta\text{ct}} \times 100$) normalized towards β -actin housekeeping gene. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, 2-way-ANOVA test with Bonferroni post-hoc test comparisons versus PBS/CFA-immunized rat derived TECs. **B.** Confocal microscopy for CK (green) and TLR4 (red) labeling on naïve (first column), PBS/CFA-(second column), R97-116/CFA-(third column) and TACHR/CFA-immunized (EAMG; fourth column) rat derived TECs in absence (upper panels) or presence (lower panels) of LPS 1 $\mu\text{g/ml}$ treatment for 24 h as in **A**. Magnification bar: 50 μm . White arrowheads point to TLR4⁺ cells.

relatively stable low V_{max} (4 $\mu\text{m/min}$), and remaining fixed to anchor points [26]. After 60 min co-culture, 65% of Teffs showed a stationary phenotype with a minimum meandering index (Table 3). Teff/Ag-DC contacts were long lasting, visually resembling immunological synapse formation (Fig. 8A, bottom panel; Supplementary Video 1).

When Tregs were added to Teffs/Ag-DCs co-culture (Fig. 8B; Supplementary Video 2), an increased Teffs V_{max} was observed at 60-min co-culture (Table 3), and only 36% of Teffs presented a stationary phenotype; 52% of Teffs maintained a motile phenotype even after 90-min co-culture. Thus, Tregs interfered in Teff/Ag-DC

stable contact formation, as confirmed by Teff high meandering index (Table 3; Supplementary Fig. 5). Treg/DC and Treg/Teff short (0.5–2 min) and medium (3–7 min) length contacts were visible throughout the imaging period, suggesting a competitor role for Tregs in forming contacts with both Teffs and DCs (Fig. 8B, bottom panel; Supplementary Video 2), as elsewhere hypothesized [31,32].

A similar motility pattern was observed when untreated TECs were added to Teffs/Ag-DCs/Tregs co-culture (Fig. 8C; Supplementary Video 3): high Teff V_{max} and low stationary Teff percentage (38%) were measured at 60-min co-culture (Table 3); 55% of Teffs maintained a motile phenotype till 90-min co-culture, and

Fig. 7. TLR4 stimulation of rat TECs induces DC activation. A. Cell cultures of antigen (Ag = R97-116) pulsed rat DCs in absence (Ag-DCs; upper left panel) or presence (Ag/LPS-DCs; upper right panel) of LPS (1 µg/ml; 24 h) stimulation, and co-cultures of Ag-DCs in presence of rat untreated TECs (Ag-DCs + nt-TEC; lower left panel) or previously LPS-stimulated TECs (1 µg/ml; 24 h; Ag-DCs + LPS-TEC). Representative images of 3 independent experiments. Magnification bar: 100 µm. B. Analysis of DC counts from microscopy images of cultures as in A. In particular, the following culture condition were analyzed: immature DCs alone (iDCs); DCs stimulated for 24 h with the antigen R97-116 10 µg/ml (Ag-DCs); DCs stimulated for 24 h with R97-116 10 µg/ml together with 1 µg/ml LPS (Ag/LPS-DCs); Ag-DCs co-cultured for 24 h with nt-TEC or with LPS-stimulated TEC. Data show means ± SEM of three independent experiments with duplicate samples. At least 3 image fields were acquired for each sample; DCs were distinguished from TECs both morphologically and via intrinsic GFP expression. Statistics show results of 1-way ANOVA comparisons plus Bonferroni correction, versus Ag-DCs sample, unless otherwise specified: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. C. and D. Real-time PCR analysis of Ag-DCs cultured for 24 h in transwell plates (3 µm pores), with untreated TECs (NT) or previously LPS-stimulated TECs for 12 or 24 h (LPS-TEC) in the bottom chamber of the transwell plate. RNA sampling was performed on the upper chamber. As control, no TEC were added on the bottom chamber (No TEC). Mean ± SEM of duplicate samples from 2 independent experiments. β -actin was used as housekeeping gene. Statistics: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, 1-way ANOVA comparisons.

an increased meandering index. These data suggest that in the complex thymic milieu fine interactions and cell-to-cell contacts may occur, dampening Teff chance to form stable synapses with DCs.

To evaluate whether an activated innate immunity microenvironment may influence Teff motility and the chances of immunological synapse formation, we added LPS-stimulated TECs to Teffs/Ag-DCs co-culture (Fig. 8D; Supplementary Video 4). TLR4-activated TECs led to a sharp decrease in Teff V_{max} in the first 20-min co-culture, related to a steep increase in the percentage of Teff stationary cells and equal decrease of Teff motile cells, showing low meandering index (Table 3). Thus, TEC LPS-stimulation facilitated cell-to-cell contacts between Teffs and DCs, with the latter showing a more pronounced activated morphology (Fig. 8D; Supplementary Video 4). When Tregs were added to LPS-stimulated TECs/Teffs/Ag-DCs co-culture, their modulatory effect was lost: indeed, most Teffs quickly acquired a stationary phenotype (60% at 30-min; Table 3) with abruptly dropped Teff V_{max} (Fig. 8E; Supplementary Video 5). Nevertheless, Teff motile cells remained more

than 10% even after 90-min co-culture (Supplementary Fig. 5), with a relatively higher displacement, sign of random movement.

3.10. The inflamed microenvironment might favor autoreactive Teff activation

To finely assess activation of AChR-specific Teff, we analyzed $[Ca^{2+}]$ level and showed that Teff $[Ca^{2+}]$ quickly increased in all co-culture conditions, consistent with the formation of TCR/Ag-MHC contacts. The presence of Tregs significantly diminished Teff $[Ca^{2+}]$ (Fig. 9A and B). LPS-stimulated TECs (LPS-TECs), but not untreated TECs (nt-TECs), increased Teff $[Ca^{2+}]$ both in the absence and in the presence of Tregs (Fig. 9A and B). However, after the initial Teff $[Ca^{2+}]$ spike, Tregs were able to reduce the level of $[Ca^{2+}]$ plateau, probably due to balancing contacts between Tregs and Teffs or Tregs and Ag-DCs (Fig. 9B).

Finally, we evaluated the inflammatory cytokine profile of the different co-culture set-ups, compared with that of Teffs and Tregs single cultures (Fig. 9C). IL17A and IFN γ mRNA levels confirmed

Fig. 8. Live-imaging of cellular thymic milieu. The figure shows the locomotion patterns of CFSE-labeled R97-116 specific effector T cells (Teff) analyzed via time-lapse video-microscopy in different co-culture set-ups with: A. antigen-pulsed DCs (DCs); B. DCs and CMRA-labeled CD4⁺CD25⁺ regulatory T cells (Treg); C. DCs, Tregs and untreated TECs (nt-TEC); D. DCs and previously LPS-treated TECs ((LPS)TEC); E. DCs, LPS-TEC and Treg. The histogram graphs in the upper row show Teff maximum velocity (μm/min) over 90 min imaging; blue bars show values referring to motile cells (displacements > 10 μm/10 min), red bars to stationary cells (displacements < 10 μm/10 min). In the second row line graphs show quantification of motile (blue line) and stationary (red line) cells (% of total Teffs) over 90 min imaging. In the third row the scatter graphs show Teff displacement along the image plan at time point 60-min: each colored scatter-line represents one analyzed cell; motility patterns confined to the axes origin indicate a minimum displacement, suggestive of stationary phenotype; motility patterns scattering throughout the axes are indicative of random movement. The inserted histograms show Teff meandering index, calculated as total cell displacement divided by the path length of a cell track, at time point 60-min (red: stationary cells; blue: motile cells). Values shown in each graph are mean quantifications ± SEM of triplicate time-lapse videos for each condition, with at least 195 Teffs analyzed for each time stack (time-lapse: 30 s; 181 time points/video). Final row shows representative pictures of typical cell-to-cell contacts observed in the co-culture conditions. Instantaneous images were taken at the end of time-lapse acquisition. Magnification bars: 10 or 50 μm; green: CFSE-labeled Teffs; red: CMRA-labeled Tregs; white arrow-heads point to sites of immunological synapse formation between activated DCs and Teffs; yellow arrow-heads indicate contacts with Tregs.

the Th1/Th17 phenotype of R97-116 specific Teffs, whereas the levels of FoxP3 and TGF-β confirmed the regulatory phenotype of naïve Tregs; IL-2Rα mRNA levels were quite high not only in Tregs but also in Teffs, as previously shown [33]. A marked CCR4 mRNA expression was observed following 12 h Teffs/Ag-DCs co-culture, accompanied by high levels of IL17A, IFN-γ, and TNF-α mRNAs. These activatory markers, together with CCR4, were instead down-regulated in Treg/Teffs/Ag-DCs co-culture. Of note, the presence of LPS-stimulated TECs determined a sharp increase in CCL17 and CCL22 mRNA expression, accompanied by reduced CCR4, suggestive of post-activatory receptor down-regulation. Similarly, IL-2Rα, IFN-γ, and IL17A mRNAs were down-regulated, while TNF-α was up-regulated. Tregs in the co-culture only partially attenuated the effect induced by LPS-TECs: the levels of CCL17, IL-17, IFN-γ, and TNF-α, but not CCL22, were less abundant (Fig. 9C). Overall, these data suggest that the TLR4-activated TEC microenvironment may result in an altered balance of cytokines and chemokines network.

4. Discussion

The thymus is the main site of autoimmunity development and maintenance in AChR-MG. Accumulating data show that chronic inflammation and innate immune activation are key features of hyperplastic MG thymus [34], pushing the need to better investigate and identify the molecular mechanisms linking the long-term inflammatory state with the autoimmune process. Abnormal recruitment of immune cells and altered Treg-mediated immunoregulation are possible mechanisms responsible for sustaining inflammation and immune response to AChR in MG thymus.

Chemokines and their receptors are key elements in the development and maintenance of innate and adaptive immune responses, orchestrating homing and interactions of DCs, T and B cells into inflammatory sites and secondary lymphoid organs [10]. CXCL13, CCL21, CCL19 and CXCL10 have been demonstrated to be up-regulated in MG thymus, thus suggesting a role in MG thymic

Table 3
Motility characteristics of AChR-specific effector T cells in TLR4-stimulated thymic milieu.

30 min co-culture	% Motile cells	Max. velocity of motile cells	Meandering index of motile cells
DC + Teff + Ag	61 ± 5	16.40 ± 2.95	0.43 ± 0.15
DC + Teff + Ag + Treg	76 ± 9*	12.88 ± 3.80	0.39 ± 0.21
DC + Teff + Ag + Treg + (nt)TEC	88 ± 5***	14.42 ± 2.17	0.69 ± 0.15*
DC + Teff + Ag + (LPS)TEC	42 ± 6**	8.63 ± 4.60**	0.63 ± 0.17*
DC + Teff + Ag + Treg + (LPS)TEC	40 ± 4***§§§	8.32 ± 4.16***§	0.60 ± 0.17
60 min co-culture			
DC + Teff + Ag	35 ± 5	5.20 ± 1.58	0.28 ± 0.08
DC + Teff + Ag + Treg	64 ± 6***	8.55 ± 3.11	0.41 ± 0.06
DC + Teff + Ag + Treg + (nt)TEC	62 ± 3***	8.74 ± 1.04	0.56 ± 0.14*
DC + Teff + Ag + (LPS)TEC	23 ± 4*	5.87 ± 1.29	0.21 ± 0.08
DC + Teff + Ag + Treg + (LPS)TEC	24 ± 3***§§	5.41 ± 1.95	0.20 ± 0.08§§§
90 min co-culture			
DC + Teff + Ag	15 ± 5	3.83 ± 0.66	0.21 ± 0.09
DC + Teff + Ag + Treg	52 ± 4***	4.33 ± 3.09	0.29 ± 0.03
DC + Teff + Ag + Treg + (nt)TEC	55 ± 2***	7.46 ± 1.76	0.54 ± 0.12**
DC + Teff + Ag + (LPS)TEC	7 ± 2	5.06 ± 1.35	0.15 ± 0.06
DC + Teff + Ag + Treg + (LPS)TEC	12 ± 2§§§	4.98 ± 1.62§§	0.15 ± 0.06§§§

Values show motile R97-116-specific Teff characteristics in each co-culture condition: percentage of motile Teffs, maximum motile Teff velocity (expressed in $\mu\text{m}/\text{min}$) and motile Teff meandering index, calculated as total cell displacement divided by the path length of a cell track, at time points 30, 60 and 90 min of imaging. Shown values are mean quantifications $\pm$ SEM of triplicate time-lapse videos for each condition, with at least 195 Teffs analyzed for each time stack (time-lapse: 30 s; 181 time points/video). Statistics show significant results of 2-way ANOVA plus Bonferroni post-hoc comparisons of each co-culture conditions versus (DCs + Teff + Ag). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistically significant post-hoc comparisons between (DCs + Teff + Ag + Treg + nt-TEC) and (DCs + Teff + Ag + Treg + LPS-TEC) conditions are shown as (§) $P < 0.05$ and (§§) $P < 0.05$.

pathology and etiology, via peripheral T- and B-cell recruitment and lymphoid formation [3,6]. Migration of peripheral immune cells in MG thymus and hyperplastic changes could be also favored by the abnormal development of lymphatic vessels and HEVs [35]. Recently, HEVs were found to express SDF-1, a chemokine supposed to drive the migration of CXCR4⁺ APCs and B cells to the thymus and to participate in hyperplastic changes [36].

Other chemokines may be involved in thymic MG pathogenesis, such as CCL17 and CCL22. They share the CCR4 receptor [14,15], expressed on functionally distinct subsets of T cells, such as Th2, Th17, Treg cells, and DCs [11,17,37]; the latter are also able to produce CCL17 and CCL22 [12,13]. This chemokine/chemokine receptor axis has been implicated in autoimmune models [38] and in human autoimmune conditions, such as multiple sclerosis [39] and rheumatoid arthritis [40].

Herein, we hypothesized a role of CCR4 and its ligands in driving abnormal recruitment of peripheral immune cells to MG thymus. The significant increase of CCL17, CCL22 and CCR4 in hyperplastic MG thymuses (Figs. 1 and 4) suggests that these chemokines might play a crucial role in thymic MG etiology. Importantly, in MG thymuses CCL17 and CCL22 were mainly expressed by medullary HCs and surrounding TECs and DCs, as confirmed by laser-capture microdissection (Figs. 1 and 2). HCs were previously described as sites of CCL22 production [41]: CCL22 is expressed by a distinct subset of medullary TECs localized to the outer part of HCs and is one of the chemokines responsible for organizing thymocyte populations into discrete microenvironments, an important process in the negative and positive selections of T-cells [41]. HCs also produce IL-17, SDF-1 and thymic stromal lymphopoietin, the latter able to stimulate thymic DCs in producing high levels of CCL17 and CCL22 [42].

Our studies co-localized CCL17 and CCL22 with TLR4 in TECs and DCs associated with HCs present in MG thymuses (Fig. 2), and allowed us to hypothesize that TLR4-mediated signaling might contribute to abnormal expression and production of the two chemokines in MG thymus. We confirmed this via in vitro studies on TECs isolated from MG and EAMG thymuses, showing a significant increase of CCL17 and CCL22 expression after TLR4 stimulation with LPS (Figs. 4 and 6).

In MG thymuses, CCL17 was also expressed in HEVs (Fig. 1D). Our observation that corticosteroid treatment tended to normalize

transcriptional levels of CCL17, but not of CCL22 (Fig. 4), could be due to the normalization of the number of HEVs in corticosteroid-treated MG thymus [36].

CCR4 is known to drive the migration of DCs and Tregs into antigen presentation sites in inflamed areas [16,17]. The observed increased proportion of DC-LAMP⁺ mature DCs, and more specifically of CCR4⁺ DC-LAMP⁺ DCs, in MG compared to normal thymic sections (Fig. 3), suggests an enhanced CCR4⁺ DCs migration to inflamed MG thymus. CCR4⁺ DC-LAMP⁺ DCs mainly localized around HCs, indicating an active role for HC-produced CCL17 and CCL22 in homing CCR4⁺ DCs around medullary HCs, where these cells could be induced to mature and therefore express DC-LAMP [42]. This event might start a feedback loop of DC infiltration, via a further CCL17/CCL22 production by DCs.

Published data demonstrated the involvement of CCR4 and its ligands on APC functions: binding of antigen-primed T cells to activated DCs was found to be dependent on CCR4 and the duration of their cognate interaction correlated with increased CCL17 production by DCs [43,44]. As CCR4⁺ mature DCs were increased in MG thymuses, we may speculate that these cells could cross-present the autoantigen to AChR-specific T cells, thus favoring maintenance of the inflammatory autoimmune process.

As far as Tregs are concerned, we neither found differences in the number of FoxP3⁺ Tregs nor in the percentage of CCR4⁺ FoxP3⁺ cells between MG thymuses and controls, in line with previous data [45]. However, Treg suppressive function is severely defective in MG thymus [9], and studies in the EAMG model showed that disequilibrium of Th1/Th2/Th17 effector cells and Tregs, and abnormal expression of cytokines affecting Th1/Th2/Th17 balance, may be implicated in MG pathogenesis [46]. The mechanisms underlying Treg dysfunction in MG thymus are still matter of debate; in particular, it is unclear whether Treg impairment is a primary event in MG pathogenesis, or a consequence of the inflammatory thymic microenvironment.

Since innate immune system activation significantly affects cytokine networks, we hypothesized that TLR4 signaling in MG thymus could alter the expression of cytokines involved in Treg function and Teff/Treg balance. Th17-related cytokines are known to play a key role in chronic inflammation and autoimmunity [47] and are mainly secreted by T-cells. However, considerable data suggest that initiation of IL-17-dependent inflammatory responses

Fig. 9. The inflamed thymic milieu favors autoreactive Teff activation. **A.** Quantification of Teff intracellular calcium elevation in co-culture set-ups as in Fig. 9. Fluo-4 loaded Teffs in co-culture with DCs (white circles), DCs and CMRA-Tregs (white squares), DCs, Tregs and nt-TECs (black triangles), DCs and previously LPS-stimulated TEC (LPS-TEC; black circles), DCs, LPS-TECs and CMRA-Tregs (white rhombs), analyzed continuously via microscopy in run-mode for 30 min. The number of Fluo-4 positive (Ca^{2+} spiking T cells) cells is shown as percentage of total Teffs in the image field at each time points. Shown are means $\pm$ SEM of triplicate measurements with at least 65 Teffs/time point. Statistics: 1-way ANOVA with Bonferroni post-hoc correction, *** P < 0.001 all comparisons versus (Teff + Ag-DCs) condition; # P at least < 0.01 starting from time point 5 min, comparison (Teff + Ag-DCs + LPS-TEC) versus (Teff + Ag-DCs + LPS-TEC + Treg). **B.** Representative images of co-culture set-ups, as in A, after 2 h imaging; green: Fluo-4⁺ Teffs; red: CMRA-labeled Tregs. Magnification bars: 50 μm . **C.** Real-time PCR analysis on RNA samples from cellular mixtures derived from 12 h co-cultures as in A–B: white bars, Teff + Ag-DCs; gray bars, Teff + Ag-DCs + Treg + ntTEC; dark gray bars, Teff + Ag-DCs + Treg + LPS-TEC; black bars, Teff + Ag-DCs + LPS-TEC; white bars with horizontal or vertical traits, RNA extracts from single cultures of Teffs and Tregs respectively. Mean $\pm$ SEM of duplicate samples from independent co-culture experiments. β -actin was used as housekeeping gene. Statistics: 1-way ANOVA with Bonferroni post-hoc correction, * P < 0.05, *** P < 0.001 all comparisons versus (Teff + Ag-DCs) condition; # P < at least 0.01, comparison (Teff + Ag-DCs + LPS-TEC) versus (Teff + Ag-DCs + LPS-TEC + Treg).

to stress, injury or pathogens involves innate immune cell populations, including invariant natural killer T-cells, lymphoid-tissue inducer-like cells, NK cells, myeloid cells and epithelial cells, in tissues specialized in immune surveillance [48]. TECs from MG patients are known to produce high levels of IL-6 and IL-1 β [3], two cytokines of the Th17-related family that may affect Treg function by driving FoxP3⁺ cells to secrete IL-17 [49]. We confirmed production of IL-6 and IL-1 β by MG TECs both in basal condition and after TLR4 stimulation (Table 2). Moreover, we found a significant increase of IL-4, IFN- γ , and IL-17F secretion by TECs stimulated with LPS for 48 h. As IL-17F and IL-17A share the IL-17RA/RC receptor and exert common biological effects [48], we suggest that IL-17F produced by MG TECs could affect immunoregulation in MG thymus. Indeed, we found that IL-17F, IL-17A and IL-6 mRNA levels were significantly increased in hyperplastic MG thymuses, accompanied by a decreased expression of TGF- β (Fig. 5), further supporting that Th17-related cytokine balance may contribute to inflammation and immunodysregulation occurring in MG thymus.

Our results seem to indicate the thymic microenvironment surrounding HCs as a privileged site for AChR autosensitization.

Indeed, all the important players in the anti-AChR autoimmune response are present into restricted medullary areas encircling MG HCs: 1) TECs and myoid cells, known to express the autoantigen [50,51]; 2) mature DC-LAMP⁺ myeloid DCs [3], that may cross-present the autoantigen; 3) B-cells, able to produce anti-AChR antibodies [52], following activation by 4) autoreactive Teffs [53]; 5) FoxP3⁺ Tregs, which are likely inefficient in dampening the autoimmune response [9]; and 6) HEVs, representing the entry site for migrating CCR4⁺ DCs [54]. Close interactions between Teffs and APCs at the medullary HC areas might play a critical role in initiation and maintenance of AChR-specific immune responses.

The best way to observe close interactions among immune cells is certainly in vivo imaging of the immune tissue, as recently reviewed [55]. T-cell adaptive immune responses [56,57] and humoral immunity induction [58] as well as the immune reactions in response to pathogens [59] and the effector functions of autoreactive encephalitogenic T cells [60] have been extensively investigated by in vivo imaging. Unfortunately, due to its anatomical position and adulthood-induced size reduction, the thymus is an organ not feasibly accessible by in vivo microscopy and, to our

Fig. 10. Proposed model for TLR4-mediated intra-thymic pathogenesis of MG. In the context of genetically susceptible backgrounds, a “danger signal” in the thymus, such as pathogen infection, may trigger the activation of TLR4-mediated signaling pathways, which in turn may lead to pathogenic and self-perpetuating inflammatory autoimmune response. Specifically, persistent activation of TLR4 in TECs and thymic DCs may promote an inflammatory state that contributes to increased expression, presentation and cross-presentation of AChR, leading to T-cell auto-sensitization and development of the autoimmune response. Close interactions between T-cells and APCs at the medullary HC areas could be critical for initiation of AChR-specific autoimmunity. Increased production of pro-inflammatory chemokines (e.g. CXCL13, CCL21, CCL17 and CCL22) may cause abnormal recruitment of DCs, T- and B-cells in the thymus, leading to hyperplastic changes and perpetuating the inflammatory process and contributing to the feedback loop that promotes maintenance of the autoimmune response in MG thymus.

knowledge, no reports on direct *in vivo* imaging of the thymus have been published. Therefore, we developed an *in vitro* model to mimic the microenvironment of thymic HCs, consisting of naïve or EAMG rat-derived TEC and DC primary cultures, and as effector cells AChR α -subunit specific Th1/Th17 lymphocytes [21]. In this model Teffs moved randomly (motile cells) until encountering the cognate antigen presented by Ag-DCs, at which point they slowed down and remained fixed to anchor sites (stationary cells), forming a relatively stable immunological synapse (Fig. 8A; Supplementary Video 1). Our *in vitro* co-culture set-up was able to reproduce the *in vivo* cell-to-cell contacts between Teffs and DCs, with timing and cell movement velocities being comparable to published data on models in live animals [26,30]. When naïve rat-derived CD127⁺CD4⁺CD25⁺ Tregs were added to our co-culture system, a reduced rate of stable contact formation between Teffs and DCs was observed, quantified by a minor percentage of stationary cells and an increased cellular displacement (Fig. 8B; Supplementary Video 2). As suggested elsewhere [31,32], this effect was likely due to physical competition between Tregs and Teffs in forming contacts with DCs, and even more amplified when a fourth cellular partner, i.e. untreated TECs, was added (Fig. 8C; Supplementary Video 3). Indeed, the co-presence of multiple cellular counterparts dampened the chances of immunological synapse formation between autoreactive Teff cells and activated DCs.

As recently reported [61], minor Teff/DC stable contacts correlated with reduced Teff calcium signals. The quick and steep elevation in Teff intracellular calcium, observed in control Teff/DC

co-culture, was significantly diminished when Tregs or Tregs/nt-TECs were added in our cellular model (Fig. 9A and B). These data suggest an effective role of naïve unspecific Tregs in attenuating specific Teff activation in a thymic microenvironment culture set-up. However, innate immunity activation via LPS-TLR4 engagement on TECs, significantly impaired Treg ability, as visualized by diminished Teff motility/displacement (Fig. 8E) and increased Teff calcium signaling (Fig. 9A and B). This is in line with published data showing LPS effect in dampening Treg functions [62], whilst inducing Th1- or Th2-specific enhanced responses [63]. Importantly, the presence of LPS-stimulated TECs induced DC activation and proliferation (Fig. 7), reminiscent of direct LPS/TLR4 ligation on DCs [64], which can account for DC increased antigen presenting capacity, leading to more stable contacts with Teffs (Supplementary Videos 4 and 5) and minor Treg suppressive capacity, in agreement with previous data [65]. Thus, in our *in vitro* inflamed model, the AChR-specific immune reaction is aided and sped-up by increased numbers of pro-activatory CCR4⁺ DCs and dampened Treg suppressive capacity. This leads to a general pro-inflammatory milieu, characterized by high IL-17, IFN- γ , TNF- α expression levels, accompanied by leukocyte trafficking inducing chemokines such as CCL17 and CCL22 (Fig. 9C), functionally resembling MG thymic inflamed microenvironment. In such activated microenvironment, we may speculate an easy B cell differentiation into anti-AChR antibody producing plasma cells, aided by activated Teffs and follicular OX40⁺ CD40L⁺ Th cells [66]. In this respect, new evidence has recently come out on the role of follicular Th cells both in MG

patients [67] and in the EAMG model [68], suggesting these cells as additional players in immune cell network, that triggers and perpetuates intrathymic autoimmunity.

5. Conclusions

Increasing data indicate that chronic inflammation and innate immune activation via TLRs characterize MG thymus. The overall findings here presented suggest a role for TLR4 signaling in driving immune cell recruitment in MG thymus and in generating an inflammatory response, that in turn may disrupt immunoregulatory mechanisms, favoring auto sensitization and autoimmunity. Our results prompted us to propose a model of intra-thymic MG pathogenesis, in which persistent TLR4 signaling, abnormal recruitment of DCs, and altered Th17-related cytokine network impairing Treg function, are key events for the initiation and perpetuation of the inflammatory autoimmune process within MG thymus (Fig. 10).

Disclosures

None of the authors have any financial conflict of interest.

Acknowledgments

We thank Drs. Antonio Lanzavecchia and Federica Sallusto (Institute for Research in Biomedicine, Bellinzona, CH) for kind gift of cyanobacterial LPS antagonist (CyP); Dr. Renato Longhi (CNR, Istituto di Chimica del Riconoscimento Molecolare, Milan, Italy) for R97-116 peptide synthesis; Dr. Naoto Kawakami (MPI-Neurobiology, Muenchen, Germany) for providing the colony founders of EGFP-transgenic rats. This work was supported by 7th Framework Programme of the European Union FIGHT-MG (Grant No. 242210) and by Italian Ministry of Health, year 2012 (annual research funding).

Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.jaut.2013.12.013>.

References

- [1] Cavalcante P, Bernasconi P, Mantegazza R. Autoimmune mechanisms in myasthenia gravis. *Curr Opin Neurol* 2012;25:621–9.
- [2] Marx A, Pfister F, Schalke B, Saruhan-Direskeneli G, Melms A, Strobel P. The different roles of the thymus in the pathogenesis of the various myasthenia gravis subtypes. *Autoimmun Rev* 2013;12:875–84.
- [3] Cavalcante P, Le Panse R, Berrih-Aknin S, Maggi L, Antozzi C, Baggi F, et al. The thymus in myasthenia gravis: site of “innate autoimmunity”? *Muscle Nerve* 2011;44:467–84.
- [4] Bernasconi P, Barberis M, Baggi F, Passerini L, Cannone M, Arnoldi E, et al. Increased toll-like receptor 4 expression in thymus of myasthenic patients with thymitis and thymic involution. *Am J Pathol* 2005;167:129–39.
- [5] Cufi P, Dragin N, Weiss JM, Martinez-Martinez P, De Baets MH, Roussin R, et al. Implication of double-stranded RNA signaling in the etiology of autoimmune myasthenia gravis. *Ann Neurol* 2013;73:281–93.
- [6] Cavalcante P, Cufi P, Mantegazza R, Berrih-Aknin S, Bernasconi P, Le Panse R. Etiology of myasthenia gravis: innate immunity signature in pathological thymus. *Autoimmun Rev* 2013;12:863–74.
- [7] Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* 2000;1:398–401.
- [8] Cavalcante P, Barberis M, Cannone M, Baggi F, Antozzi C, Maggi L, et al. Detection of poliovirus-infected macrophages in thymus of patients with myasthenia gravis. *Neurology* 2010;74:1118–26.
- [9] Gradolatto A, Nazzari D, Foti M, Bismuth J, Truffault F, Le Panse R, et al. Defects of immunoregulatory mechanisms in myasthenia gravis: role of IL-17. *Ann N Y Acad Sci* 2012;1274:40–7.
- [10] Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunol Today* 1998;19:568–74.
- [11] Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 1999;11:81–8.
- [12] Vulcano M, Albanesi C, Stoppacciaro A, Bagnati R, D'Amico G, Struyf S, et al. Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. *Eur J Immunol* 2001;31:812–22.
- [13] Penna G, Vulcano M, Sozzani S, Adorini L. Differential migration behavior and chemokine production by myeloid and plasmacytoid dendritic cells. *Hum Immunol* 2002;63:1164–71.
- [14] Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. *J Biol Chem* 1997;272:15036–42.
- [15] Imai T, Chantry D, Raport CJ, Wood CL, Nishimura M, Godiska R, et al. Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4. *J Biol Chem* 1998;273:1764–8.
- [16] Yuan Q, Bromley SK, Means TK, Jones KJ, Hayashi F, Bhan AK, et al. CCR4-dependent regulatory T cell function in inflammatory bowel disease. *J Exp Med* 2007;204:1327–34.
- [17] Stutte S, Quast T, Gerbitzki N, Savinko T, Novak N, Reifemberger J, et al. Requirement of CCL17 for CCR7- and CXCR4-dependent migration of cutaneous dendritic cells. *Proc Natl Acad Sci U S A* 2010;107:8736–41.
- [18] Fu W, Chen W. Roles of chemokines in thymopoiesis: redundancy and regulation. *Cell Mol Immunol* 2004;1:266–73.
- [19] Waite JC, Skokos D. Th17 response and inflammatory autoimmune diseases. *Int J Inflamm* 2012;2012:819467.
- [20] Baggi F, Antozzi C, Toscani C, Cordiglieri C. Acetylcholine receptor-induced experimental myasthenia gravis: what have we learned from animal models after three decades? *Arch Immunol Ther Exp (Warsz)* 2012;60:19–30.
- [21] Baggi F, Annoni A, Ubiali F, Milani M, Longhi R, Scaioni W, et al. Breakdown of tolerance to a self-peptide of acetylcholine receptor alpha-subunit induces experimental myasthenia gravis in rats. *J Immunol* 2004;172:2697–703.
- [22] Inoue H, Ohsawa I, Murakami T, Kimura A, Hakamata Y, Sato Y, et al. Development of new inbred transgenic strains of rats with LacZ or GFP. *Biochem Biophys Res Commun* 2005;329:288–95.
- [23] Macagno A, Molteni M, Rinaldi A, Bertoni F, Lanzavecchia A, Rossetti C, et al. A cyanobacterial LPS antagonist prevents endotoxin shock and blocks sustained TLR4 stimulation required for cytokine expression. *J Exp Med* 2006;203:1481–92.
- [24] Marolda R, Ruocco C, Cordiglieri C, Toscani C, Antozzi C, Mantegazza R, et al. Differential targeting of immune-cells by Pixantrone in experimental myasthenia gravis. *J Neuroimmunol* 2013;258:41–50.
- [25] Flugel A, Willem M, Berkowicz T, Wekerle H. Gene transfer into CD4+ T lymphocytes: green fluorescent protein-engineered, encephalitogenic T cells illuminate brain autoimmune responses. *Nat Med* 1999;5:843–7.
- [26] Kawakami N, Nagerl UV, Odoardi F, Bonhoeffer T, Wekerle H, Flugel A. Live imaging of effector cell trafficking and autoantigen recognition within the unfolding autoimmune encephalomyelitis lesion. *J Exp Med* 2005;201:1805–14.
- [27] Cordiglieri C, Odoardi F, Zhang B, Nebel M, Kawakami N, Klinkert WE, et al. Nicotinic acid adenine dinucleotide phosphate-mediated calcium signalling in effector T cells regulates autoimmunity of the central nervous system. *Brain* 2010;133:1930–43.
- [28] Parker LC, Whyte MK, Vogel SN, Dower SK, Sabroe I. Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. *J Immunol* 2004;172:4977–86.
- [29] Miller MJ, Hejazi AS, Wei SH, Cahalan MD, Parker I. T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. *Proc Natl Acad Sci U S A* 2004;101:998–1003.
- [30] Miller MJ, Safrina O, Parker I, Cahalan MD. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *J Exp Med* 2004;200:847–56.
- [31] Tadokoro CE, Shakhar G, Shen S, Ding Y, Lino AC, Maraver A, et al. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J Exp Med* 2006;203:505–11.
- [32] Tomiyama T, Ueda Y, Katakai T, Kondo N, Okazaki K, Kinashi T. Antigen-specific suppression and immunological synapse formation by regulatory T cells require the mst1 kinase. *PLoS One* 2013;8:e73874.
- [33] Flugel A, Berkowicz T, Ritter T, Labour M, Jenne DE, Li Z, et al. Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. *Immunity* 2001;14:547–60.
- [34] Le Panse R, Berrih-Aknin S. Autoimmune myasthenia gravis: autoantibody mechanisms and new developments on immune regulation. *Curr Opin Neurol* 2013;26:569–76.
- [35] Le Panse R, Bismuth J, Cizeron-Clairac G, Weiss JM, Cufi P, Dartevielle P, et al. Thymic remodeling associated with hyperplasia in myasthenia gravis. *Autoimmunity* 2010;43:401–12.
- [36] Weiss JM, Cufi P, Bismuth J, Eymard B, Fadel E, Berrih-Aknin S, et al. SDF-1/CXCL12 recruits B cells and antigen-presenting cells to the thymus of autoimmune myasthenia gravis patients. *Immunobiology* 2013;218:373–81.
- [37] Lim HW, Lee J, Hillsamer P, Kim CH. Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. *J Immunol* 2008;180:122–9.
- [38] Poppensieker K, Otte DM, Schurmann B, Limmer A, Dresing P, Drews E, et al. CC chemokine receptor 4 is required for experimental autoimmune

- encephalomyelitis by regulating GM-CSF and IL-23 production in dendritic cells. *Proc Natl Acad Sci U S A* 2012;109:3897–902.
- [39] Galimberti D, Fenoglio C, Comi C, Scalabrini D, De Riz M, Leone M, et al. MDC/CCL22 intrathecal levels in patients with multiple sclerosis. *Mult Scler* 2008;14:547–9.
- [40] Flytlie HA, Hvid M, Lindgreen E, Kofod-Olsen E, Petersen EL, Jorgensen A, et al. Expression of MDC/CCL22 and its receptor CCR4 in rheumatoid arthritis, psoriatic arthritis and osteoarthritis. *Cytokine* 2010;49:24–9.
- [41] Annunziato F, Romagnani P, Cosmi L, Lazzeri E, Romagnani S. Chemokines and lymphopoiesis in human thymus. *Trends Immunol* 2001;22:277–81.
- [42] Watanabe N, Wang YH, Lee HK, Ito T, Wang YH, Cao W, et al. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature* 2005;436:1181–5.
- [43] Wu M, Fang H, Hwang ST. Cutting edge: CCR4 mediates antigen-primed T cell binding to activated dendritic cells. *J Immunol* 2001;167:4791–5.
- [44] Henry CJ, Ornelles DA, Mitchell LM, Brzoza-Lewis KL, Hiltbold EM. IL-12 produced by dendritic cells augments CD8+ T cell activation through the production of the chemokines CCL1 and CCL17. *J Immunol* 2008;181:8576–84.
- [45] Matsui N, Nakane S, Saito F, Ohigashi I, Nakagawa Y, Kurobe H, et al. Undiminished regulatory T cells in the thymus of patients with myasthenia gravis. *Neurology* 2010;74:816–20.
- [46] Gertel-Lapter S, Mizrahi K, Berrih-Aknin S, Fuchs S, Souroujon MC. Impairment of regulatory T cells in myasthenia gravis: studies in an experimental model. *Autoimmun Rev* 2013;12:894–903.
- [47] Hemdan NY, Birkenmeier G, Wichmann G, Abu El-Saad AM, Krieger T, Conrad K, et al. Interleukin-17-producing T helper cells in autoimmunity. *Autoimmun Rev* 2010;9:785–92.
- [48] Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* 2010;10:479–89.
- [49] Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, et al. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A* 2009;106:4793–8.
- [50] Wakkach A, Guyon T, Bruand C, Tzartos S, Cohen-Kaminsky S, Berrih-Aknin S. Expression of acetylcholine receptor genes in human thymic epithelial cells: implications for myasthenia gravis. *J Immunol* 1996;157:3752–60.
- [51] Schluep M, Willcox N, Vincent A, Dhoot GK, Newsom-Davis J. Acetylcholine receptors in human thymic myoid cells in situ: an immunohistological study. *Ann Neurol* 1987;22:212–22.
- [52] Hill ME, Shiono H, Newsom-Davis J, Willcox N. The myasthenia gravis thymus: a rare source of human autoantibody-secreting plasma cells for testing potential therapeutics. *J Neuroimmunol* 2008;201–202:50–6.
- [53] Melms A, Schalke BC, Kirchner T, Muller-Hermelink HK, Albert E, Wekerle H. Thymus in myasthenia gravis. Isolation of T-lymphocyte lines specific for the nicotinic acetylcholine receptor from thymuses of myasthenic patients. *J Clin Invest* 1988;81:902–8.
- [54] Miyasaka M, Tanaka T. Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nat Rev Immunol* 2004;4:360–70.
- [55] Germain RN, Robey EA, Cahalan MD. A decade of imaging cellular motility and interaction dynamics in the immune system. *Science* 2012;336:1676–81.
- [56] Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 2002;296:1869–73.
- [57] Stoll S, Delon J, Brotz TM, Germain RN. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* 2002;296:1873–6.
- [58] Qi H, Egen JG, Huang AY, Germain RN. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science* 2006;312:1672–6.
- [59] Chtanova T, Han SJ, Schaeffer M, van Dooren GG, Herzmark P, Striemen B, et al. Dynamics of T cell, antigen-presenting cell, and pathogen interactions during recall responses in the lymph node. *Immunity* 2009;31:342–55.
- [60] Bartholomaeus I, Kawakami N, Odoardi F, Schlager C, Miljkovic D, Ellwart JW, et al. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature* 2009;462:94–8.
- [61] Lodygin D, Odoardi F, Schlager C, Korner H, Kitz A, Nosov M, et al. A combination of fluorescent NFAT and H2B sensors uncovers dynamics of T cell activation in real time during CNS autoimmunity. *Nat Med* 2013;19:784–90.
- [62] Adjobimey T, Satoguina J, Oldenburg J, Hoerauf A, Layland LE. Co-activation through TLR4 and TLR9 but not TLR2 skews Treg-mediated modulation of Igs and induces IL-17 secretion in Treg:B cell co-cultures. *Innate Immun* 2014;20(1):12–23. [Epub 2013 Mar 25].
- [63] Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002;196:1645–51.
- [64] Cavanagh LL, Weninger W. Dendritic cell behaviour in vivo: lessons learned from intravital two-photon microscopy. *Immunol Cell Biol* 2008;86:428–38.
- [65] Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 2003;299:1033–6.
- [66] Gomez-Martin D, Diaz-Zamudio M, Romo-Tena J, Ibarra-Sanchez MJ, Alcocer-Varela J. Follicular helper T cells poise immune responses to the development of autoimmune pathology. *Autoimmun Rev* 2011;10:325–30.
- [67] Luo C, Li Y, Liu W, Feng H, Wang H, Huang X, et al. Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. *J Neuroimmunol* 2013;256:55–61.
- [68] Xie X, Mu L, Yao X, Li N, Sun B, Li Y, et al. ATRA alters humoral responses associated with amelioration of EAMG symptoms by balancing Tfh/Tfr helper cell profiles. *Clin Immunol* 2013;148:162–76.