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Central Role of Macrophages and Nucleic Acid Release in Myasthenia Gravis Thymus

Cloé A. Payet, PhD,¹ Axel You, MSc,¹ Odessa-Maud Fayet, MSc,¹ Edouard Hemery, MSc,¹ Frederique Truffault, MSc,¹ Vincent Bondet, PhD,² Darragh Duffy, PhD,² Frédérique Michel, PhD,³ Elie Fadel, MD,⁴ Julien Guihaire, MD,⁴ Sophie Demeret, MD,⁵ Sonia Berrih-Aknin, PhD,¹ and Rozen Le Panse, PhD ¹

Objective: Myasthenia gravis (MG) is a neuromuscular disease mediated by antibodies against the acetylcholine receptor (AChR). The thymus plays a primary role in AChR-MG and is characterized by a type I interferon (IFN) signature linked to IFN- β . We investigated if AChR-MG was characterized by an IFN-I signature in the blood, and further investigated the chronic thymic IFN-I signature.

Methods: Serum levels of IFN- β and IFN- α subtypes, and mRNA expression for IFN-I subtypes and IFN-stimulated genes in peripheral mononuclear blood cells (PBMCs) were analyzed. The contribution of endogenous nucleic acids in thymic expression of IFN-I subtypes was investigated in human thymic epithelial cell cultures and the mouse thymus. By immunohistochemistry, thymic CD68⁺ and CD163⁺ macrophages were analyzed in AChR-MG. To investigate the impact of a decrease in thymic macrophages, mice were treated with an anti-CSF1R antibody.

Results: No IFN-I signature was observed in the periphery emphasizing that the IFN-I signature is restricted to the MG thymus. Molecules mimicking endogenous dsDNA signalization (Poly(dA:dT) and 2'3'-cGAMP), or dexamethasone-induced necrotic thymocytes increased IFN- β and α -AChR expression by thymic epithelial cells, and in the mouse thymus. A significant decrease in thymic macrophages was demonstrated in AChR-MG. In mice, a decrease in thymic macrophages led to an increase of necrotic thymocytes associated with IFN- β and α -AChR expression.

Interpretation: These results suggest that the decrease of thymic macrophages in AChR-MG impairs the elimination of apoptotic thymocytes favoring the release of endogenous nucleic acids from necrotic thymocytes. In this inflammatory context, thymic epithelial cells may overexpress IFN- β , which specifically induces α -AChR, resulting in self-sensitization and thymic changes leading to AChR-MG.

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Introduction

Myasthenia gravis (MG) is a neuromuscular disease characterized by disabling muscle weaknesses due to autoantibodies targeting the acetylcholine receptor (AChR), mainly the α -AChR subunit.¹ In early-onset AChR-MG (EOMG), the thymus is the effector organ and its removal

alleviates the symptoms of the disease.² The MG thymus displays functional and morphological abnormalities characterized by abnormal B-cell infiltration leading to ectopic germinal center development (thymic follicular hyperplasia). Moreover, the MG thymus includes all the components of the anti-AChR response: the expression of AChR

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Address correspondence to Dr Le Panse, Center of Research in Myology, Sorbonne University—INSERM—AIM, 105, Boulevard de l'Hôpital, 75013 Paris, France. E-mail: rozen.lepanse@upmc.fr

Axel You, Odessa-Maud Fayet these authors contributed equally to this work.

From the ¹Sorbonne University, INSERM, Association Institute of Myology, Center of Research in Myology, UMRS, Paris, France; ²Translational Immunology Unit, Institut Pasteur, Université Paris Cité, Paris, France; ³Cytokine signaling unit, INSERM U1224, Institut Pasteur, Paris, France; ⁴Marie Lannelongue Hospital, Paris-Sud University, Le Plessis-Robinson, France; and ⁵Department of Neurology, Neuro Intensive Care Unit, Hôpital Pitié-Salpêtrière, APHP, Paris, France

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subunits by thymic epithelial cells (TECs) and myoid cells,³ the presence of anti-AChR auto-reactive T and B cells.^{4–7}

In AChR-MG, a persistent interferon (IFN)-I signature is detected in the thymus. This is especially linked to the overexpression of IFN- β ⁸ that seems to be the main orchestrator of thymic changes observed in patients: (1) IFN- β favors the selective sensitization against AChR by specifically inducing α -AChR expression in TECs, and increasing TEC death and the uptake of TEC proteins by dendritic cells.⁹ (2) IFN- β increases thymic expression of CXCL13 and CCL21, two chemokines involved in germinal center development and overexpressed in the EOMG thymus.¹⁰ (3) IFN- β induces the overexpression of the B-cell activating factor (BAFF).⁹ (4) IFN- β stimulates the development of pathogenic Th17 cells in the thymus.^{11,12} In addition, a causative role of IFN-I is supported by a mouse model where injections of Poly(I:C) induce thymic changes that lead to an anti- α -AChR response.^{8,9}

The discovery of the etiological mechanisms involved in AChR-MG lies in the understanding of signals that drive this abnormal thymic IFN-I signature.¹³ The thymus is a common target organ for infectious diseases¹⁴ and IFN-I subtypes are transiently produced in response to viral infections. However, the IFN-I signature is detected in the MG thymus, even long after disease onset.⁸ In 2011, Crow et al, defined a new group of Mendelian disorders characterized by the chronic overexpression of IFN-I: interferonopathies.¹⁵ Some autoimmune diseases tend to share features with interferonopathies with an IFN-I signature in peripheral blood and target tissues, such as in systemic lupus erythematosus, dermatomyositis, and Sjögren's syndrome.^{16,17}

We investigated whether EOMG shared some features with interferonopathies by analyzing the IFN-I signature in the blood and thymus of MG patients. Our results showed no alteration of IFN-I subtypes or interferon-stimulated genes (ISGs) in the blood, indicating that the IFN-I signature is restricted to the thymus in EOMG. Using *in vitro* and *in vivo* approaches, we demonstrated that endogenous nucleic acids (NAs) released by necrotic thymocytes induced IFN- β and α -AChR expression by TECs. In patients with AChR-MG, we observed a decreased number of thymic macrophages. This could alter the proper efferocytosis of apoptotic thymocytes, and necrotic thymocytes would release their intracellular content, such as NAs. Finally, endogenous NAs may activate innate immunity pathways involved in the elevated IFN- β expression at the basis of thymic changes leading to the EOMG.

Methods

Human Blood and Thymic Samples

Serum (n = 43, 17–47 years old) and peripheral mononuclear blood cells (PBMCs; n = 23, 20–45 years old) were collected from EOMG patients with anti-AChR antibodies that were not under immunosuppressive therapies and with only cholinesterase inhibitors (Tables S1A, S1B). For blood samples collected after thymectomy, the delay after thymectomy was variable ranging from 6 months to 21 years. Control serum (n = 14, 17–50 years old) and PBMCs (n = 10, 23–45 years old) from healthy donors were obtained from the French blood establishment.

For immunohistochemistry, thymic biopsies were obtained from the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France), where AChR-positive MG patients (n = 10, 7–44 years old) underwent thymectomy (Table S2), and from age-/sex-matched non-MG controls (n = 6, 6–44 years old) from cardiovascular surgery. TECs were prepared from infant thymus obtained from cardiovascular surgery.

None of the patients with MG displayed thymoma or infectious diseases. Studies on blood and thymic samples were approved by local ethics committees (RCB 2006-A00164-47 and RCB 2010-A00250-39).

Serum Cytokine Measurement

IFN- α serum levels were measured using a Quanterix Simoa homebrew assay developed as previously described.¹⁶ Samples were diluted threefold, and this assay allowed for the detection of all IFN- α subtypes with a limit of detection (LOD) of 0.04 fg/ml. IFN- β serum levels were assessed by an enzyme-linked immunosorbent assay (ELISA; PBL-41415, Enzo Life Science, Villeurbanne, France) on serum samples diluted to half.

TEC Cultures

TECs were obtained from thymic explant cultures as previously described.⁸ After 1 week, TECs were seeded overnight at 1.4×10^5 cells/cm² in RPMI-5% horse serum. TECs were treated for 24 hours with Poly(I:C) 200 μ g/ml (ttrl-pic), Poly(dA:dT) 100 μ g/ml (ttrl-patn), CpG 5 μ g/ml (ttrl-2216), 2'3'-cGAMP 100 μ g/ml (ttrl-nacga23), or HSV60 100 μ g/ml (ttrl-hsv60n; Invivogen) in RPMI-0.5% horse serum. Interferon- α/β receptor subunit 2 (IFNAR2) blocking antibody 10 μ g/ml (PBL-21385-1, Enzo Life Science) was added 1 hour before the other reagents.⁸

For co-culture experiments, TECs were prepared as above and co-cultured with thymocytes induced into apoptosis (thymocytes were obtained from infant thymus biopsies). The induction was carried out by incubating thymocytes for 4 hours with 50 μ M dexamethasone

(D4902, Sigma-Aldrich, Saint-Quentin-Fallavier, France). Thymocytes were washed to remove dexamethasone and seeded with TECs for 24 hours (Thymocyte/TEC ratio = 20:1). Before TECs were collected, the co-culture was thoroughly washed with D-PBS to get rid of the thymocytes.

Reverse Transcription and Quantitative Polymerase Chain Reaction

Total RNA from human TECs or mouse thymic fragments was extracted in Trizol (15596018, Life Technologies, Courtaboeuf, France). Then, 500 ng of RNA was retro-transcribed using AMV (10109118001, Roche Life Science, Saint-Quentin-Fallavier, France) for 1 hour at 42°C with oligo-dT (18418012, Life Technologies, Courtaboeuf, France). Quantitative PCR reactions were performed with the LightCycler 480 SYBR Green Master Mix (4887352001, Roche Life Science, Saint-Quentin-Fallavier, France) on the LightCycler 480 System. Primer sequences are listed in Supplementary Table S3. Relative mRNA expressions were normalized with GAPDH or 28 S.

Immunohistochemistry

Cryosections of thymic samples (7 µm) were fixed in ice-cold acetone for 20 minutes and unspecific binding sites were blocked with 5% BSA for 20 minutes. Sections were stained overnight at 4°C with antibodies against keratin 5 (905501, Biolegend) for medullary TECs, CD68 (MAB20401, R&D Systems) or CD163 (556017, BD Pharmingen) for macrophages, CD208 (IM3448, Beckman Coulter) for mature dendritic cells or CD123 (555642, Becton Dickinson) for plasmacytoid dendritic cells. Next, sections were stained with fluorescent secondary antibodies: Donkey anti-mouse alexa-594 or chicken anti-rabbit alexa-488 (A-21203 and A-21441, Life Technologies) for 1 hour 30°C at room temperature. Images were acquired with a ZeissAxio Observer R1 Inverted Microscope. The number of cells was counted by Zen Software in 3 to 4 mosaic pictures containing 25 fields of each thymic section. Representative sections were selected distinguishing cortical or medullary regions based on Keratin 5 staining.

Animals

Four to 5 weeks old C57/bl6J mice (Janvier Labs) were treated by intraperitoneal (i.p.) injections of Poly(I:C) 200µg/injection, dexamethasone 5mg/kg/injection, Poly(dA:dT) 100µg/injection, 2'3'-cGAMP 25µg/injection, or saline solution 3 times per week. Mice were weighed and euthanized after 1 week. To analyze the impact of a decreased number of thymic macrophages, 4 to 5 week

old C57/bl6J mice were treated with an anti-CSF1R antibody 300µg/injection (NBP2-43363, BioTechne) every 3 days for 4 weeks. Thymic cells were analyzed by flow cytometry and a piece of the thymus was stored at -80°C for reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Flow Cytometry

Mouse thymuses were crushed and filtered through 60 µm nylon. Human or mouse thymocytes were labeled with Annexin-V and propidium iodide (PI) (556419 and 51-66211E, Becton Dickinson, Le point de Claix, France) in Annexin-V binding buffer for 15 minutes and monitored using the Beckman Coulter Cytoflex S apparatus.

Statistical Analysis

For 2-by-2 comparisons, the non-parametric Mann-Whitney test was applied. For multiple comparisons, a one-way analysis of variance (ANOVA) test was applied with Dunn's post hoc tests. Correlation analyses were performed using Spearman's correlation coefficient for non-Gaussian distributed variables, with $p < 0.05$ considered significant.

Results

No IFN-I Signature in the Blood of MG Patients

First, we investigated the serum levels of IFN-β (Fig 1A) and all IFN-α subtypes (Fig 1B) by ELISA and Simoa, respectively. No differences were observed between controls and patients with AChR-MG. Furthermore, among patients with AChR-MG, no differences were observed between thymectomized patients or not (Fig 1A, 1B).

In PBMCs from controls and patients with AChR-MG, we also measured mRNA expression for IFN-β (Fig 1C), IFN-α2 (Fig 1D), and their receptor subunits IFNAR1 (Fig 1E) and IFNAR2 (Fig 1F). No significant differences were detected for these 4 genes in MG patients whether or not patients were thymectomized. We measured the mRNA expression of ISGs: OAS1, MX1 but also 6 ISGs previously described for diagnosis of interferonopathies.¹⁸ No significant differences were observed (Fig 1G–N) in thymectomized or not MG patients as compared to controls, except for RSAD2 (Fig 1N).

Overall, these results indicate no IFN-I signature in the serum or PBMCs of AChR-MG patients.

Synthetic Molecules Mimicking Endogenous dsDNA Induced IFN-β and α-AChR Expression in TECs and the Thymus

We previously demonstrated that Poly(I:C), a double-stranded(ds)RNA strongly induces the expression of IFN-β and α-AChR, the main autoantigen in AChR-MG, in

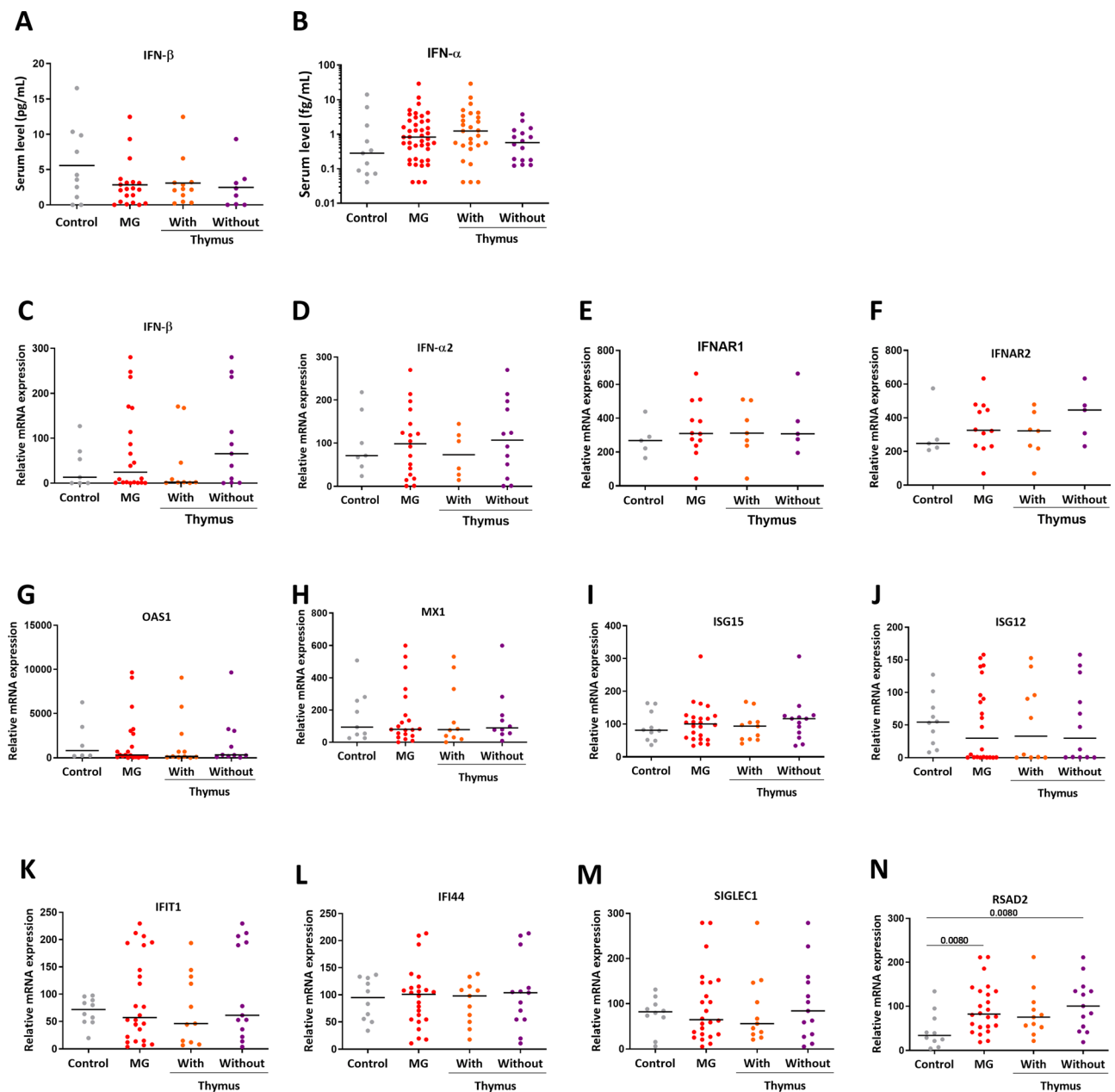


FIGURE 1: Search for an IFN-I signature in serum and PBMCs of AChR-MG patients. Measurement of serum levels of IFN- β by ELISA (A) and IFN- α subtypes⁵² by Simoa (B) in AChR-MG patients ($n = 20$ for IFN- β , $n = 43$ for IFN- α) and healthy donors ($n = 10$ for IFN- β and $n = 11$ for IFN- α). The mRNA expression of IFN- β (C), IFN- α 2 (D), IFNAR1 (E), and IFNAR2 (F) and ISGs: OAS1 (G), MX1 (H), ISG15 (I), IFIT1 (J), ISG12 (K), IFI44L (L), SIGLEC1 (M), and RSAD2 (N) in PBMCs of AChR-MG patients ($n = 12$ – 23 , depending on PCR) and healthy donors ($n = 6$ – 10 depending on PCR). The mRNA expression was normalized on 28 S. The p values were determined by the Mann–Whitney test for 2-by-2 comparisons for each subgroup. The p values were indicated only when $p < 0.05$. ELISA = enzyme-linked immunosorbent assay; MG = myasthenia gravis; PBMCs = peripheral mononuclear blood cells. [Color figure can be viewed at www.annalsofneurology.org]

TEC cultures.⁸ To determine if this effect is specific to dsRNA or if it could be also elicited by dsDNA, we investigated on TECs the in vitro effect of molecules mimicking dsDNA of bacterial (unmethylated CpG), viral (HSV-60, a synthetic herpes simplex viral DNA), or mammalian (Poly(dA:dT)) origin, and a molecule involved in intracellular signalization of dsDNA (2'3'-

cGAMP). The expression of IFN- β (Fig 2A) and α -AChR (Fig 2B) mRNA was observed in TECs treated for 24 hours with Poly(dA:dT) or 2'3'-cGAMP but not with molecules mimicking pathogen infections. Of note, if Poly(dA:dT) and 2'3'-cGAMP induced IFN- β and α -AChR in TECs, they were not as potent as Poly(I:C), a dsRNA (IFN- β and α -AChR mRNA induction rate as

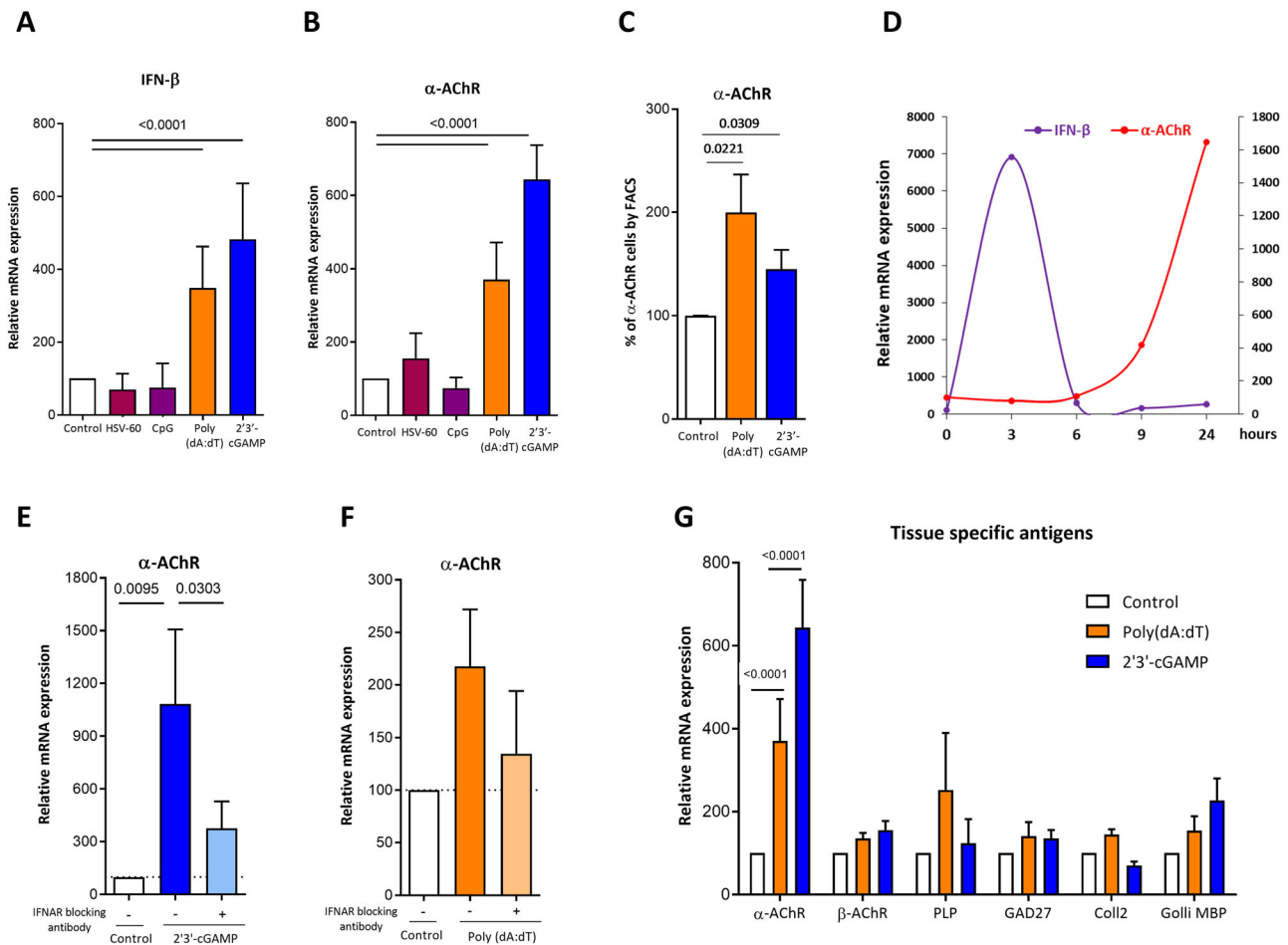


FIGURE 2: Effects of endogenous nucleic acids on TECs. mRNA expression of IFN- β (A) and α -AChR (B) by TECs measured 24 hours after stimulation with HSV-60 100 μ g/ml (n = 3), CpG 5 μ g/ml (n = 3), Poly(dA:dT) 100 μ g/ml (n = 8), or 2'3'-cGAMP 100 μ g/ml (n = 16). Analysis by flow cytometry of the percentage of α -AChR expression by TECs after 24 hours in culture with Poly(dA:dT) 100 μ g/ml (n = 3) or 2'3'-cGAMP 100 μ g/ml (n = 4) (C). Representative time course effect of 2'3'-cGAMP on IFN- β and α -AChR mRNA expression by TECs (D). Effects of 2'3'-cGAMP (E) and Poly(dA:dT) (F) on α -AChR expression by TECs pretreated with or without 10 μ g/ml of an IFNAR2 blocking antibody. mRNA expression was normalized on GAPDH, and for each experiment, the mRNA level of untreated control cells was fixed at 100. The *p* values were determined by the Mann-Whitney test for 2-by-2 comparisons for each treatment as compared with untreated control (A, B, D, G) and to treatment with the IFNAR2 blocking antibody (E, F). The *p* values were indicated only when *p* < 0.05. TEC = thymic epithelial cell. [Color figure can be viewed at www.annalsofneurology.org]

compared with control cells: 28.3 \pm 11.3 (n = 16) and 24.3 \pm 9.9 (n = 21), respectively). Experiments were done using TECs deriving from infant thymuses but using 3 to 4 adult control or MG thymuses, similar results were obtained for TECS treated with 2'3'-cGAMP or Poly(I:C) (data not shown). Nevertheless, we confirmed at the protein level that Poly(dA:dT) and 2'3'-cGAMP were able to induce α -AChR (Fig 2C). We also analyzed the kinetic expression of IFN- β and α -AChR mRNA upon activation of the dsDNA signaling pathway with 2'3'-cGAMP. We observed that IFN- β expression peaked at 3 hours and decreased afterward, whereas α -AChR expression peaked at 24 hours (Fig 2D). This shift suggests that α -AChR expression was mediated in an autocrine manner by IFN- β . To test this hypothesis,

TECs were pretreated with an IFNAR-blocking antibody before being stimulated with either 2'3'-cGAMP (Fig 2E) or Poly(dA:dT) (Fig 2F) for 24 hours. The results showed a strong decrease in the expression of α -AChR mRNA when IFN-I signaling was blocked. The induction of α -AChR expression upon stimulation of TECs with Poly(dA:dT) or 2'3'-cGAMP was specific as no inductions were observed for β -AChR or other tissue-specific antigens (Fig 2G).

The effects of Poly(dA:dT) and 2'3'-cGAMP were also tested in vivo. Mice were i.p. injected every 2 days and the thymus was analyzed at day 7. We did not detect any thymic involution (Fig 3A,3F). However, we observed an increased thymic expression of IFN- β and IFN- α 2 mRNA (Fig 3B,C,G,H) associated with an increase in

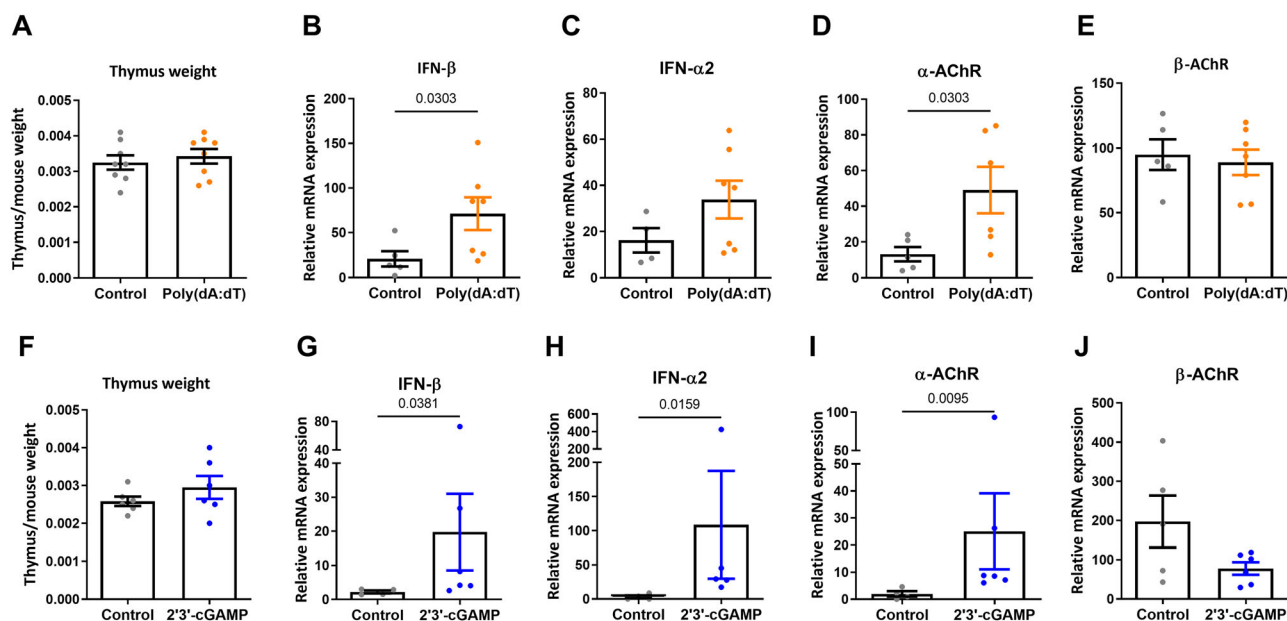


FIGURE 3: In vivo analyses of the effects of Poly(dA:dT) and 2'3'-cGAMP on the thymus of mice. Mice were ip injected every other day with Poly(dA:dT) 100 μ g/kg/injection (A–E) or 2'3'-cGAMP 25 μ g/kg/injection (F–J). Mice were euthanized at day 7, 24 hours after the last i.p. injection. Thymus weight was normalized on the mouse weight (A and F) and mRNA was extracted to analyze the expression of IFN- β (B and G), IFN α -2 (C and H), α -AChR (D and I), and β -AChR (E and J). The mRNA expression was normalized on GAPDH. The *p* values were assessed by the Mann–Whitney test and indicated only for *p* < 0.05. [Color figure can be viewed at www.annalsofneurology.org]

α -AChR (Fig 3D,I) but not in β -AChR mRNA expression (Fig 3E,J).

Altogether, these in vitro results suggest a specific implication of synthetic molecules mimicking endogenous dsDNA in the expression of IFN- β that further elicited α -AChR in TECs. In addition, in mice, the injection of these molecules induced an IFN- β and IFN- α 2 thymic expression associated with α -AChR expression.

Necrotic Thymocytes Induced IFN- β and α -AChR Expression in TECs

Endogenous NAs are known to be released in the cellular environment by necrotic cells. To determine whether “natural” NAs have the same effects as Poly(I:C), Poly(dA:dT), and 2'3'-cGAMP, we investigated the impact of co-culturing necrotic thymocytes with TECs. Thymocytes were induced in apoptosis/necrosis by a 4 hours pretreatment with dexamethasone¹⁹ that increased the percentage of PI⁺ cells corresponding to late apoptotic and necrotic cells (Fig 4A, 4B). Thymocytes were then washed to get rid of dexamethasone and co-cultured for 24 hours with TECs. We observed that apoptotic/necrotic thymocytes increased the expression of IFN- β and α -AChR mRNA as compared with co-cultures with control thymocytes (Fig 4C, 4D). The effect of apoptotic/necrotic thymocytes was specific to α -AChR and not observed for β -AChR or ϵ -AChR (Fig 4E, 4F).

These data suggest that the release of the intracellular content of necrotic thymocytes containing NAs could induce the expression of IFN- β and, subsequently, that of α -AChR by TECs.

Necrotic Thymocytes Induced IFN- β and α -AChR Expression in the Thymus

Dexamethasone injections can induce an acute thymic involution due to thymocytes apoptosis.²⁰ We investigated the impact of thymocyte apoptosis on the thymus of mice. Seven days after dexamethasone treatment, the thymus weight was significantly decreased in treated mice (Fig 5A), but not the total weight of the mice (data not shown). Dexamethasone treatment led to a strong increase in the percentage of late apoptotic and necrotic thymic cells (Fig 5B) that was associated with an important decrease in the relative number of thymocytes (data not shown) but the thymic stroma was not affected, as no change in keratin 5 mRNA expression was observed (Fig 5C). Consequently, acute thymic involution was thus due to the induction in thymocyte apoptosis/necrosis in dexamethasone-injected mice.

In parallel, we found an increased thymic expression of IFN-I subtypes, IFN- β (Fig 5D), and IFN- α 2 (Fig 5E) mRNA, and also of α -AChR mRNA (Fig 5F) as well as the ISGs: IFI44, ISG12, IFIT1, and MX1 (Fig 5H). No increased expression was measured for β -AChR mRNA (Fig 5G). Furthermore, a strong correlation between IFN-

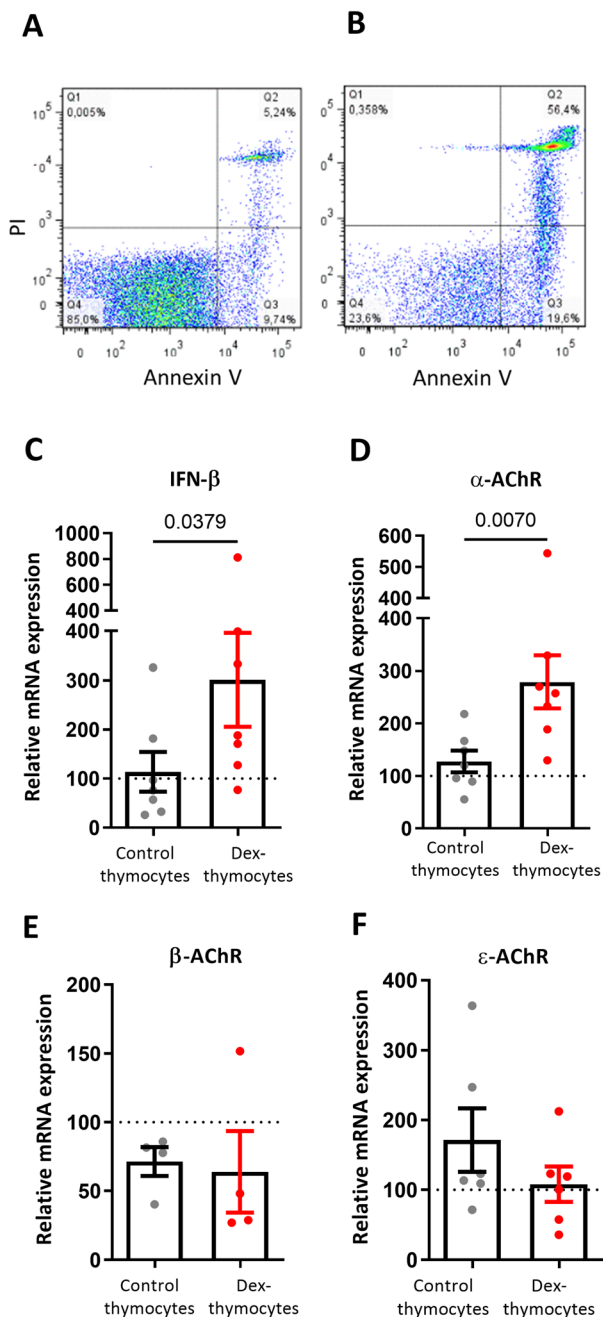


FIGURE 4: Analysis of the effects of necrotic thymocytes on TECs. Representative staining of thymocyte apoptosis/necrosis by FACS with Annexin V and PI labeling, before (A) and after (B) a 4 hours dexamethasone treatment (representative labeling). The mRNA expression of IFN- β (C), α -AChR (D), β -AChR (E), and ε -AChR (F) by TECs after a 24 hours co-culture with thymocytes induced or not in necrosis by an initial 4 hours pretreatment with dexamethasone. The mRNA expression was normalized on GAPDH, and for each experiment, the mRNA level of untreated control TECs was fixed at 100 (dotted line on graphs). The *p* values were determined by the Mann-Whitney test and *p* values were indicated only when *p* < 0.05. FACS = fluorescent activated cell sorting; PI = propidium iodide; TECs = thymic epithelial cells. [Color figure can be viewed at www.annalsofneurology.org]

β or IFN- α 2, and α -AChR mRNA expression in the thymus of mice injected with dexamethasone was observed (Fig 5I, 5J).

These results suggest that necrotic thymocytes induced upon dexamethasone treatment could trigger the expression of IFN-I leading to α -AChR expression in the thymus.

Decreased Number of Macrophages in the Thymus of AChR-MG Patients

Macrophages play a major role in the efferocytosis of apoptotic thymocytes.^{21,22} By immunofluorescence, we detected a significant decrease in the number of CD68+ and CD163+ macrophages in the thymus of patients with AChR-MG as compared to controls. This decrease was observed in both cortical and medullary regions (Fig 6A, 6B). CD68 or CD163 are classically used as macrophage markers, nevertheless, they can also stain other cells. To exclude the possibility that the observed decrease was due to other cells than macrophages, we analyzed the number of CD208+ mature dendritic cells and CD123+ plasmacytoid dendritic cells.²³ No significant differences were observed between control and AChR-MG thymuses for mature or plasmacytoid dendritic cells (data not shown).

Decreased Thymic Macrophages Might Result in Increased IFN-I and α -AChR in Mice

To determine if a decrease in thymic macrophages could induce an IFN-I expression, mice were injected with an anti-CSF1R antibody for 4 weeks. CSF1 is a molecule implicated in the renewal and differentiation of tissue macrophages, and prolonged blocking of its receptor induces macrophage depletion.²⁴ We observed a decrease in the thymus weight (Fig 7A) associated with a slight increase in the percentage of late apoptotic and necrotic thymic cells (Fig 7B) but no change regarding the expression of keratin 5 (Fig 7C). We confirmed that the prolonged treatment with the anti-CSF1R antibody decreased the proportion of macrophages as measured by CD68 mRNA expression (Fig 7D). In parallel, we observed an increased thymic expression of IFN- β (Fig 7E), IFN- α 2 (Fig 7F), and of α -AChR (Fig 7G) but not of β -AChR mRNA (Fig 7H). These results suggest that in mice a reduced number of thymic macrophages may induce the expression of IFN-I subtypes and a higher expression of α -AChR.

Discussion

The thymus of early-onset AChR-MG patients is characterized by a persistent IFN-I signature, associated with a high expression of IFN- β and ISGs.⁸ We wondered

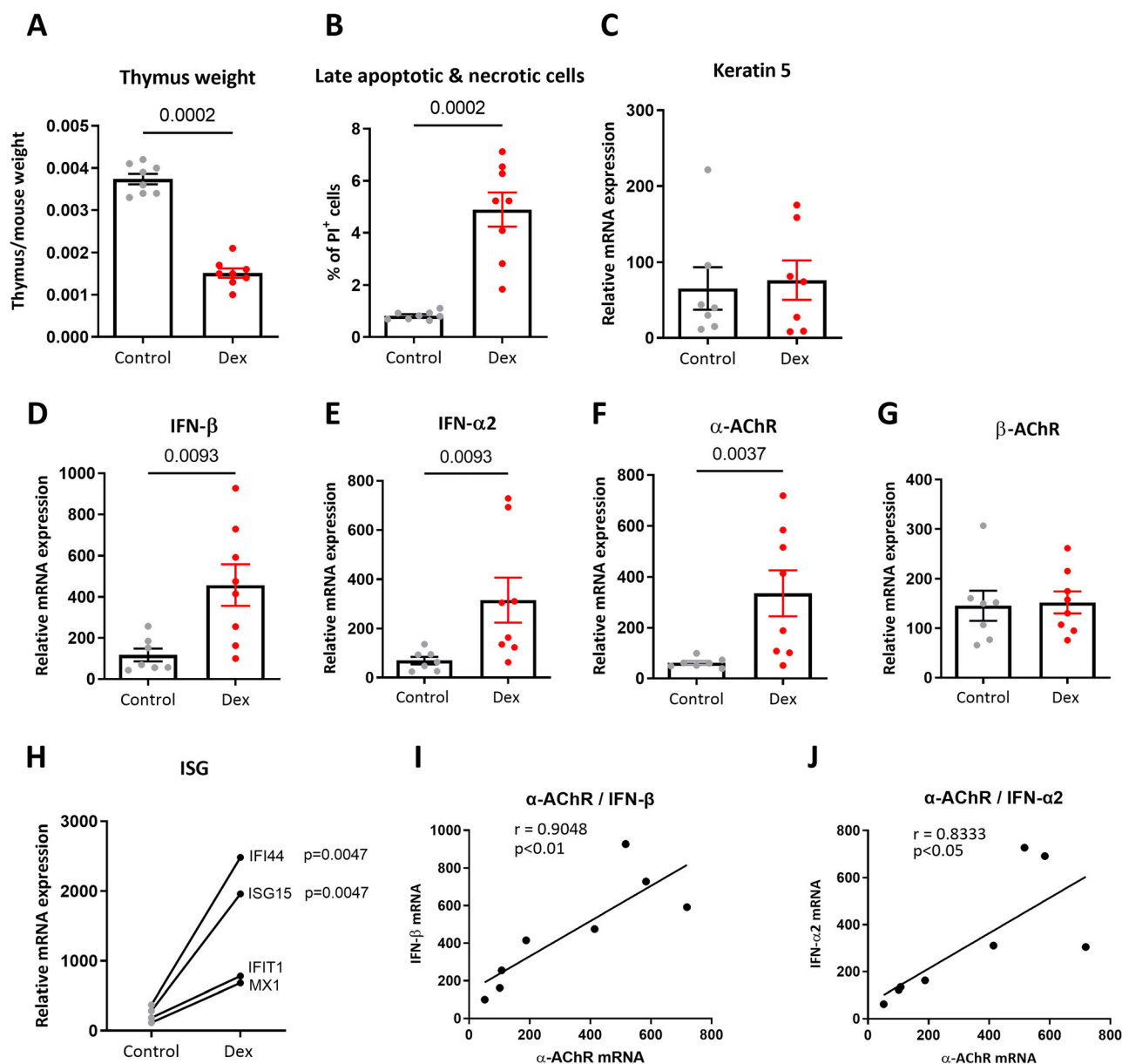


FIGURE 5: Analysis of the effects of acute thymic involution due to necrotic thymocytes in mice. Mice were injected with dexamethasone 5 mg/kg/injection every 2 days. After a week, mice were euthanized. The thymuses were weighted (A) and the percentages of late apoptotic/necrotic thymocytes were measured by FACS with Annexin V and PI labeling (B). Thymic mRNA expression was measured and normalized to GAPDH for keratin 5 (C), IFN- β (D), IFN- $\alpha 2$ (E), α -AChR (F), β -AChR (G), and 4 ISG (IFI44, ISG15, IFIT1, and MX1) (H). The p values were assessed by the Mann-Whitney test and p values were indicated only when $p < 0.05$ (A–H). Correlations between IFN- β (I) or IFN- $\alpha 2$ mRNA (J) and α -AChR expression were performed for dexamethasone-treated mice using Spearman's correlation test, with a $p < 0.05$ considered significant. FACS = fluorescent activated cell sorting; PI = propidium iodide. [Color figure can be viewed at www.annalsofneurology.org]

whether an IFN-I signature could also be detected in periphery in EOMG patients. However, no significant increased serum levels of IFN- α subtypes or IFN- β were detected, and no IFN-I signature was observed in PBMCs of patients with EOMG. In a whole-transcriptome sequencing study on PBMCs from EOMG patients, Barzago et al detected enriched functional pathways related to “infectious disease” and “inflammatory response-associated” categories. However, from further in-

depth analysis, no obvious IFN-I signature was found.²⁵ Both in our study and that of Barzago et al, no changes were detected for thymectomized or non-thymectomized patients with EOMG, indicating that the IFN-I signature is contained in the thymus of patients. In contrast to EOMG, chronic peripheral activation of the IFN-I pathway seems to be linked to systemic autoimmune diseases and characterized by the presence of anti-nuclear antibodies, such as in systemic lupus erythematosus,

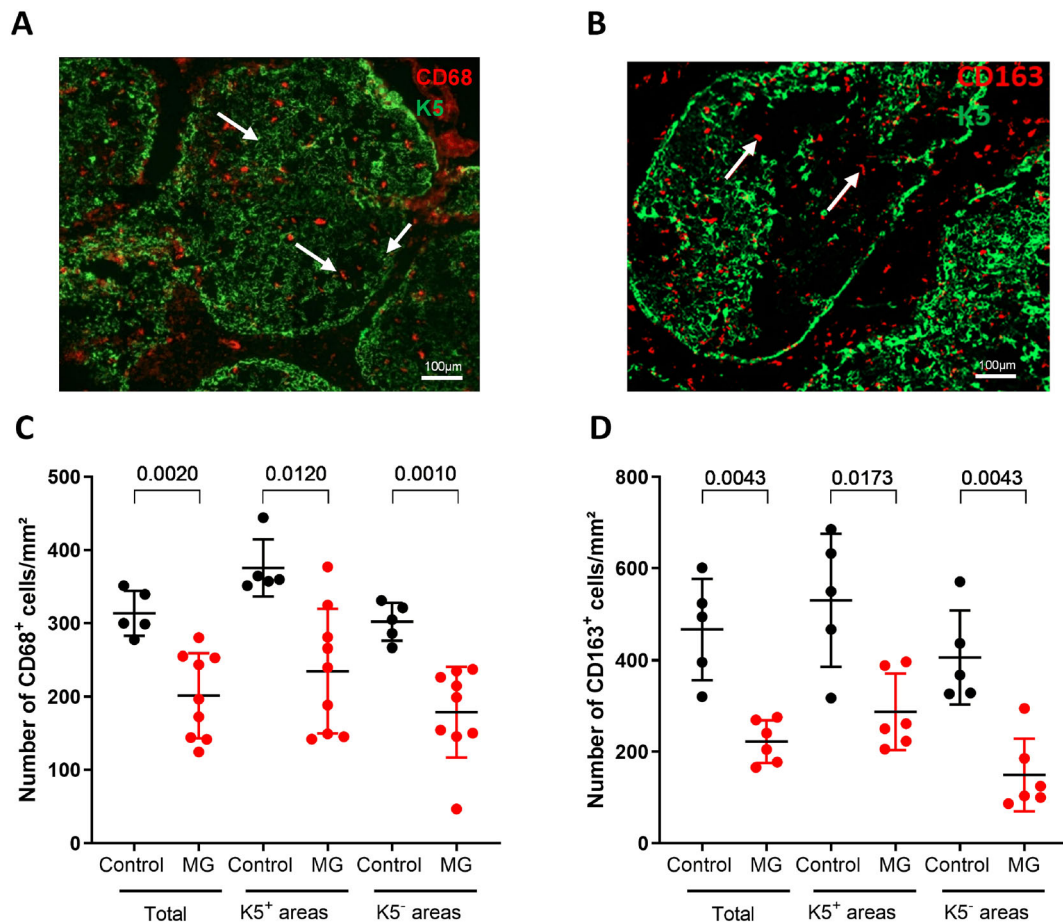


FIGURE 6: Analyses of thymic macrophages in AChR-MG patients. Immunohistochemical analyses of thymic macrophages. Thymic sections from AChR-MG patients and controls were labeled with antibodies against CD68 or CD163 for macrophages (in red) and keratin 5 (K5) for medullary TECs (in green). Representative image of an adult control labeled with CD68 (A) and CD163 (B) and with K5 (A, B). For each donor, the number of cells was counted by Zen Software in 3 or 4 mosaic pictures containing 25 fields of each thymic section. Representative sections were selected distinguishing cortical or medullary regions based on Keratin 5 staining and CD68⁺ (C) and CD163⁺ (D) cells were counted. The *p* values were determined by the Mann-Whitney test for 2-by-2 comparisons for MG patients as compared with controls. MG = myasthenia gravis; TECs = thymic epithelial cells.

dermatomyositis, and Sjögren's syndrome.^{16,17,26,27} Some patients with EOMG without autoimmune comorbidities can have anti-nuclear antibodies and it would be interesting to measure serum or thymic levels for IFN- α and IFN- β for these patients. In EOMG, the IFN-I signature is thus specifically detected in the thymus.⁸ IFN-I subtypes are also increased in the thymus of thymoma-associated MG²⁸ without a peripheral increase in IFN- α subtypes and IFN- β .²⁹ Altogether, this leads to consider that MG could correspond to a thymus-restricted interferonopathy.

As the thymus is thought to initiate the anti-AChR response associated with EOMG, a key issue is the understanding of signals that drive the chronic expression of IFN- β . We previously demonstrated that Poly(I:C), a dsRNA molecule, induces the overexpression of IFN- β by TECs that, in turn, leads to the specific expression of α -AChR.⁸ Here, we demonstrated that Poly(dA:dT) and

2'3'-cGAMP induced the expression of IFN-I, in particular IFN- β , and α -AChR by TECs. The expression of IFN- β peaked at 3 hours and that of α -AChR was time-lagged. Blocking the IFN-I receptor strongly inhibited α -AChR expression. These results suggest that dsDNA signalization could induce the IFN- β release by TECs and the subsequent specific overexpression of α -AChR. Similarly, Poly(dA:dT) and 2'3'-cGAMP injections to mice led to thymic expression of IFN-I subtypes and α -AChR expression, as observed with Poly(I:C) injections.⁸

We did not observe any effect of HSV-60 or CpG, synthetic molecules mimicking dsDNA from viral or bacterial origin, respectively. In contrast, Poly(dA:dT) mimics mammalian canonical B-DNA that specifically activates cGAS.³⁰ Poly(I:C) is usually considered as a molecule mimicking viral infection but it can also mimic endogenous dsRNA.³¹ This suggests that endogenous dsDNA/dsRNA could be responsible for the IFN- β expression

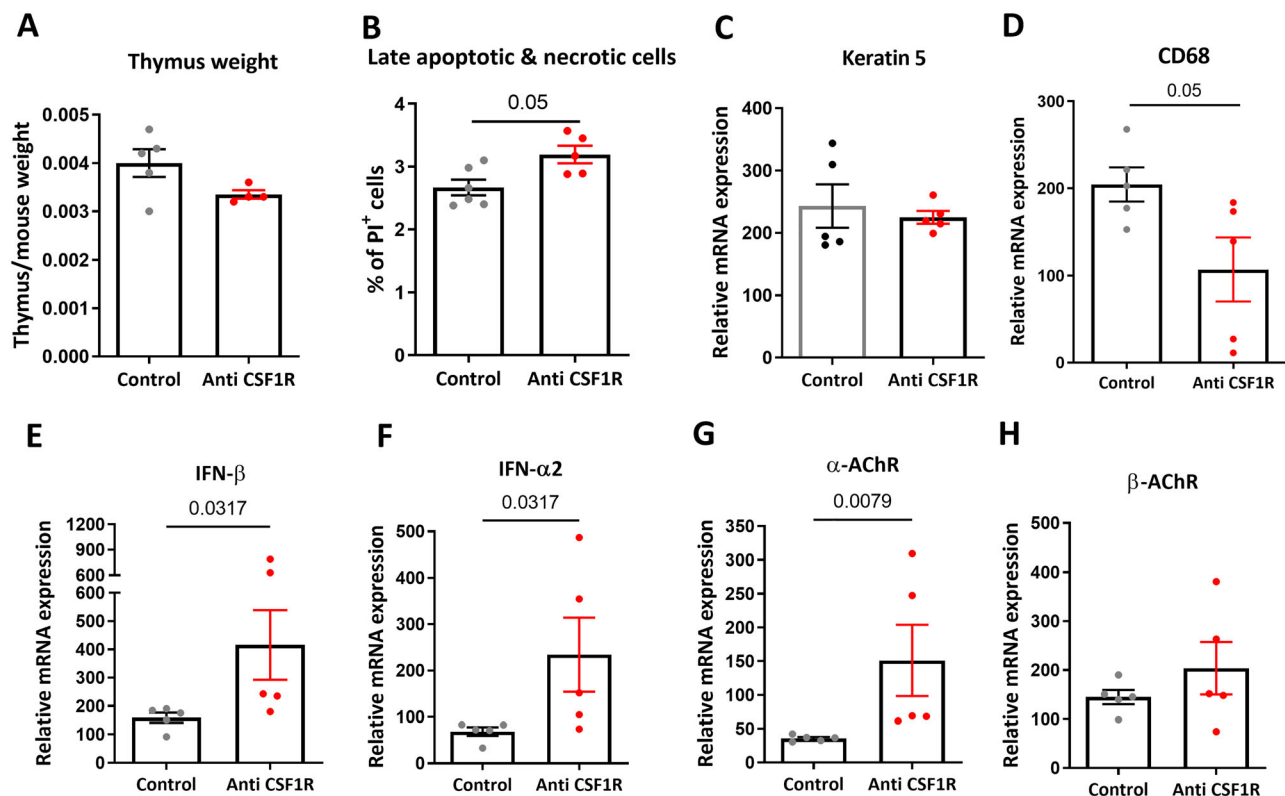


FIGURE 7: Analysis of the impact of a decreased number of thymic macrophages. Mice were injected with an anti-CSF1R antibody to decrease thymic macrophages. After 4 weeks, control and treated mice were euthanized. The thymuses were weighed (A) and the percentages of late apoptotic/necrotic thymocytes were measured by FACS with Annexin V and PI labeling (B). Thymic mRNA expression was measured and normalized to GAPDH for keratin 5 (C), CD68 (D), IFN- β (E), IFN- α 2 (F), α -AChR (G), and β -AChR (H). The *p* values were assessed by the Mann–Whitney test and *p* values were indicated only when *p* \leq 0.05 (A–H). FACS = fluorescent activated cell sorting; PI = propidium iodide. [Color figure can be viewed at www.annalsofneurology.org]

in the AChR-MG thymus. The fact that endogenous dsRNA and dsDNA use innate signaling pathways³² may explain the long-standing hypothesis that MG may be linked to pathogen infection. Except for debates regarding Epstein–Barr virus (EBV),³³ no clear evidence supports a causal link between a specific pathogen and the onset of MG.³⁴

Under physiological conditions, apoptotic cells are taken over by phagocytes, such as macrophages, through a process called efferocytosis. If apoptotic cells are not eliminated, they progress to a secondary necrosis stage and locally release their intracellular content, such as endogenous NAs that lead to inappropriate activation of innate signaling pathways.³² We demonstrated that the co-culture of apoptotic/necrotic thymocytes with TECs induced IFN- β and α -AChR expression, similar to what we observed with synthetic NAs. These *in vitro* observations were reproduced *in vivo* by inducing a thymic acute stress. Dexamethasone injections are known to lead to thymic involution due to the huge induction of thymocyte death.¹⁹ We showed that the increase in the number of apoptotic/necrotic thymocytes led to the expression of

IFN-I subtypes, such as IFN- β and IFN- α 2, and of α -AChR. The implication of endogenous NAs in inflammatory disorders and autoimmunity is increasingly suspected.^{35,36} For example, defective clearance of endogenous NAs from dying cells is implicated in the initiation of systemic lupus erythematosus.³⁷ Endogenous NAs can also induce IFN-I-associated responses in keratinocytes and are involved in the pathogenesis of cutaneous lupus erythematosus.³⁸

The thymus is essential to shape the T cell repertoire to optimize the immune response and avoid autoimmunity.³⁹ This is a site of intense cell death in the context of positive and negative selection during which about 95% of the thymocytes are eliminated.⁴⁰ In addition, thymocyte apoptosis is strongly induced upon acute stress.⁴¹ It is thus clear that efferocytosis of apoptotic thymocytes is a critical physiological process to maintain thymic homeostasis. We observed a clear decreased number of macrophages in the thymus of patients with AChR-MG. We do not have a clear explanation for this decrease in thymic macrophages. Deficiencies of macrophages in human diseases are relatively rare. However, changes in macrophage

number or function could contribute to disease processes such as autoimmunity, inflammatory diseases, and cancer.⁴² We observed that a decreased number of thymic macrophages in mice was associated with an IFN-I signature and the induction of α -AChR expression. In studies on MG thymus, Salakou et al measured the apoptotic body index on thymic sections from controls and MG patients. The apoptotic body index is higher in MG patients suggesting an increased number of apoptotic thymocytes in MG.^{43,44} Kanatli et al also observed an increase in the apoptotic body index in the thymus of MG patients compared to adult controls.⁴⁵ Fas and its ligand (FasL) are 2 molecules involved in the regulation of cell death, and Fas expression is increased in apoptotic cells.⁴⁶ A study on isolated thymocytes showed a higher proportion of thymocytes with a strong Fas expression in AChR-MG patients. This was specifically observed in the thymus but not in peripheral T cells.⁴⁷ Of note, the thymus of AChR-MG patients also displays fewer myoid cells⁴⁸ that normally protect thymocytes from apoptosis.⁴⁹

We previously showed that IFN- β plays a central role in thymic events leading to MG by triggering the overexpression of α -AChR in TECs but also inducing a slight increase in TEC death, likely leading to thymic dendritic cell sensitization against this autoantigen, the abnormal recruitment of peripheral cells and germinal center formation, the differentiation of pathogenic Th17 cells, and the survival of autoreactive B cells.^{9,11,12} Here, investigating the cause of this IFN- β signature, we suggest that an altered efferocytosis process in the thymus due to a decreased number of macrophages favors the accumulation of apoptotic/necrotic thymocytes and the subsequent release of endogenous NAs. This would lead to the activation of innate signaling pathways and the chronic IFN- β signature in EOMG. A normal thymus is known to rapidly recover upon acute stress.^{50,51} Consequently, the persistent IFN-I signature observed in the AChR-MG thymus suggests that in addition to a decrease in the number of macrophages, an abnormal resolution of inflammation might also be implicated in EOMG pathogenesis.

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Author Contributions

C.P., F.T., and R.L.P. conception and design of the study. C.P., A.Y., O.-M.F., F.T., V.B., D.D., S.D., E.F., J.G., and R.L.P. acquisition and analysis of data. C.P., V.B., D.D., F.M., S.B.-A., and R.L.P. drafting the text or preparing the figures.

Potential Conflicts of Interest

The authors declare that they have no competing interests.

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