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Granzyme B: Evidence for a Role in the Origin of Myasthenia Gravis

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Introduction

Myasthenia gravis (MG) is currently the best understood human autoimmune disease. The basic abnormality is a reduction in the number of available acetylcholine receptors (AChRs) at neuromuscular junctions, due to an antibody-mediated autoimmune attack (1, 2). In the great majority of patients the antibodies are directed against epitopes on the AChR subunits (3), while a minority of patients have autoantibodies to a protein adjacent to the AChR at the neuromuscular junction (muscle specific kinase) (4, 5). Although a great deal is known about the immune response and the antibodies' pathogenic mechanisms in MG, the steps leading to the origin of the autoimmune response are still unclear. There is considerable evidence that the thymus is intimately involved in the initiation of the autoimmune response in MG (6). Abnormalities of the thymus are known to be present in ~75% of MG patients; of these hyperplasia (ie- prominent germinal centers) occurs in ~85%, and thymoma in ~15% (7). The antigen AChR is present on muscle-like "myoid" cells (8, 9) and some epithelial cells in the thymus (10), in the context of surrounding immune cells (11, 12). However, the mechanism by which this intrathymic protein becomes recognized as an autoantigen and triggers an autoimmune response is not yet known.

An intriguing possibility that has been suggested by studies of other autoimmune diseases is that proteolytic cleavage by Granzyme B (GrB) - a protease present in cytolytic T cells and NK cells - can alter an otherwise innocuous antigen so as to produce novel "neoantigens" that are recognized as foreign, and thereby trigger an autoimmune response (13). Indeed, a

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unifying feature of autoantigens in SLE and other systemic autoimmune diseases is their susceptibility to cleavage by GrB. In contrast, non-autoantigens are either not cleaved by GrB, or are cleaved by GrB at the same sites as caspases, thus generating fragments identical to those formed in other forms of apoptosis (14). In this regard, it is noteworthy that GrB is absent in mature thymocytes in the normal adult thymus (15). In order to assess the possible role of GrB in the pathogenesis of MG, we tested its ability to cleave the subunits of human AChR, and performed biochemical, immunohistochemical and molecular studies to compare GrB expression in thymus glands from MG patients with that in thymus glands from non-MG individuals. The results show that GrB cleaves subunits of AChR, especially the ϵ subunit. GrB is present in thymus glands from MG patients but not in thymus from normal adult subjects. These findings provide evidence supporting the potential role of GrB in the initiation of MG, and are consistent with the concept of an immunodominant ϵ epitope.

Methods

In vitro transcription translation (IVTT) of human AChR subunits and mutagenesis of AChR-epsilon (AChR- ϵ) subunit

pcDNA3.1 plasmids containing cDNA encoding human AChR subunits α , δ , ϵ , and γ were gifts of David Beeson, University of Oxford, UK. These cDNAs were used in coupled IVTT reactions to generate [^{35}S]-methionine-labeled α and ϵ subunits, according to the manufacturer's protocol (Promega). To evaluate the specificity of the GrB susceptible cleavage site, the point mutation Asp¹⁹⁵ to Ala was generated in the ϵ subunit by site-directed mutagenesis (Stratagene, La Jolla, CA) and was sequence verified. [^{35}S]-methionine-labeled mutant protein was generated by IVTT as described above.

Expression of the transfected AChR subunits

The pcDNA3.1 plasmid containing a cDNA construct encoding wild type human AChR- ϵ , with a V5 tag at the C terminus, was transfected transiently into HEK 293 cells. The cells were washed with PBS, then lysed in Buffer A (1% Nonidet P40, 20 mM Tris pH 7.4, 150 mM NaCl) and a protease inhibitor cocktail of antipain, pepstatin, leupeptin and phenylmethylsulfonyl fluoride. Transfected AChR- ϵ subunit and the cleavage fragment generated after GrB incubation were detected by immunoblotting with a monoclonal anti-V5 antibody and enhanced chemiluminescence (Pierce). HEK cells transfected with control pcDNA3.1 only did not immunoblot any bands when probed with the anti-V5 antibody (data not shown).

Cleavage reactions with GrB

We examined the ability of GrB to cleave the α and ϵ AChR subunits expressed either as [^{35}S]-methionine-labeled proteins by IVTT, or endogenously expressed by transfection in HEK293 cells. Cleavage reactions using IVTT subunits were performed as previously described (16) (and see Figs 1A & B). Briefly, [^{35}S]-methionine-labeled IVTT products were incubated at 37°C for 1 hr in the absence or presence of 56 to 100 nM human GrB (kind gift of Nancy Thornberry, Merck, Rahway, NJ). Ca^{2+} , Mg^{2+} , Mn^{2+} or Zn^{2+} (all at final concentrations of 1 mM except for Zn^{2+} , which was at 0.1 mM) were added to some of the reactions (see Fig 1). Reaction volumes were made to 20 μl using Buffer B (1% Nonidet P40, 10 mM Hepes pH 7.4). After terminating the reactions with gel application buffer and boiling, the samples were electrophoresed on 12% SDS-polyacrylamide gels. Intact AChR- ϵ subunit and the 28 kDa cleavage fragment were visualized by fluorography. Catalytic constant ($k_{\text{cat}}/K_{\text{m}}$) values for the cleavage of the α and ϵ were calculated from the densitometrically scanned data as described (17).

Endogenous V5-tagged AChR- ϵ subunit (in lysates made from HEK 293 cells transiently transfected with the appropriate cDNA construct as described above) was incubated with 135 nM GrB in the absence or presence of 1 mM added Ca^{2+} for 1 hr at 37°C. After terminating the reactions, the samples were electrophoresed on 12% SDS-polyacrylamide gels (equal cell numbers were loaded in each gel lane), transferred to nitrocellulose, and intact AChR- ϵ subunit and the cleaved fragment were detected by immunoblotting with a monoclonal antibody to V5, followed by visualization with enhanced chemiluminescence (Pierce, Femto kit).

Detection of GrB in human thymuses by immunoblotting

All studies on human materials were performed on samples provided in compliance with IRB and HIPAA regulations. Surgical procedures were performed for patient management, and the research tissue samples were in excess of thymus tissue required for routine diagnostic purposes. The myasthenic patients ranged in age from 13 to 38 at the time of thymectomy. The control subjects were adults, but HIPAA requirements for anonymity prevent determination of their ages. The tissue was snap frozen immediately and stored in liquid N_2 . Lysates were made from frozen thymus tissue pieces obtained from 5 non-myasthenic patients and 5 MG patients, essentially as described (18). Briefly, 120 mg of thymus was manually homogenized on ice in 1 ml of Buffer A supplemented with 1 mM EDTA. The homogenates were centrifuged (16,000 $\times$ g, 10 min at 4°C), and the protein concentrations of the resulting lysates (supernatants) were typically in the 1.5–8 mg/ml range. Equal protein amounts were loaded on 12% SDS-polyacrylamide gels. When probing for GrB in lysates, GrB-containing granule contents (GC), prepared from YT cells as described previously (17), were loaded as a positive control for GrB in the blots. The samples were transferred to nitrocellulose and immunoblotted. GrB was detected using polyclonal rabbit antibody R1593, raised against whole recombinant human GrB (Fig 2A) or a monoclonal antibody (Sigma, Fig 2B). To confirm the specificity of the 32 kDa band detected by blotting with R1593 polyclonal antibody, competition immunoblots were also performed by preincubating R1593 serum with GC (1 hr at 4°C) before using it as the source of primary antibody in the blot. Vinculin was probed with monoclonal anti-vinculin antibody (Sigma) and AChR ϵ subunit was probed with a polyclonal rabbit antibody to the peptide KTINKIDID (amino acids 187 – 195 of the ϵ subunit) prepared by Covance Research Products, Denver PA).

RT-PCR

RNA was prepared using standard procedures from frozen thymus biopsies obtained from MG patients, as well as cultured K562 cells (negative control) and PBMCs isolated from human blood from a normal donor (positive control). RT-PCR was performed with the following human GrB-specific primers: ATCAGAAGTCTCTGAAGAGG (forward) and TGCACGTGTCATCTTCACCTC (reverse) using supermix® POLYMERASE (Invitrogen) with the following PCR conditions: 94°C for 5 min; 36 cycles at 95°C for 1 min; 50°C for 1 min and 72°C for 2 min followed by a final extension at 72°C for 5 min. Aliquots of the PCR products were analyzed on 1.5% agarose gels and bands were visualized with ethidium bromide staining.

Immunohistochemistry

Immunohistochemical studies to evaluate the presence of GrB in myasthenic thymuses were performed using tissue obtained from 6 MG patients. Paraffin sections were stained with R1593 serum (1:100 dilution) and GrB was visualized with diaminobenzidine per the manufacturer's directions (Dako, Carpinteria, CA). Specificity of the staining was confirmed (i) by staining serial sections with R1593 anti-GrB antibody in the absence or presence of GC competition as described above and (ii) staining with preimmune serum.

Results

The AChR- α and ϵ subunits are cleaved by GrB

Since a unifying feature of autoantigens recognized in SLE and other systemic autoimmune diseases is susceptibility to cleavage by GrB (14), we tested whether the AChR, which is targeted in MG, is cleaved by this protease. We initially tested this using the individual [^{35}S]-methionine-labeled α , γ , δ , and ϵ AChR subunits, generated by IVTT. When these proteins were incubated *in vitro* with 56 to 100 nM human GrB for 1 hr at 37°C, cleavage of the α and ϵ subunits was detected by a significant reduction (30 – 42%) of the subunit and/or by the presence of cleaved fragments. By contrast, there was minimal or no cleavage of the δ and γ subunits. Using these densitometrically scanned data, the catalytic constant (k_{cat}/K_m) values for cleavage of the α and ϵ subunits were found to be 0.4×10^3 and $1.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, respectively. Since the ϵ subunit was cleaved most efficiently, we focused on this subunit in our subsequent studies. Cleavage efficiency of AChR- ϵ was increased in the presence of Ca^{2+} , Mg^{2+} or Mn^{2+} but not Zn^{2+} (Fig 1A). These findings, made with IVTT AChR- ϵ only, were subsequently confirmed using endogenously expressed protein in cell lysates from HEK 293 cells transiently transfected with AChR- ϵ tagged with V5 at the C-terminus. The lysates were incubated with GrB as described in the Methods section and intact AChR- ϵ and the C-terminal cleavage fragment were detected by blotting with an anti-V5 monoclonal antibody (Fig 1C). Consistent with the IVTT data shown in Fig 1A, (i) endogenously expressed AChR- ϵ was cleaved by GrB, and (ii) this cleavage was more efficient in the presence of Ca^{2+} . Based on the size of the cleavage fragment and the known amino acid preferences for human GrB (16), we identified a putative GrB cleavage site in the AChR- ϵ sequence at IDID¹⁹⁵. Using site-directed mutagenesis, Asp¹⁹⁵ was converted to Ala, and after sequence verification of the mutation, the cDNA was used for IVTT. Cleavage by GrB of the mutated AChR- ϵ was abolished (Fig 1B), confirming that this is indeed the GrB cleavage site.

GrB is detected in thymus from patients with MG

Since the AChR subunits α and ϵ are cleavable by GrB, and because they are targeted by the autoimmune response in MG (19-22), we investigated whether GrB is present in the thymus of MG patients. Gel samples were prepared from homogenates made from frozen MG thymus tissue obtained at therapeutic surgery, and immunoblotted with a polyclonal rabbit antibody (R1593) raised against whole recombinant GrB. The antibody detected a 32 kDa band which was present in substantial amounts in the MG thymus samples (Fig 2A, lanes 1-4). Note that the levels vary in amount in samples from different patients; this is typical of inter-patient tissue variation. The identity of the 32 kDa band as GrB was confirmed using the following 3 different approaches: (i) Human GC (which consist predominantly of GrB) were electrophoresed on the same gel as the thymus samples. Immunoblotting with the anti-GrB antibody revealed a 32 kDa band that co-migrated with that detected in the thymus samples (Fig 2, compare lanes 1, 4 & 5). (ii) Competition immunoblots were performed by preincubating the anti-GrB antibody with GrB-containing GC. This abolished detection of the 32 kDa band in the thymus and the GC samples. Of note, the 30 kDa nonspecific band detected by R1593 serum was not competed away by preincubation with GC, further underscoring the specificity of the 32 kDa blotted band (Fig 2A, lanes 1-4 and 6-9). (iii) When a different antibody against GrB was used to perform the immunoblots (monoclonal Clone GrB7, purchased from Sigma), a 32 kDa band was detected in lysates made from MG thymuses; this band co-migrated with that detected in the GrB-containing GC sample (Fig 2B, lanes 11-14). Of note, GrB was not detected when immunoblots were similarly performed using lysates prepared from normal thymuses (Fig 2B, lanes 15-17).

We subsequently confirmed these findings of GrB expression in MG thymus at the level of mRNA by performing RT-PCR. To facilitate direct comparison of the results, RNA was prepared from the same frozen thymus fragments as were used to generate the lysates depicted in Fig 2A. RT-PCR was performed using GrB primers on RNA obtained from the thymus samples, as well as message prepared from K562 cells (negative control) and human PBMCs (positive control). After electrophoresis on agarose gels and ethidium bromide staining, a 379 bp product, consistent with the size of GrB, was detected in the same thymus samples as had shown GrB by blotting. The identity of this message as GrB was further confirmed because (i) it was absent in K562 cells but present in PBMCs (Fig 2C, lanes 22 & 23) and (ii) it was not detected in the thymus samples after performing the RT-PCR minus the RT (Fig 2C, lanes 24 B 27).

To assess GrB expression in the thymus directly, immunohistochemistry was performed. Cytoplasmic GrB staining was detected in paraffin sections made from thymuses obtained from 6 different MG patients; the data shown in Fig 2D & F are typical of the staining detected in all the MG patient thymuses. Specificity of the staining was confirmed as follows: (i) it was competed away by preincubation of the anti-GrB antibody with GC (Fig 2, compare panels D & E); (ii) no staining was detected when preimmune serum from rabbit 1593 was used for staining serial sections (data not shown) and (iii) identical staining patterns were detected using sera from 2 different rabbits immunized with whole recombinant GrB (R1593 and R1592), as well as a monoclonal antibody to TIA1 (Abcam) that stains cytoplasmic granules (Fig 2 D-F, and data not shown).

AChR- ϵ subunit is expressed at elevated levels in thymus obtained from MG patients compared to normal thymus

Recent findings have documented that the autoantigen expression levels in specific tissues may be elevated in the autoimmune disease in which they are targeted. For example, Mi-2 is elevated in muscle from patients with dermatomyositis, and is targeted by the autoimmune response only in this autoimmune muscle disease (18). We therefore examined the levels of AChR- ϵ expression in myasthenic thymus compared to normals. Equal amounts of protein were electrophoresed in each gel lane and immunoblotted with antibody to AChR- ϵ (Fig 3, upper panel). Levels of expression were elevated 2.8 fold. Immunoblotting with vinculin (Fig 3, lower panel) was used as a loading control. Indeed, normalizing the data in the upper panel relative to vinculin gave an even higher extent of AChR- ϵ expression in MG thymus (3.8 fold).

Discussion

In this study, we have found evidence consistent with the concept that GrB may play a key role in the autoimmune response to AChR in MG. This is of particular interest because the present extensive knowledge of the immunopathogenesis of MG provides a platform for examining the role of proteolytic cleavage of the autoantigen - AChR - by GrB in a strictly humorally - mediated human autoimmune disease, in the context of the likely site of initiation (the thymus).

Certain stringent criteria must be fulfilled for potential autoantigens to be selected for a primary and sustained response by the immune system: The actual autoantigenic components must (i) be non-tolerized molecules, (ii) that can be presented to immune responder cells at suprathreshold concentrations, (iii) in a pro-immune context, and (iv) preferably in individuals that are genetically predisposed to the generation and presentation of these novel neoantigens (23). Under normal circumstances, potential autoantigens - including AChR - are undoubtedly seen as "self" by the immune system, which has access only to dominant determinants (23). However, when proteolytic cleavage of these antigens

produces novel fragments, the immune system can react to newly exposed cryptic determinants to which it has not previously been tolerized, setting the stage for an autoimmune response. Cleavage by GrB has been shown to produce such novel neoantigenic fragments in several autoimmune diseases, and the present results provide the first evidence that it may do so in MG as well.

GrB is a serine protease that is present in cytoplasmic granules of cytotoxic lymphocytes (CTL) and NK cells. When somatic cells are targeted by CTL or NK cells, they may release GrB-containing granules, which can enter either through pre-made openings created by perforin release, or more likely by endocytosis without perforin introduction (13). Cleavage of proteins by GrB requires a rather restricted amino acid sequence, with aspartic acid in the P₁ position, Ile or Val in P₄, and Glu, Gly or Ser in P₃ (13). It can induce apoptosis by activation of caspases, or by caspase independent cleavage. During the past several years, evidence implicating GrB in the production of such novel fragments, and in the initiation of autoimmune disease has accumulated. The majority of autoantigens targeted in human systemic autoimmune diseases are efficiently cleaved by GrB during CTL granule-induced death, generating unique fragments not observed during any other form of apoptosis. Conversely, it is rare for non-autoantigens to be cleavable by GrB, and if cleaved, their fragments are identical to those produced by other forms of apoptosis (14). Diseases in which presumptive autoantigens can be cleaved by GrB include lupus erythematosus, rheumatoid arthritis, scleroderma, myositis, Sjögren's syndrome, and Rasmussen's encephalitis (24-28). More than 20 defined autoantigens are known to be cleaved by GrB (14).

In the present study, we have shown that AChR, which is the autoantigen for the majority of patients with MG, can be cleaved by GrB *in vitro*. We demonstrated that the α and ϵ AChR subunits expressed by IVTT were cleaved, whereas the δ and γ subunits were not. Since the ϵ subunit was cleaved most efficiently, we focused our subsequent studies on this subunit. Cleavage by GrB occurred in AChR subunits expressed both by IVTT and in lysates made from ϵ subunit-transfected HEK 293 cells, and the efficiency of cleavage was enhanced by the presence of divalent cations. Using site-directed mutagenesis, we showed that IDID¹⁹⁵ is the GrB cleavage site for the AChR- ϵ subunit. This site is actually within the epitope (ϵ 188 – 229) that was recognized by T cells from patients with early (recent) onset MG, and just upstream from a shorter nested epitope recognized by some of them (ϵ 201-219) (22). These peptides have been identified as “immunodominant”. Because they are close to the first trans-membrane domain of the ϵ subunit, it is possible that GrB cleavage may be required in order for the epitopes to be liberated, and recognized by the immune system.

Since there is abundant evidence that the thymus may be the site of origin of MG (6, 8, 29, 30), we explored the possibility that GrB cleavage of AChR could occur in thymus glands of MG patients. We have shown that GrB is expressed in adult thymus glands that had been removed surgically from myasthenic patients for therapeutic indications, as demonstrated by immunoblotting, immunohistochemistry, and RT-PCR. In contrast, GrB was not detected in thymus tissue from adult non-MG patients. We also examined thymus tissue from MG patients, and control non-MG adults and infants for the presence of AChR, using our anti- ϵ subunit antibody. Thymus tissue from the MG patients had markedly elevated levels of ϵ subunit protein, compared with absent or minimal ϵ protein in thymus tissue from non-MG adults. It is of interest that the ϵ subunit is present in thymic tissue, both by our demonstration of the protein, and by previous studies of mRNA in thymus tissue from myasthenic patients and normal subjects (31-33), and in cultured myoid cells from rat thymus (34). Messenger RNA for the ϵ subunit was higher in thymus tissue from MG patients than in controls, relative to the message for the housekeeping protein actin, and ϵ mRNA was higher than γ mRNA in thymic tissue from both MG patients and controls (33).

The presence of ϵ in the thymus is somewhat counterintuitive, since the γ subunit would be expected to be expressed exclusively by the non-innervated myoid cells. The AChR is comprised of five subunits, 2 α and one each of β , δ , and γ or ϵ . The γ subunit is present in AChR of skeletal muscles that are immature, non-innervated or denervated (2). It is replaced by the ϵ subunit when the muscle is innervated. Therefore, the presence of the ϵ subunit in thymus tissue, which presumably is not innervated, had not been anticipated. A question has been raised as to whether AChR is expressed only by the myoid cells or whether thymic epithelial cells also express both the ϵ subunit and other subunits of the AChR (10, 31). In any case, our results not only confirm the presence of the ϵ subunit, but further implicate it as a candidate autoantigen. As noted above, previous studies have demonstrated that T cells from recent onset myasthenic patients recognize primarily the ϵ subunit (19, 22).

Our findings are consistent with the concept that GrB may play a role in the immunopathogenesis of MG, as in many other autoimmune diseases. Both GrB and the autoantigen AChR are present at elevated levels in thymuses from MG patients, and the fact that subunits of the AChR are cleavable by GrB is a hallmark of autoantigens. The GrB-cleaved fragments of AChR subunits likely are asynchronously generated and rapidly scavenged within the thymus, making it challenging to detect them with available reagents. It is well established that the specific humoral response to AChR is T cell dependent (35, 36), and T cells from thymic tissue of myasthenic patients are more responsive to AChR than T cells from peripheral blood (37). Although both AChR and T cells normally coexist in the thymus, the trigger that breaks tolerance, and thereby leads to an autoimmune attack against AChRs has not previously been identified. The present evidence suggests that proteolysis by GrB provides a missing link. However, it is unlikely that the existence of GrB in cells within the thymus is sufficient by itself to account for initiation of the autoimmune response. Other factors must also interact, including a pro-immune context, and in some cases a genetic predisposition to responsiveness to AChR and/or to other autoimmune responses as well. The presence of proinflammatory cytokines, chemokines and their receptors in thymic tissue of myasthenic patients (38-41) may provide stimuli for initiating activation of the GrB mediated trigger of the autoimmune response to AChR, and have also been shown to up-regulate expression of AChR in the thymus. Genetic factors may also predispose to the development of MG (42, 43).

In conclusion, we propose that initiation of the autoimmune response in MG may occur when novel structures are generated by GrB cleavage of AChR in a pro-immune context within the thymus. It is particularly intriguing that the GrB cleavage site corresponds closely with the epitopes on the ϵ subunit that are recognized by T cells in recent onset MG, and that have been identified as immunodominant. Our findings extend the concept that GrB may have an important role in the pathogenesis of autoimmune diseases, since MG is an exclusively antibody-mediated autoimmune disease.

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References

1. Drachman DB. Myasthenia gravis. *N Engl J Med.* 1994; 330(25):1797–810. [PubMed: 8190158]
2. Vincent A, Drachman DB. Myasthenia gravis. *Adv Neurol.* 2002; 88:159–88. [PubMed: 11908224]
3. Lindstrom JM, Seybold ME, Lennon VA, Whittingham S, Duane DD. Antibody to acetylcholine receptor in myasthenia gravis Prevalence, clinical correlates, and diagnostic value. *Neurology.* 1976; 26(11):1054–9. [PubMed: 988512]

4. Hoch W, McConville J, Helms S, Newsom-Davis J, Melms A, Vincent A. Autoantibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat Med.* 2001; 7(3):365–8. [PubMed: 11231638]
5. Zhou L, McConville J, Chaudhry V, Adams RN, Skolasky RL, Vincent A, et al. Clinical comparison of muscle-specific tyrosine kinase (MuSK) antibody-positive and -negative myasthenic patients. *Muscle Nerve.* 2004; 30(1):55–60. [PubMed: 15221879]
6. Conti-Fine, BM.; Diethelm-Okita, B.; Ostlie, N.; Wang, W.; Milani, M. Immunopathogenesis of Myasthenia Gravis. In: Kaminski, HJ., editor. *Myasthenia Gravis and Related Disorders*. Totowa, NJ: Humana Press; 2003. p. 53-91.
7. Castleman B. The pathology of the thymus gland in myasthenia gravis. *Ann N Y Acad Sci.* 1966; 135:496–503. [PubMed: 5221360]
8. Kao I, Drachman DB. Thymic muscle cells bear acetylcholine receptors: possible relation to myasthenia gravis. *Science.* 1977; 195(4273):74–5. [PubMed: 831257]
9. Wekerle H, Ketelsen UP, Zurn AD, Fulpius BW. Intrathymic pathogenesis of myasthenia gravis: transient expression of acetylcholine receptors on thymus-derived myogenic cells. *Eur J Immunol.* 1978; 8(8):579–82. [PubMed: 699955]
10. 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(8):3752–60. [PubMed: 8871679]
11. Schluep M, Willcox N, Ritter MA, Newsom-Davis J, Larche M, Brown AN. Myasthenia gravis thymus: clinical, histological and culture correlations. *J Autoimmun.* 1988; 1(5):445–67. [PubMed: 3254184]
12. Kirchner T, Hoppe F, Schalke B, Muller-Hermelink HK. Microenvironment of thymic myoid cells in myasthenia gravis. *Virchows Arch B Cell Pathol Incl Mol Pathol.* 1988; 54(5):295–302. [PubMed: 2895542]
13. Andrade F, Casciola-Rosen LA, Rosen A. Granzyme B-induced cell death. *Acta Haematol.* 2004; 111(1-2):28–41. [PubMed: 14646343]
14. Casciola-Rosen L, Andrade F, Ulanet D, Wong WB, Rosen A. Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. *J Exp Med.* 1999; 190(6):815–26. [PubMed: 10499920]
15. Held W, MacDonald HR, Mueller C. Expression of genes encoding cytotoxic cell-associated serine proteases in thymocytes. *Int Immunol.* 1990; 2(1):57–62. [PubMed: 2150921]
16. Casciola-Rosen L, Garcia-Calvo M, Bull HG, Becker JW, Hines T, Thornberry NA, et al. Mouse and human granzyme B have distinct tetrapeptide specificities and abilities to recruit the bid pathway. *J Biol Chem.* 2007; 282(7):4545–52. [PubMed: 17179148]
17. Andrade F, Roy S, Nicholson D, Thornberry N, Rosen A, Casciola-Rosen L. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. *Immunity.* 1998; 8(4):451–60. [PubMed: 9586635]
18. Casciola-Rosen L, Nagaraju K, Plotz P, Wang K, Levine S, Gabrielson E, et al. Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy. *J Exp Med.* 2005; 201(4):591–601. [PubMed: 15728237]
19. Wang ZY, Okita DK, Howard JF Jr, Conti-Fine BM. CD4+ T cell repertoire on the epsilon subunit of muscle acetylcholine receptor in myasthenia gravis. *J Neuroimmunol.* 1998; 91(1-2):33–42. [PubMed: 9846817]
20. Manfredi AA, Protti MP, Dalton MW, Howard JF Jr, Conti-Tronconi BM. T helper cell recognition of muscle acetylcholine receptor in myasthenia gravis. Epitopes on the gamma and delta subunits. *J Clin Invest.* 1993; 92(2):1055–67. [PubMed: 7688757]
21. Vincent A, Willcox N, Hill M, Curnow J, MacLennan C, Beeson D. Determinant spreading and immune responses to acetylcholine receptors in myasthenia gravis. *Immunol Rev.* 1998; 164:157–68. [PubMed: 9795773]
22. Hill M, Beeson D, Moss P, Jacobson L, Bond A, Corlett L, et al. Early-onset myasthenia gravis: a recurring T-cell epitope in the adult- specific acetylcholine receptor epsilon subunit presented by the susceptibility allele HLA-DR52a. *Ann Neurol.* 1999; 45(2):224–31. [PubMed: 9989625]

23. Rosen A, Casciola-Rosen L. Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. *Cell Death Differ.* 1999; 6(1):6–12. [PubMed: 10200542]
24. Casciola-Rosen LA, Pluta AF, Plotz PH, Cox AE, Morris S, Wigley FM, et al. The DNA mismatch repair enzyme PMS1 is a myositis-specific autoantigen. *Arthritis Rheum.* 2001; 44(2):389–96. [PubMed: 11229471]
25. Nagaraju K, Cox A, Casciola-Rosen L, Rosen A. Novel fragments of the Sjogren's syndrome autoantigens alpha-fodrin and type 3 muscarinic acetylcholine receptor generated during cytotoxic lymphocyte granule-induced cell death. *Arthritis Rheum.* 2001; 44(10):2376–86. [PubMed: 11665980]
26. Schachna L, Wigley FM, Morris S, Gelber AC, Rosen A, Casciola-Rosen L. Recognition of Granzyme B-generated autoantigen fragments in scleroderma patients with ischemic digital loss. *Arthritis Rheum.* 2002; 46(7):1873–84. [PubMed: 12124872]
27. Gahring L, Carlson NG, Meyer EL, Rogers SW. Granzyme B proteolysis of a neuronal glutamate receptor generates an autoantigen and is modulated by glycosylation. *J Immunol.* 2001; 166(3):1433–8. [PubMed: 11160179]
28. Skoldberg F, Ronnblom L, Thorne M, Lindahl A, Bird PI, Rorsman F, et al. Identification of AHNK as a novel autoantigen in systemic lupus erythematosus. *Biochem Biophys Res Commun.* 2002; 291(4):951–8. [PubMed: 11866458]
29. Simpson JA. Myasthenia gravis, a new hypothesis. *Scott Med J.* 1960; 5:419.
30. Wekerle H, Hohlfeld R, Ketelsen UP, Kalden JR, Kalies I. Thymic myogenesis, T-lymphocytes and the pathogenesis of myasthenia gravis. *Ann N Y Acad Sci.* 1981; 377:455–76. [PubMed: 6978664]
31. MacLennan CA, Beeson D, Willcox N, Vincent A, Newsom-Davis J. Muscle nicotinic acetylcholine receptor mRNA expression in hyperplastic and neoplastic myasthenia gravis thymus. *Ann N Y Acad Sci.* 1998; 841:407–10. [PubMed: 9668266]
32. Kaminski HJ, Fenstermaker RA, Abdul-Karim FW, Clayman J, Ruff RL. Acetylcholine receptor subunit gene expression in thymic tissue. *Muscle Nerve.* 1993; 16(12):1332–7. [PubMed: 8232389]
33. Navaneetham D, Penn AS, Howard JF Jr, Conti-Fine BM. Human thymuses express incomplete sets of muscle acetylcholine receptor subunit transcripts that seldom include the delta subunit. *Muscle Nerve.* 2001; 24(2):203–10. [PubMed: 11180203]
34. Kobayashi T, Mishina M, Michikawa M. Alpha- beta- gamma-, delta- and epsilon-subunit specific mRNAs of acetylcholine receptors (AChRs) in cultured rat thymus myoid cells. *J Neurol Sci.* 1990; 98:299.
35. Ragheb S, Lisak RP. Immune regulation and myasthenia gravis. *Ann N Y Acad Sci.* 1998; 841:210–24. [PubMed: 9668243]
36. Lennon VA, Lindstrom JM, Seybold ME. Experimental autoimmune myasthenia gravis: cellular and humoral immune responses. *Annals of the New York Academy of Sciences.* 1976; 274:283–289. [PubMed: 1085587]
37. Sommer N, Willcox N, Harcourt GC, Newsom-Davis J. Myasthenic thymus and thymoma are selectively enriched in acetylcholine receptor-reactive T cells. *Ann Neurol.* 1990; 28(3):312–9. [PubMed: 2241114]
38. Saito R, Onodera H, Tago H, Suzuki Y, Shimizu M, Matsumura Y, et al. Altered expression of chemokine receptor CXCR5 on T cells of myasthenia gravis patients. *J Neuroimmunol.* 2005; 170(1-2):172–8. [PubMed: 16214223]
39. Suzuki Y, Onodera H, Tago H, Saito R, Ohuchi M, Shimizu M, et al. Altered expression of Th1-type chemokine receptor CXCR3 on CD4+ T cells in myasthenia gravis patients. *J Neuroimmunol.* 2006; 172(1-2):166–74. [PubMed: 16427705]
40. Meraouna A, Cizeron-Clairac G, Panse RL, Bismuth J, Truffault F, Tallaksen C, et al. The chemokine CXCL13 is a key molecule in autoimmune myasthenia gravis. *Blood.* 2006; 108(2):432–40. [PubMed: 16543475]
41. Le Panse R, Cizeron-Clairac G, Bismuth J, Berrih-Aknin S. Microarrays reveal distinct gene signatures in the thymus of seropositive and seronegative myasthenia gravis patients and the role

- of CC chemokine ligand 21 in thymic hyperplasia. *J Immunol.* 2006; 177(11):7868–79. [PubMed: 17114458]
42. Janer M, Cowland A, Picard J, Campbell D, Pontarotti P, Newsom-Davis J, et al. A susceptibility region for myasthenia gravis extending into the HLA-class I sector telomeric to HLA-C. *Hum Immunol.* 1999; 60(9):909–17. [PubMed: 10527401]
43. Niks EH, Kuks JB, Roep BO, Haasnoot GW, Verduijn W, Ballieux BE, et al. Strong association of MuSK antibody-positive myasthenia gravis and HLA-DR14-DQ5. *Neurology.* 2006; 66(11):1772–4. [PubMed: 16769963]

Figure 1. The AChR-ε is cleaved by GrB

(A). [³⁵S]-methionine-labeled AChR-ε subunit, generated by IVTT, was incubated for 60 min at 37°C in the absence or presence of 56 nM purified human GrB. The reactions were performed with the following cations, as indicated on the figure: Ca²⁺, Mg²⁺, Mn²⁺ or Zn²⁺. After terminating the reactions, the gel samples were electrophoresed on 12% SDS-polyacrylamide gels. Intact AChR-ε subunit and the 28 kDa cleavage fragment were visualized by fluorography. (B) Wild type (WT) and D¹⁹⁵A mutated AChR-ε subunit were cleaved with GrB and processed as described in (A); the reactions were performed in the absence of added cations. (C) Lysates made from HEK 293 cells transfected with V5 tagged AChR-ε subunit were incubated for 60 mins at 37°C in the absence or presence of 56 nM GrB. The reactions were performed without added Ca²⁺ (2 left lanes), or with 1 mM added Ca²⁺ (right 2 lanes). Intact and cleaved AChR-ε subunit were detected by immunoblotting with an anti-V5 monoclonal antibody.

Figure 2. GrB is detected in thymuses from MG patients

(A) Detection by immunoblotting. Lysates made from 4 different MG patients' thymuses were immunoblotted with a rabbit polyclonal antibody to GrB (R1593, lanes 1-4). GrB-containing human GC were electrophoresed as a reference to mark the migration of GrB (lane 5). A duplicate set of samples, processed simultaneously, was immunoblotted with R1593 preincubated with GrB-containing GC ("competition immunoblot", lanes 6-10). Preincubation of R1593 serum with GrB-containing GC competed out the ability of the antibody to immunoblot 32 kDa GrB (upper band in lanes 1-5). The data presented are from a single autorad that has been spliced between lanes 4 & 5, and 9 & 10. (B) Lysates made from 3 MG patients' thymuses (lanes 12-14), 3 normal thymuses (lanes 15-17) and GC (lane 11) were immunoblotted with a monoclonal antibody to GrB. GrB was detected in lysates made from myasthenic but not normal thymuses. (C) RT-PCR. RNA, prepared from the same MG patients' thymuses used in (A) (lanes 18-21 and 24-27) as well as K562 cells and human PBMCs (lanes 22 & 23) was used to perform RT-PCR. These reactions were performed in the absence (lanes 24-27) or presence (lanes 18-23) of reverse transcriptase, and the DNA products were electrophoresed on agarose gels. The arrow on the left marks GrB DNA. The data presented is from a single gel run; intervening lanes in the image have been spliced between lanes 23 & 24. (D-F) Immunohistochemistry. Paraffin sections were cut from the thymus of an MG patient and stained with anti-GrB antibody R1593 (D & F), or R1593 preincubated with GC (E). D & F are the same section, photographed at 10x and 63x, respectively; D and E are serial sections.

Figure 3. The human AChR- ϵ subunit is expressed at elevated levels in thymuses from myasthenic patients compared to normal thymuses

Lysates made from normal and myasthenic thymuses were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose and immunoblotted with a polyclonal anti-AChR- ϵ antibody (top panel) or a monoclonal antibody to vinculin (lower panel). Equal amounts of protein were loaded in each lane. The data shown was obtained from a single immunoblot, which was spliced between the normal and myasthenic lanes.