# *Autoimmunizing Mechanisms in Thymoma and Thymus**

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**Autoimmunizing mechanisms are very hard to study in humans, so we have focused on vital clues in thymomas and hyperplastic thymuses in myasthenia gravis (MG). According to our multistep hypothesis: thymic epithelial cells (TEC) present epitopes from the isolated acetylcholine receptor (AChR) subunits they express, and autoimmunize helper T cells; subsequently, these evoke "early antibodies" that then attack rare thymic myoid cells expressing intact AChR; in the resulting germinal centers, autoantibodies diversify to recognize native AChR. We have studied: 1) thymomas, to identify autoimmunizing cell types, focusing on IFN-α, against which many patients have high titer autoantibodies, as in another highly informative autoimmune syndrome. Although IFN-α is much easier to label than the sparse and delicate AChR subunits, we have not yet located obviously autoimmunizing micro-environments; 2) hyperplastic MG thymuses, where we find (a) upregulation of complement receptors and regulators on hyperplastic TEC and deposition of activated C3b complement component on them, (b) absence of complement regulators from almost all myoid cells, indicating vulnerability to attack, and (c) deposition of C3b, and even of the terminal membrane attack complex, especially on the myoid cells close to the infiltrating germinal centers. The changes are very similar in over 50% of the so-called seronegative patients with generalized MG (SNMG) but without detectable autoantibodies against AChR or MuSK, consistently with other evidence that they belong to the spectrum of AChR-seropositive MG. Together, moreover, our findings implicate both myoid cells and TEC in autoimmunization, and thus strongly support our hypothesis.**

*Key words:* **myasthenia gravis; acetylcholine receptor; autoantibody; thymoma; thymus; thymic deletion; self-tolerance; thymic hyperplasia; germinal center; complement; immunopathology; autoimmunization; autoimmune polyendocrine syndrome 1 (APS1 or APECED); AIRE; type I interferon; tissue-restricted autoantigen**

## Introduction

Mechanisms of autoimmunization are extremely hard to study, even in the best animal models. Here we summarize our search for clues in the human thymic neoplasia and hyperplasia that so regularly associate with myasthenia gravis (MG). MG is a particularly informative prototype because of its very well-defined

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patient subgroups and target—the acetylcholine receptor (AChR)—and because autoantibodies against it are so clearly pathogenic. These high-affinity, highly mutated IgGs must be T helper cell (Th) dependent.

There are several crucial *a priori* considerations. First, the autoantibodies in MG are almost entirely specific for the AChR in its native conformation and very rarely, if ever, recognize unfolded subunits or synthetic peptides.[1,2] Second, native AChR has been detected only in muscle and in the rare thymic myoid cells[3] (see below). However, isolated AChR subunits are also expressed by medullary thymic epithelial cells (mTEC), presumably to induce self-tolerance in maturing T cells.[4–10] Indirect evidence for such tolerance is the much easier selection of Th that recognize contaminants in the stimulating AChR antigen preparations than epitopes naturally processed from the intact AChR itself.[11–13] Third, whereas there are almost no signs of autoimmunization in muscle,[14] ~10% of patients have thymomas, and the thymus is usually "hyperplastic" in another ~30% of patients with early-onset MG (EOMG)[15,16] (before age 40), showing lymph node–type infiltrates in the medulla[17,18] (see Section II). Since these latter include mature peripheral T and B cells, antigen-presenting cells (APC), such as macrophages and dendritic cells (DC), germinal centers (GC), and high-endothelial venules, they constitute a fully professional lymphoid environment.

Where are the pathogenic Th first primed, and how can their recognition of linear AChR epitopes eventually lead to production of the conformation-specific autoantibodies? In EOMG thymuses, our findings have led to a multi-step hypothesis[18,19] that implicates mTEC—in hyperplastic or neoplastic thymus—in priming specific Th, and thymic myoid cells in provoking the subsequent B cell responses specific for the native AChR. We have found novel clues to the earlier steps in patients with thymomas and in the striking parallels with a monogenic autoimmune syndrome. Since these concern T cell priming, we focus on them in Section I, and turn to thymic hyperplasia in Section II.

## Results and Discussion

### *I. Unexpected Findings in Thymoma/ MG and Other Autoimmune Syndromes*

#### *Clues from Autoantibodies against Cytokines in Thymoma Patients*

Thymomas are epithelial neoplasms of cortical or mixed TEC and often generate abundant maturing thymocytes.[20] The patients nearly always have autoantibodies that recognize other striational muscle proteins in addition to AChR, notably titin[21] and ryanodine receptor.[22] Because thymomas generate Th in a disorganized environment that is often HLA-class II-deficient, some workers believe that they merely export T cells that have not been properly tolerized and happen to react predominantly against muscle in the periphery.[23] However, whereas ~30% of thymoma patients develop MG, other autoimmune associations are rare,[24]which argues for some more specific selection and/or activation of autoreactive T cells within these tumors,[25] such as by the isolated AChR subunits, titin, or ryanodine receptor epitopes expressed by the neoplastic TEC.[5,6,26]

About 10 years ago, we were surprised to find high-titer neutralizing autoantibodies against interferon-α (IFN-α; all its 12 subtypes), and the 60% identical IFN-ω, in ~70% of MG/ thymoma patients; independently also against IL-12 in ~50% (summarized in TABLE 1). Mostly present already at diagnosis, they persist over many years, despite treatment with immunosuppressive drugs in many cases.[27] We found no such antibodies against a wide range of other cytokines, including the distantly related IL-10 or the unrelated IFN-γ, IL-2, IL-4, or IL-6. Since APC, including plasmacytoid ($CD123^+$) dendritic cells (pDC), are the professional producers of both IFN-α and IL-12, they seemed likely autoimmunizing cell types. Notably, titers of these antibodies usually increase strikingly when thymomas recur or metastasize.[28] This hint that they are boosted by IFN-expressing cell types in the tumors is supported by their spontaneous production by thymoma cells in culture.[29]

These responses are clearly highly selective; neutralizing antibodies are very rare in numerous other infectious, neoplastic, or autoimmune diseases.[27] However, as with antibodies against titin,[21] they appear surprisingly similar in 20–30% of nonthymoma patients with late-onset MG (after age 40), making us wonder if these patients could have previously rejected occult thymomas that are never detected. We have also found neutralizing antibodies, especially against IFN-α8, in occasional patients with systemic lupus erythematosus (SLE) (TABLE 1), where IFN-α is heavily implicated in autoimmunization.[30–32]

It is instructive to consider the many diseases where we did *not* find these autoantibodies. They include newly diagnosed type 1 diabetes,[27] although upregulation of IFN-α is one of the earliest changes noted in recent-onset diabetic islets.[33] Nor did we find them in recent-onset polymyalgia rheumatica or giant cell arteritis (TABLE 1), even though DCs are heavily implicated in autoimmunization here too.[34] Perhaps a key difference in MG is the concomitant involvement of the thymus/thymoma.

**TABLE 1. Anti-IFN and other anti-cytokine autoantibodies in a range of disorders**

| | | ← Neutralizing anti-IFN antibodies → | | | | | ← Binding anti-interleukin antibodies → | | |
|---|---|---|---|---|---|---|---|---|---|
| Disorder | n | IFN-α | IFN-ω | IFN-β | IFN-λ1 | IFN-λ2 | IL-12 | IL-4 | IL-10 |
| **MG/thymoma** | **170** | ~65% | ~60% | ~7% | <5% | <5% | ~50% | <<5% | <<5% |
| **Late-onset MG** | **60** | ~25% | ~20% | ~4% | nd | nd | ~20% | <<5% | <<5% |
| | | | | n = 28 | | | | n = 28 | n = 28 |
| **APS1** | **127** | 98% | 100% | ~20% | ~15% | ~8% | <<5% | nd | <<5% |
| | | | | | n = 75 | n = 75 | | | n = 70 |
| **Polymyalgia rheumatica** | | 0/8 | 0/8 | nd | 0/8 | 0/8 | 0/8 | 1/8 | 1/8 |
| (OD in ELISA) | **8** | | | | | | | (0.2) | (0.3) |
| **Giant cell arteritis** | **9** | 0/9 | 0/9 | nd | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 |
| **Rheumatoid arthritis** | | <<5% | <<5% | <<5% | <5% | <5% | 1/18 | 4/17 | 2/18 |
| (OD in ELISA) | **44** | | | | n = 18 | n = 18 | (0.17) | (0.32–0.64) | (0.45, >3.0) |
| **SLE** | | 2/ 47 | 1/47 | <<5% | <<5% | <<5% | <<5% | 1/7 | 1/7 |
| (OD in ELISA) | **47** | | | n = 40 | | | | (1.1) | (0.29) |
| **Osteoarthritis** | | 0/9 | 0/9 | nd | 0/9 | 0/9 | 0/9 | 2/9 | 2/9 |
| (OD in ELISA) | **9** | | | | | | | (0.53, 0.24) | (0.24, 0.53) |

Most of the patients with MG/thymoma, late-onset MG, RA, and SLE feature in Ref. 27, and those with APS1 in Ref. 35 (where the antibody assays are also described). In the other patient groups, the onset of polymyalgia rheumatica, or giant cell arteritis was within the year before sampling, and diagnostic criteria were standard, as for osteoarthritis. n, represents the number of sera that were tested at the time that particular IFN (etc.) was available for testing. Values in brackets are the actual OD values in ELISA assays.

## *Clues from Informative Parallels in a Monogenic Autoimmune Syndrome: a Digression*

Anti-IFN (but not anti-IL-12) antibodies proved to be most striking of all in Autoimmune Polyendocrinopathy Syndrome 1 (APS1) patients,[35] who have recessive mutations in the Autoimmune Regulator gene (*AIRE*). This monogenic disease (also called APECED) typically manifests first in childhood with chronic mucocutaneous candidiasis (CMC), an occasional thymoma association that is still unexplained in both settings. In APS1, the autoimmune attack usually focuses on the adrenal cortex and parathyroids; often also on the skin, nails, teeth, and/or gastrointestinal tract. Above all, it is extremely variable, both in the organs targeted and in their timing.[36]

We found even higher titers of neutralizing antibodies against all the IFN-αs, and especially IFN-ω, in 100% of >100 APS1 patients, regardless of their country of origin, of their precise AIRE mutation, or of their exact clinical phenotype[35] (TABLE 1). Undetectable in unaffected heterozygous relatives, titers of anti-IFN antibodies were already high in the earliest samples from APS1 patients, and consistently remained high for decades afterwards. Moreover, they appear highly APS1-specific; since they are not found in patients with isolated (nonsyndromic) CMC or adrenal or parathyroid autoimmunity, they clearly must have diagnostic value, especially in early or atypical cases.

Despite these high neutralizing titers, persistent infections are surprisingly rare—either in APS1 or even in thymoma patients with additional anti-IL-12 antibodies[37]—even though they are usually treated with corticosteroids for their MG. However, there are occasional exceptions.[19,27,37,38] Furthermore, the antibodies preceded the candidiasis or autoimmunity in the informative APS1 cases, so they are clearly not an effect of any one of these disease components. Since they are very much commoner than candidiasis in thymoma patients, they seem not to confer a high risk for this particular infection.

Moreover, in APS1, they evidently behave like a heritable trait, which is extremely unusual for any antibody response, even after deliberate immunization in inbred mice.[39] Their precocious ~100% prevalence suggests that they are a "smoking gun" implicating some key (dendritic?) cell type at early stages in autoimmunization. Since AIRE normally governs thymic expression of peripheral tissue-specific autoantigens by mTEC and their induction of self-tolerance,[10] it is tempting to invoke parallels between the APS1 thymus and MG thymoma and to propose "central autoimmunization" in each site.

## *Searching for Autoimmunizing Cell Types*

Because APS1 thymuses were not available, we next compared sections of normal thymus and thymomas, hoping to identify potentially autoimmunizing microenvironments/cell types. We focused on IFN-α, which

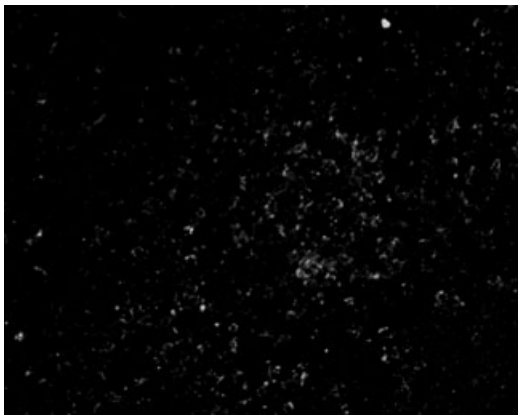

**FIGURE 1.** Double labeling for IFN-α (green) and MxA (red) in a thymoma (WHO type B2) from an MG patient. In general, the areas that label most densely for each molecule overlap, but very few cells label with both antibodies. We stained paraffin sections with polyclonal sheep anti-human IFN-α[33,35] and an anti-MxA mAb.[40] The donor was aged 35 at thymectomy and had high-titer autoantibodies against IFN-α and a low titer against IL-12. (In color in *Annals* online.)

is a much simpler (~18 kd), more robust, and abundant molecule than any AChR subunit. We could test only limited staining combinations, because we could detect IFN-α only in paraffin and not in frozen sections (and IL-12 in neither). We also stained for the type I IFN-inducible protein MxA.[40]

In general, we never detected any IFN-α in epithelial cells. In the normal thymus, we found numerous IFN-$\alpha^+$ cells both in the cortex and medulla, which were mainly $CD68^+$ macrophages. Though minimal in the cortex, MxA labeling was abundant in the medulla, implying that type I IFNs are normally secreted there, but not in the cortex.[35]

In most thymomas, we again found numerous IFN-$\alpha^+$ cells, which were all $CD68^+$, many with macrophage morphology (FIG. 1A). There was usually also plentiful labeling for MxA, but, as with IFN-α, it was very variable. Some of the IFN-$\alpha^+$ cells were clearly HLA-class $II^+$, and some were in/near occasional B cell clusters (not shown).

In frozen sections, most of the $CD68^+$ macrophages were clearly $CD14^+$ too. In cell suspension, more of them were $CD1a^+$ than in control thymuses, with moderate levels of HLA-class II and of the CD86 co-stimulatory molecule, implying antigen-presenting potential. Staining for DCs, which also express CD68, we found small numbers of $CD11c^+$ (myeloid) DC, often expressing activation markers, and slightly more $CD123^+$ (plasmacytoid) DCs, some of which again expressed HLA-class II and costimulatory molecules.

**TABLE 2. Antigen-presenting potential of macrophages ($CD14^+$) and pDCs ($CD123^+$) enriched from normal infant thymus or thymoma, assessed by their stimulation of mixed lymphocyte responses by unmatched naïve cord blood T cells**

| Stimulators → | Normal infant thymus | | Type AB thymoma | |
|---|---|---|---|---|
| $CD14^+$ cells/well → | $5 \times 10^3$ | $5 \times 10^4$ | $5 \times 10^3$ | $5 \times 10^4$ |
| ↓ pre-activation | ←– – $^3$H-thymidine uptake; cpm – –→ | | | |
| – | 3,631 | 22,173 | 17,003 | 23,450 |
| LPS | 4,987 | 21,140 | 11,339 | 20,411 |
| $CD123^+$ cells | | | | |
| – | 8,675 | 22,967 | 25,454 | 38,365 |
| Influenza virus (A/PR8) | 13,295 | 22,502 | 23,506 | 37,554 |

Cryopreserved thymic cells were thawed, depleted of dead cells on Lymphoprep® (Axis Shield PoC AS, Oslo, Norway), and enriched for $CD14^+$ or $CD123^+$ cells with Miltenyi immuno-magnetic beads (Miltenyi-Biotech, Milton Keynes, UK) (purity >70%; the contaminants were almost all $CD1^+$ thymocytes). They were then pre-activated for 40 h with the indicated stimulus before coculture with $5 \times 10^4$ thawed cord blood lymphocytes. After 5 days, cocultures were pulsed with $^3$H-thymidine; their uptake was measured after a further 18 h; the counts per min (cpm) for cord blood cells cultured alone ($10 \times 10^4$ cells/well) were $303 \pm 54$. The HLA-disparity with the responder cells may differ between the normal and thymoma stimulators.

Disappointingly, we saw no consistent differences between thymomas from patients with or without anti-IFN antibodies (Y. Kadota *et al*, in preparation), or even between primary and recurrent thymomas from individuals whose antibody titers had increased strikingly, clearly showing that they truly were responders.

To assess their antigen-presenting potential, we next enriched APC from cryopreserved thymoma cell suspensions (by magnetic bead selection), and measured their IFN-α productivity. In one preliminary experiment, we first checked for viability/function (after pre-activating with influenza virus for 40 h). Unmatched naïve cord blood responder cells showed even stronger mixed lymphocyte reactions against $CD123^+$ cells enriched from an MG thymoma than from a nonmyasthenic infant thymus (TABLE 2). We next pre-activated APC subsets enriched similarly from 10 MG thymomas ± influenza virus or lipo-polysaccharide (LPS) for 40 h. The $CD14^+$ macrophages from one infant thymus produced no detectable IFN-α over the subsequent 48 h: from the thymomas, only 6 of the 10 samples produced even small amounts (TABLE 3). Levels were much higher with the virus-stimulated $CD123^+$ pDCs from the infant thymus, as expected.

**TABLE 3. IFN-α production in culture by APC enriched from infant thymus or thymoma**

| $CD14^+$ cells from → | Normal infant thymus | | | MG thymomas | | |
|---|---|---|---|---|---|---|
| Stimulus → | – | Influenza virus | LPS | – | Influenza virus | LPS |
| IFN-α in supernatant:- | | | | | | |
| >1000 pg/mL | 0/1 | 0/1 | 0/1 | 0/10 | 0/10 | 0/10 |
| 100–1000 | | | | 0/10 | 1/10 | 1/10 |
| 10–100 | | | | 1/10 | 0/10 | 4/10 |
| $CD123^+$ cells | | | | | | |
| IFN-α in supernatant:- | – | Influenza virus | LPS | – | Influenza virus | LPS |
| >1000 pg/mL | | 1/1 | | 0/10 | 6/10[a] | |
| 100–1000 | nd | | nd | 3/10[a] | 4/10[a] | nd |
| 10–100 | | | | 3/10[a] | 0/10 | |

Cryopreserved thymic cells were enriched for $CD14^+$ or $CD123^+$ cells as for TABLE 2. We tested 3 thymomas of WHO type AB and 7 of type B2. They were then incubated for 48 h with the indicated stimulus before supernatants were collected for ELISA assays for a broad range of IFN-α subtypes (cat # 41,105, Biomed Labs, Piscataway, NJ).

[a]Some of the patients in each group were positive for serum anti-IFN antibodies and others negative.

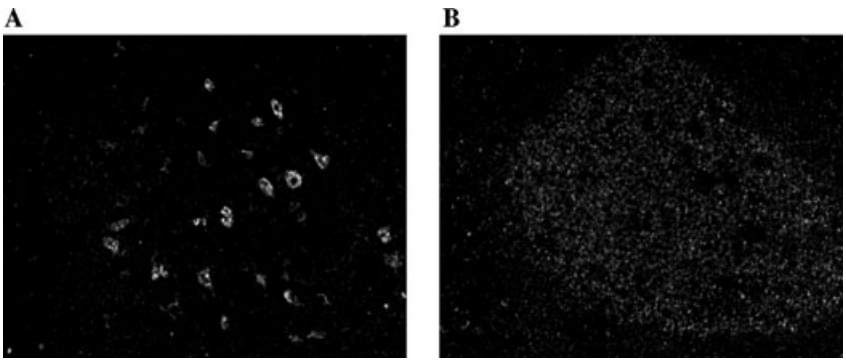


**FIGURE 2.** Double labeling for IFN-α (**A**) and MxA (**B**) in a GC in an EOMG thymus. The donor was female, aged 24 at thymectomy, positive for anti-AChR antibodies, and not taking corticosteroids. The appearances are essentially similar in normal (reactive) tonsils. (In color in *Annals* online.)

Notably, they were also substantial—or even high—with 6 of the 10 thymomas, whether of WHO AB or B2 types, and also regardless of the donor's anti-IFN sero-positivity or -negativity (TABLE 3). The generally lower but significant production by "unstimulated" cells could reflect either prior induction *in vivo* or some level of activation *in vitro* by dead cell debris (after thawing), by the isolation procedure or by culture constituents.

Paradoxically, AIRE is almost undetectable in >90% of thymomas,[41,42] but, even so, the patients rarely develop APS1-type diseases/autoantibodies. Conversely, there are no reports of MG in APS1 patients. Apart from their thymic involvement, the one feature common to these two syndromes is their striking anti-IFN responses. Curiously, too, AChR-α-subunit expression by normal human mTECs now appears to be enhanced by AIRE,[7] as well as by IFN response factor 8 (IRF-8), activation that is modulated by polymorphisms in the AChR-α-promoter that predispose to particularly early-onset MG.[8,9] Since IRF-8 is a signaling factor involved in induction of—and responses to—both type I IFNs and the type II IFN-γ, it is tempting to suggest some role for type I IFNs in normal self-tolerance, though it is not yet clear how the various pieces in the puzzle fit together.

Although these results yield more questions than answers, we did find a likely explanation for the long-term persistence of the anti-IFN antibodies. In any GC—whether in "normal" tonsils or in the EOMG thymus—we found numerous IFN-$\alpha^+$ "tingible body macrophages," whose function is to dispose of any dying B cells (such as those with unsuccessful Ig gene rearrangements/mutations). The abundant nearby MxA labeling indicates secretion of at least some of this IFN-α (FIG. 2). That implies that, once an antibody response has been initiated against IFN-α, there will always be strategically located antigen

available to perpetuate it and select for the somatic mutations that we observed in MG/thymoma patients.[29]

In conclusion, we suggest that autoimmunizing micro-environments may well be focal or transient in thymomas, and thus easily overlooked. Nevertheless, our independent evidence from cultured thymoma cells[29] implies that these tumors include cell types—possibly DCs—that autoimmunize both Th and B cells against IFN-α (from macrophages?) and IL-12 (from DCs?), but only Th against unfolded AChR subunits (from mTEC). If so, the B cells must be immunized against native AChR elsewhere, possibly by myoid cells in the adjacent uninvolved thymus or even in muscle/draining lymph nodes.

It is widely assumed that autoimmunization in APS1 is a default process that proceeds autonomously.[43–45] We feel that there are equally strong arguments for some central process in the thymus, involving not only mTEC but also DCs and macrophages, which show enhanced APC activity in *Aire*$^{-/-}$ mice.[45] We suspect that the contrasting autoimmune responses in these two syndromes reflect the distinct repertoires of peripheral autoantigens expressed by the aberrant TEC, which seem biased toward muscle in thymomas and endocrine and ectodermal tissues in APS1. Importantly, some "AIRE-dependent" antigens are still expressed, though at lower levels, even when AIRE is absent.[43–45] We suspect that these are key primary targets in APS1, and, more generally, that thymic expression of peripheral tissue autoantigens in the absence of AIRE is "dangerous." Whatever the case, the striking parallels with thymomas must hold clues to autoimmunizing cell types/mechanisms. So, too, must the bias toward IFN-ω (and, to a lesser extent, IFN-β and IFN-λ) in APS1 versus IL-12 in thymomas—clues that could be pursued further when we understand more about the actions and regulation of the different IFN subtypes[32] and can test more discriminating monoclonal antibodies (mAbs).

## II. Autoimmunization against Native AChR: Clues from Thymic Hyperplasia in EOMG

By definition, anti-AChR antibodies are always found in EOMG, often at high titers. In general, however, these correlate poorly with MG severity. This long-standing puzzle was partly resolved by the discovery of anti-MuSK (muscle-specific kinase) antibodies in many patients with severe generalized MG but with no detectable anti-AChR antibodies.[46] However, there remain another ~10% of clinically typical patients whose (generalized) MG clearly responds to plasma exchange and/or immunosuppressive drug treatments, but who are negative against MuSK and have either borderline (AChR-Ab$^{lo}$) or undetectable anti-AChR antibodies in standard radio-immuno-assays—so called "seronegatives" (SNMG). These latter patients are seldom thymectomized, but, very fortunately, our colleagues in Leiden, London, Paris, Porto, and Würzburg kindly helped us to collect paraffin sections from a total of 30 such thymuses which enabled us to study a variety of markers systematically.[47,48]

Our previous studies showed that few myoid cells are HLA-class II$^{+}$, even in the EOMG thymus,[3] and they also have an unpromising costimulatory phenotype, so they seemed unlikely to prime specific Th.[18] However, they apparently were implicated in GC formation.[18] Because their numbers and disposition are extremely variable in MG,[3,18] and because complement (C) is such an important effector at the endplates in MG,[49] we next focused on its receptors C3aR and C5aR, on the cell surface regulators of C3 convertase (CD46 and CD55), on CD59, which blocks assembly of the membrane attack complex (MAC), and on the activated C components, particularly the pivotal C3b and the terminal (pore-forming) MAC.[48] Starting with EOMG thymuses, we also compared the other MG subgroups mentioned above and a series of age-matched controls.

In normal adult thymuses, labeling for C3aR is generally minimal, even in blood vessels; for C5aR, it is somewhat stronger and more widespread on some of the mTEC.[48] The main cell-surface regulators of C3 activation are CD46 and CD55; both are sparsely distributed on normal mTEC, but we noted stronger labeling for CD59. We also saw minimal deposition elsewhere of the early components C1q and C3b (in fact, only in blood vessels), and none at all of C9, a marker for the MAC.[48]

In EOMG, the cortex and some medullary regions appear normal for the donor's age. In other areas, infiltrates expanding in the perivascular spaces compress the adjacent true thymic parenchyma into characteristic medullary epithelial cells bands (MEB), where the mTEC appear hyperplastic.[16,17] The infiltrates include lymph node–type T cell areas and GC. Our main findings were: 1) In most EOMG thymuses, the mTEC in the MEB show strong but patchy upregulation of C5aR.[48] 2) The three C regulators, which were much less obvious in the more normal medullary areas, are also upregulated. There was increased C3aR staining too, mostly on DCs, macrophages, and B cells, but it was much stronger within the infiltrates, and especially the GC. 3) We also saw striking deposition of C1q and C3b on mTEC in the MEB, again in a patchy distribution, but C9/MAC was almost undetectable.[48]

**TABLE 4. Patient Ig binding to AChR-δ-subunit, and image analysis of the bands detected by immunoblotting**

| Patient subgroup | *n* | % of samples positive | | integrated optical density (IgG) % of positive control mAb median% (range) |
|---|---|---|---|---|
| | | IgM | IgG | |
| Control | 12 | 0.0 | 8.3 | 0.0 (0.0–13) |
| AChRAb$^+$ | 53 | 9.4 | 55.0 | 30.0 (0.0–100) |
| SNMG | 25 | 4.0 | 52.0 | 27.0 (0.0–98) |
| MuSKAb$^+$ | 45 | 2.3 | 29.5 | 0.0 (0.0–71) |

AChR-δ-subunits were expressed in *E. coli* and purified from inclusion bodies as previously described.[65]

To us, these findings imply: (a) a response to attack on mTEC by the autoantibodies reported previously[50] (possible targets are considered below in TABLE 4), whose effects are (b) restrained by C regulators, which (c) effectively prevent generation of the MAC, but (d) stimulate not only the increased expression of C5aR and of the C regulators but also the hyperplastic changes in the mTEC. It is well known that sub-lytic C deposition can lead to proliferation of various epithelial cell types.[51–53]

Myoid cells have long been implicated in autoimmunization in MG.[54] They are haphazardly distributed, even in the normal medulla. In EOMG, they are very often found at the borders of the MEB where they are aberrantly exposed to the infiltrates and their GC (because of breakdown of the intervening laminin borders[17,18]). We next compared these "exposed myoid cells" with those in the more normal medullary areas.[48] In either site, 4) their expression of C receptors and regulators was rare/weak, and we never saw significant CD59 staining.[48] Conversely, 5) we noted deposition of C1q and especially of C3b on many of the exposed myoid cells (FIG. 3), particularly on those within the infiltrates or even the GC. About 10% were C9/MAC$^+$ (FIG. 3), a few even showing signs of damage (FIG. 3). By contrast, both components were seen much less frequently on the myoid cells deeper in the MEB in the more normal areas (FIG. 3; middle panels).

Comparing the different MG subgroups, we noted very few abnormalities in thymuses from MuSKAb$^+$ patients; only 4 of 14 had even small infiltrates/GC, and C3b was rarely seen on myoid cells. Similarly, thymuses from 10 of the 30 SNMGs also appeared largely normal.[47,48,55] Remarkably, however, in the other 20, they were very similar to those with EOMG in all the respects 1–5 described above, as also in most AChRAb$^{lo}$ thymuses, though the GC tended to be smaller and less frequent in SNMG than in EOMG. Notably too, the above changes, both in the mTEC and myoid cells, were evident in recent-onset as well as long-standing cases and are clearly not just late effects.[48]

## Conclusions

Together, these findings argue very strongly that the AChRAb$^{lo}$ and many SNMG thymuses belong to the EOMG (AChRAb$^+$) spectrum. Very interestingly, we now find evidence of low-affinity antibodies that bind preferentially to AChR when it is clustered on transfected cell surfaces. We find them in 100% of the AChRAb$^{lo}$ patients and in ~60% in the SNMG subgroup,[56] especially those with larger thymic infiltrates and more C3b$^+$ myoid cells.[48] Their fewer/smaller GC seem consistent with more limited affinity and/or diversity of their antibodies, both of which could readily explain their inability to immunoprecipitate soluble AChR efficiently at the low concentrations currently available in standard radio-immuno-assays. Could the poor GC responses be a consequence of genetic variants in C components or receptors or Fc receptors? Other candidates include tumor necrosis factor and Lymphotoxin, which are involved in formation of ectopic lymphoid tissue[57,58] and whose genes are located very close to the strongly EOMG-associating locus MYAS-1.[9,59]

### Our multi-step hypothesis

Initiating mechanisms in EOMG remain a mystery. Is the evident involvement of mTEC a primary event, or is it an effect of some earlier provocation? There is much recent evidence for major influences on self-tolerance from quite modest quantitative variations in thymic expression levels:1) of peripheral tissue antigens such as insulin[60,61] and apparently also AChR subunits[7–9]; 2) of AIRE itself, which vary considerably[7–9]; and 3) of IL-2, on which regulatory T cells are highly dependent.[62] One could easily imagine that, without any external insults, unlucky combinations of genetic, hormonal, and/or stochastic influences could tip the balance against self-tolerance and possibly even convert the differently expressed self antigen(s) into an autoimmunogenic stimulus.

Once T cells begin to react against AChR subunit(s), one might expect direct attack on myoid

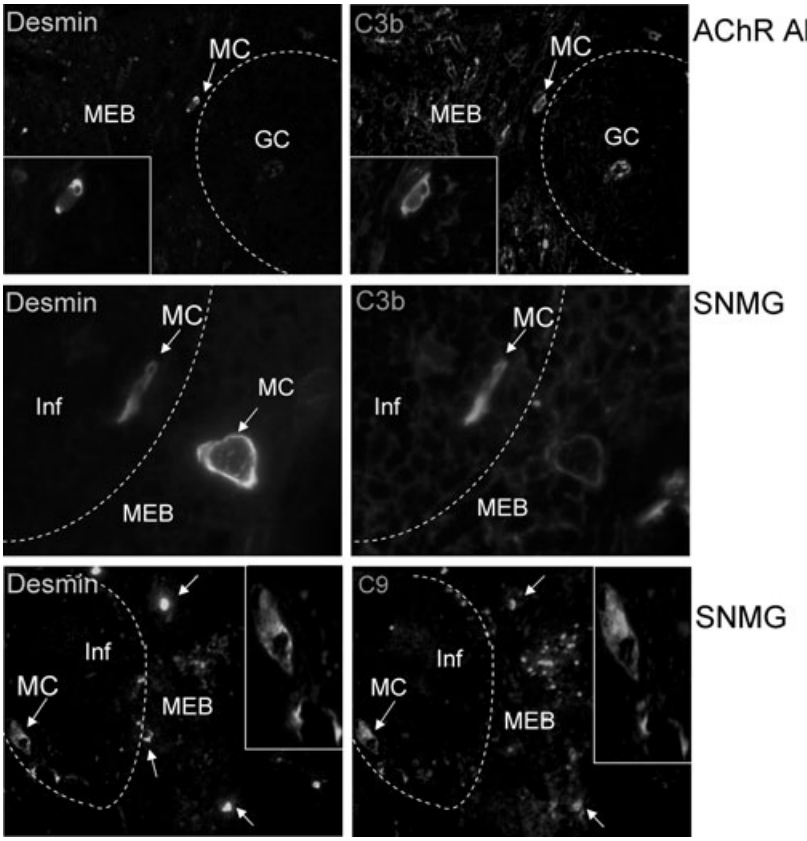


**FIGURE 3**. Double labeling for myoid cells (Desmin; originally green) and the indicated activated C components (originally red) in thymic sections from the indicated MG subgroups.[48] In each case, labeling for C3b or C9/MAC is more obvious on the myoid cells (MC) 'exposed' to the infiltrates (Inf) and their GC than on those within the MEB. In the upper and lower panels, the myoid cells labeled 'MC' are shown enlarged in the insets. The MG donors were:(top) AChR$^+$ female aged 25 y, with MG duration 18 months at thymectomy; (middle) SNMG #1 aged 22 y, with MG duration 6 months at thymectomy; (bottom) SNMG #2 male aged 38 y, with MG duration 12 months at thymectomy, also taking corticosteroids. (In color in *Annals* online.)

cells—especially by any specific HLA-class I–restricted CD8$^+$ T cells.[63] Moreover, concomitant induction of autoantibodies seems very likely too. We therefore looked for antibodies against unfolded subunits (by immuno-blotting), first focusing on the δ, because some SNMG sera previously mimicked mAbs directed at this subunit.[64] We did, indeed, detect mainly IgG antibodies in ~50% of the EOMG and SNMG patients tested (summarized in TABLE 2), though they were not especially prevalent in those with the most recent MG onset. The preferential expression of the AChR-ε subunit by mTEC in MG[6]—and its recognition by Th in EOMG[65]—demand analogous studies on this adult-specific subunit, which is apparently also expressed by myoid cells. These should be accessible/vulnerable to attack by such "early antibodies," and might well break down and present surplus unfolded AChR subunits/epitopes. If we are correct, these antibodies must focus the response onto extracellular/fetal AChR epitopes rather than the other muscle antigens that myoid cells also express but are seldom recognized in EOMG.

By this stage, the reaction against the mTEC would already have provoked lymph node–type infiltration into the thymic medulla,[66] and so the unprotected myoid cells would be exposed–in a professional lymphoid environment—to attack by C. The resulting antibody–antigen complexes are a potent stimulus to formation of GC[67,68]; indeed, we previously found evidence that α-bungarotoxin-binding AChR is trapped in MG

thymic GC.[19] These, in turn, are the sites of antibody diversification/affinity maturation, for which there is abundant evidence in the EOMG thymus.[69–71]

Finally, therefore, these responses culminate in autoantibodies specific for the native conformation of the intact AChR. Our findings support the hypothesized involvement of mTEC, perhaps abetted by DCs in priming AChR-reactive Th and then of myoid cells in autoimmunizing B cells specific for native AChR. They also show that many seronegative MG patients belong to the EOMG spectrum, and thus imply that they, too, might benefit from thymectomy. Nevertheless, there remains a hard core of about 5% of clinically similar patients whose target autoantigen(s) have still to be identified.

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### Conflicts of Interest

The authors declare no conflicts of interest.

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