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Cathepsin S Is Required for Murine Autoimmune Myasthenia Gravis Pathogenesis

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Because presentation of acetylcholine receptor (AChR) peptides to T cells is critical to the development of myasthenia gravis, we examined the role of cathepsin S (Cat S) in experimental autoimmune myasthenia gravis (EAMG) induced by AChR immunization. Compared with wild type, Cat S null mice were markedly resistant to the development of EAMG, and showed reduced T and B cell responses to AChR. Cat S null mice immunized with immunodominant AChR peptides showed weak responses, indicating failed peptide presentation accounted for autoimmune resistance. A Cat S inhibitor suppressed in vitro IFN-γ production by lymph node cells from AChR-immunized, DR3-bearing transgenic mice. Because Cat S null mice are not severely immunocompromised, Cat S inhibitors could be tested for their therapeutic potential in EAMG. The Journal of Immunology, 2005, 174: 1729–1737.

*yasthenia gravis is an autoimmune neuromuscular disease characterized by T cell-dependent autoantibody responses to the muscle nicotinic acetylcholine receptor (AChR)1. AChR-specific T cells help B cells to produce anti-AChR Abs. Complement activating IgG2 subclass of Abs bind AChR at the neuromuscular junction and accelerate AChR destruction, thus culminating in neuromuscular transmission failure, muscle weakness, and fatigue (1). Experimental autoimmune myasthenia gravis (EAMG) can be induced in mice by immunization with purified AChR from Torpedo californica (T-AChR) (2). Cellular analysis has revealed that AChR-specific lymphocyte proliferation following immunization with AChR depends on MHC class II-restricted CD4+ Th cells and APC (3). MHC class II-deficient mice fail to develop cellular and humoral immune responses to AChR and clinical EAMG (4). HLA-DQβ and HLA-DR3 polymorphisms have been linked to myasthenia gravis (5) and MHC class II has been shown to restrict immune response to AChR in humans as well (6). In vivo treatment with anti-CD4 mAb, GK1.5, not only suppressed autoimmune responses to AChR, but also helped to prevent and delay development of muscular weakness characteristic of EAMG (7). GK1.5, in vivo, also induced clinical remission when given after established clinical disease (7). In EAMG susceptible C57BL/6 (I-Aβ) mice immunized with T-AChR, the sequence region 146–162 of the T-AChR α subunit forms an immunodominant epitope for CD4+ T cell sensitization (8–10), and tolerance to this peptide suppresses EAMG development (11, 12). Moreover, EAMG could be induced in HLA-DQ8 and HLA-DR3 transgenic mice with either T-AChR or human AChR immunization (13, 14), and dominant human AChR T cell epitopes have been mapped for AChR immune T cells of HLA-DQ8 and HLA-DR3 transgenic mice (14).

Ag presentation by APC requires both Ag processing and maturation of MHC class II αβ heterodimers to a functional state. MHC class II molecules are synthesized along with a chaperone, Ii, which controls the MHC class II trafficking and access of its peptide groove to peptides. The αβ-Ii complex in the endoplasmic reticulum is transported through the Golgi complex to an acidic endosomal or lysosomal compartment, where Ii is removed from αβ chain by stepwise proteolysis (15, 16). Proteolysis of αβ-Ii isolated from cells generates αβ-CLIP. Within endosomal compartments, CLIP rapidly dissociates from MHC class II dimers aided by H-2M molecule, allowing loading of exogenous peptide from endocytosed protein and subsequent surface expression of MHC class II molecules with antigenic peptides (17–19). Evidence from protease inhibitor studies and in vitro digestion of purified class II-Ii complexes implicates endosomal cysteine proteases as important in Ii degradation. Recent work shows that the cysteine protease cathepsin S (Cat S) is required for the terminal step in CLIP formation in B cells and most dendritic cells (DCs), and in vitro can mediate all steps of digestion of class II-Ii complexes (20–25). Cat S is a potent endoprotease highly expressed in professional APCs, e.g., B cells and DCs (26, 27). However, not all MHC class II-restricted Ags require processing and some antigenic peptides appear to load onto MHC class II molecules independently of Ii (28, 29). Consequently, the influence of specific proteases on MHC class II-dependent immune responses is somewhat variable and likely Ag, MHC haplotype, and APC dependent (21, 23, 30–35).

Because of these uncertainties we explored the role of Cat S in the medically important autoantigen, AChR. The initial step in the...
pathogenesis of EAMG in C57BL/6 and HLA-DR3 transgenic mice following immunization with T-AChR or human AChR is presentation of the AChR dominant peptide *Torpedo* α_{146–162} or human α_{320–337} respectively by MHC class II molecules to CD4\(^+\) T cells (8, 11, 14). We hypothesized that Cat S has a critical role in the development of Ab and complement-mediated EAMG. To test this hypothesis, Cat S\(^+/−\) and wild-type mice in the C57BL/6 background were immunized with AChR in CFA. They were then examined for cellular and humoral immune responses to AChR, and development of clinical EAMG. The findings we report provide the first direct genetic evidence for a key role of Cat S in autoimmune responses to AChR and in EAMG pathogenesis.

**Materials and Methods**

**AChR and mice**

AChR was purified from the electric organ of *Torpedo californica* or TE671 cell line expressing human AChR by α-neurotoxin affinity column (14, 36). T-AChR α-chain peptide α_{146–162} (immunodominant in C57BL/6 mice immunized with T-AChR (8–12)) and human AChR α-chain peptide. α_{320–337} (immunodominant in HLA-DR3 transgenic mice immunized with human AChR), was synthesized in M. D. Anderson Cancer Center and Jeevan Sciences, Houston, TX. C57BL/6 mice 7– to 8-week-old were purchased from The Jackson Laboratory. C5-deficient mice were generated in a 129/SV1 background as previously described (37) and bred into a C57BL/6 background for over 10 generations. The percentage of CD4\(^+\) and CD8\(^+\) cells in the PBL of Cat S\(^+/−\) mice and the wild-type mice before immunization were analyzed by flow cytometry. This analysis revealed normal ratios of CD19\(^+\) B cells, CD3\(^+\) T cells and CD4\(^+\) and CD8\(^+\) T cells in both wild-type (C57BL/6) and Cat S\(^+/−\) mice. We also assessed cell surface class II expression, and found no significant differences between Cat S\(^+/−\) mice and wild-type mice. Therefore, the immune system in Cat S\(^+/−\) mice developed normally. All of the animals were housed in the viral Ab-free barrier facility at the University of Texas Medical Branch and maintained and experimented performed according to the Animal Care and Use Committee Guidelines.

**Induction and clinical evaluation of EAMG**

Cat S\(^+/−\) mice and wild-type mice were anesthetized and immunized with 20 μg of T-AChR emulsified in CFA (Difco) s.c. at four sites (two hind footpads and shoulders) on day 0. Control Cat S\(^+/−\) and wild-type mice received PBS in CFA. All the T-AChR immunized and control mice were boosted with 20 μg of T-AChR or PBS in CFA s.c. at four sites on the back on day 30 and day 60. Mice were screened for clinical EAMG on a daily basis. Clinical muscle weakness was graded (clinical scores) as follows: Grade 0, mouse with normal muscle strength; Grade 1, normal at rest, with muscle weakness characteristicly shown by hunched-back posture, and difficulty to raise the head after exercise, consisting of 20–30 paw grips on cage top grid; Grade 2, mouse showed grade 1 symptoms without exercise; Grade 3, dehydrated and moribund with grade 2 weakness; and Grade 4, dead.

**Immunopathological evaluation of EAMG**

The primary pathology of myasthenia gravis and EAMG in mice is the loss of muscle AChR due to Ab- and complement-mediated attack (1). The total concentration of AChR per muscle was determined according to previously published methods and expressed in picomoles of α-bungarotoxin (α-BT) binding sites (36). Preimmune serum and postimmune serum after 14, 45, and 90 days were collected from individual mice. The serum anti-mouse AChR Ab was measured by α-BT (Amersham) radioimmunoassay (36), and the anti-mouse AChR IgM and IgG (IgG1, IgG2b, IgG2c) subclasses were measured by ELISA (14).

**Lymph node cell and B cell expansion in AChR immunized mice**

Inguinal and axillary lymph nodes from AChR in CFA-immunized and PBS in CFA-immunized mice were harvested on day 7 and 90 (after boosting with AChR in CFA on day 30 and 60), and single cell suspension made in RPMI 1640 medium. Live cells were counted under the microscope by trypan blue exclusion of dead cells. Lymph node cells were also analyzed for CD3, CD4, CD8, CD19, CD40, and I-A\(^b\) surface markers after 30 min incubation with the following Abs: CD3 (Cy-Chrome-conjugated), CD4 and CD8 (PE-conjugated), CD19 (FITC-conjugated), CD40, (PE-conjugated), BD Pharmingen), and FITC-conjugated I-A\(^b\) (Caltag Laboratories) anti-mouse mAbs. PE- or FITC-conjugated isotypes were used as controls. Cells were washed twice and then were fixed with 2% paraformaldehyde, and analyzed by FACStation flow cytometry (Becton Dickinson).

**Lymphocyte proliferative and cytokine assays**

For studies on early immune response to AChR, Cat S\(^+/−\) mice and wild-type mice were divided into two groups, each group of mice were anesthetized and immunized s.c. with 20 μg of T-AChR or 50 μg of T-AChR α subunit peptide 146–162 emulsified in CFA (Difco). Seven days later, the mice were euthanized and the draining lymph node cells (popliteal and inguinal) plated in triplicate were stimulated in vitro with T-AChR (2.5 ng/ml) or the peptide (20 μg/ml). The extent of cell proliferation was determined from the incorporation of [\(^{3}H\)Jthymidine (11). Culture supernatants were analyzed for IFN-γ, IL-2, and IL-10 by ELISA (11, 14).

Wild-type and Cat S\(^+/−\) mice were immunized with AChR (20 μg/ml) in CFA with subsequent boosts with same amount of AChR in CFA on day 30 and 60. Inguinal and axillary lymph node cells were collected at termination of the evaluation on day 90 and plated 4×10\(^5\) cells in triplicate. These cells were stimulated in vitro with T-AChR (2.5 μg/ml) or α_{146–162} peptide (20 μg/ml) and on day 5 lymphocyte proliferative response was measured (11). Culture supernatants were analyzed for IFN-γ, IL-2, and IL-10 by ELISA (11, 14).

**Ag presentation assay**

C57BL/6 female mice 6– to 7-week-old were immunized s.c. in the hind footpad with 20 μg of T-AChR in CFA. After day 9, inguinal and popliteal lymph nodes were removed and single cell suspensions prepared after RBC lysis. Lymph node cells were more than stimulated with 20 μg/ml α_{146–162} peptide and 10 ng/ml mouse recombinant IL-2 (Endogen) for 3–4 days. On day 4 residual IL-2 and α_{146–162} peptide were removed from the medium and the cells rested for 2–3 days. After washing cells and blocking with Fc block (0.5 μg/1×10\(^5\) cells), and stained for CD4\(^+\) using anti-CD4 cytochrome Ab (BD Pharmingen). The cells were then sorted for CD4\(^+\) T cells by high throughput sorting (CytoFusion). Splenocytes from C57BL/6 and Cat S\(^+/−\) mice were treated with 50 μM minocyclin (Sigma-Aldrich) for 30 min at 37°C, washed, and used as APCs. The sorted CD4\(^+\) T cells were seeded in triplicates with APCs at 1:40 and 1:10 of T cell to APC ratios and stimulated in vitro with different concentrations of AChR (0, 0.062, 0.25, and 1 μg/ml). AChR immunodominant peptide α_{146–162} was used at 20 μg/ml. Alternatively, Ag presentation was also performed on lymph node cells from AChR immunized mice that were not expanded in vitro with peptide α_{146–162} or IL-2 and instead sorted directly for CD4\(^+\) T cells. Proliferation of AChR specific CD4\(^+\) T cell was assessed by measuring [\(^{3}H\)] incorporation with a Beckman Coulter beta scintillation counter. Cells supernatants were analyzed for IFN-γ by ELISA.

**Dendritic cell migration**

In situ CFSE labeling was done as described recently (38). Briefly, CFSE (Molecular Probes) was dissolved at 25 mM, diluted in Iscove’s media to 0.0625 μM and 0.25 μM. Cat S was inhibited by 10 nM of the inhibitor and LPS (10 μg/ml). Cells were seeded in triplicates with AChR plated at 1:40 and 1:10 of T cell to APC ratios and stimulated in vitro with different concentrations of AChR (0, 0.062, 0.25, and 1 μg/ml). AChR immunodominant peptide α_{146–162} was used at 20 μg/ml. Alternatively, Ag presentation was also performed on lymph node cells from AChR immunized mice that were not expanded in vitro with peptide α_{146–162} or IL-2 and instead sorted directly for CD4\(^+\) T cells. Proliferation of AChR specific CD4\(^+\) T cell was assessed by measuring [\(^{3}H\)] incorporation with a Beckman Coulter beta scintillation counter. Cells supernatants were analyzed for IFN-γ by ELISA.
Results

AChR-immunized Cat S\(^{-/-}\) mice are resistant to the development of clinical EAMG

To evaluate the role of Cat S in the development of EAMG, Cat S\(^{-/-}\) and wild-type mice were immunized with T-AChR in CFA on day 0 and boosted with AChR in CFA on day 30 and 60 in two independent experiments. In the first experiment, 9 of 12 (75\%) wild-type mice, compared with 2 of 12 (17\%) Cat S\(^{-/-}\) mice developed clinical EAMG. The onset of EAMG in the wild-type mice was around day 22 which progressively increased during the evaluation. Therefore, Cat S\(^{-/-}\) mice were remarkably less susceptible to development of EAMG (Fig. 1). In the second experiment, 1 of 10 (10\%) Cat S\(^{-/-}\) mice and 8 of 10 (80\%) wild-type mice developed clinical EAMG. In both the experiments, Cat S\(^{-/-}\) mice had a delayed onset, lower total incidence, and less severe clinical EAMG (\(p < 0.005\)) compared with wild-type mice. The data demonstrate the first direct genetic evidence for the involvement of Cat S in the development of clinical EAMG.

The primary pathology in EAMG is a significant reduction of muscle AChR due to Ab and complement-mediated attack of the neuromuscular junction (1). The numbers of \(\alpha\)-BT binding sites in the muscles, which reflect the amount of functionally available muscle AChR, were measured in AChR-immunized wild-type and Cat S\(^{-/-}\) mice. The functional AChR in AChR-immunized Cat S\(^{-/-}\) mice was significantly higher (\(p < 0.05\)) than that of wild-type mice (Fig. 2A). Therefore, the lower incidence of EAMG in Cat S\(^{-/-}\) mice correlated with higher available functional muscle AChR. Control group wild-type and Cat S\(^{-/-}\) mice immunized with PBS in CFA had similar amounts of muscle AChR (Fig. 2A). This indicates that Cat S does not play a role in AChR expression and high AChR availability correlates with significantly reduced pathogenesis of EAMG in the Cat S null mice.

Reduced serum anti-AChR Ab level in AChR-immunized Cat S\(^{-/-}\) mice

To examine the effect of Cat S on anti-AChR Ab responses, wild-type and Cat S\(^{-/-}\) mice were immunized with AChR. Sera from individual mice were collected on days 14, 45, and 90 and evaluated for anti-mouse AChR Ab concentration by an \(\alpha\)-BT radioimmunoassay. Compared with AChR-immunized wild-type mice, AChR-immunized Cat S\(^{-/-}\) mice had significantly lower concentrations of serum anti-mouse AChR Ab (Fig. 2B). Two independent sets of experiments showed similar suppression of anti-AChR Abs in Cat S\(^{-/-}\) mice. No anti-AChR Abs were detected in PBS/CFA immunized Cat S\(^{-/-}\) and wild-type mice (Fig. 2B).
Serum anti-AChR IgM and IgG subclasses were analyzed by ELISA. After the first boost with AChR at day 30 a significant number of wild-type mice developed clinical EAMG. The anti-AChR Abs belonging to the IgM and IgG subclasses IgG1, IgG2b, IgG2c, were markedly reduced in T-AChR-immunized Cat S⁻/⁻ mice compared with T-AChR-immunized wild-type mice (Fig. 3). Similar suppression of anti-AChR IgM and IgG subclasses was observed in the second experiment. The Cat S deficiency led to defective anti-AChR IgM and IgG subclasses production. This could be due to a defect in T cell help provided for effective B cell development and Ab production, leading to the resistance to clinical EAMG.

Reduced B cell expansion in AChR immunized Cat S⁻/⁻ mice

B cell expansion was evaluated by analyzing the expression of cell surface markers MHC class II (I-Aβ), CD3, CD4, CD8, CD19, and CD40 in Cat S⁻/⁻ and wild-type mice axillary and inguinal lymph node cells collected 7 days after a single immunization with T-AChR and at termination of the long-term experiment, (90 days after the first immunization with T-AChR in CFA). Increased numbers of lymph node cells were observed in both day 7 and 90 following AChR immunization (Fig. 4A and B). Also at these time points CD19, CD40, and IAb positive subsets were lower in Cat S⁻/⁻ mice compared with wild-type mice (Fig. 4, C and D) indicating that Cat S is required for B cell expansion in AChR-immunized mice. The expanded B cell population (CD19) in wild-type mice following AChR immunization is presumably the anti-AChR IgG Ab-producing population of B cells. The reduction in CD19 expressing B cells could account for the reduction in the expression of CD40 and IAb molecules and the overall reduction in the lymph node cell counts.

AChR-immunized Cat S⁻/⁻ mice have reduced cellular immune responses to AChR and its dominant peptide α₁₄₆–₁₆₂

To examine whether the reduced humoral responses in T-AChR immunized Cat S⁻/⁻ mice were due to defective cellular responses in these mice we explored the proliferative and cytokine responses in wild-type and Cat S⁻/⁻ mice immunized with T-AChR. Lymph node cells from T-AChR immunized Cat S⁻/⁻ and wild-type mice were stimulated in vitro with T-AChR and α₁₄₆–₁₆₂ peptide. At conclusion of the 7 days (Fig. 5A) and 90 days (Fig. 5B) experiments, Cat S⁻/⁻ mice had a reduced proliferative response to T-AChR and α₁₄₆–₁₆₂ peptide, compared with wild-type mice. In vitro stimulation with AChR and α₁₄₆–₁₆₂ peptide had significantly suppressed production of IFN-γ, IL-2, and IL-10 in Cat S⁻/⁻ mice compared with wild-type mice (Fig. 5, A and B). The data suggest that the early and established lymphocyte responses to T-AChR were suppressed in Cat S⁻/⁻ mice. Further, the data implicate Cat S having an important role in T-AChR-specific IFN-γ, IL-2, and IL-10 production. IFN-γ and IL-10 contribute to EAMG pathogenesis (39–41), therefore, reduction in the anti-AChR Ab response in Cat S⁻/⁻ mice could be due to suppressed AChR specific IFN γ, IL-2, and IL-10 production by AChR immune lymph node cells.

To investigate whether the suppression of proliferative response in Cat S⁻/⁻ mice could be due to defective Ag processing, we also immunized Cat S⁻/⁻ and wild-type mice with the dominant peptide α₁₄₆–₁₆₂ in CFA (Fig. 5A). Seven days later lymph node cells were stimulated in vitro with T-AChR and α₁₄₆–₁₆₂ peptide. Compared with wild-type mice, the α₁₄₆–₁₆₂ peptide-specific lymphocyte proliferation and production of IFN-γ, IL-10, and IL-2 were all significantly suppressed in peptide α₁₄₆–₁₆₂ immunized Cat S⁻/⁻ mice. Therefore, the suppression of AChR and α₁₄₆–₁₆₂ Peptide-specific responses in Cat S⁻/⁻ mice is not due to defective processing of AChR protein because α₁₄₆–₁₆₂ peptide itself is not effectively presented to α₁₄₆–₁₆₂ peptide immune T cells of Cat S⁻/⁻ mice. Of note, Ab production sufficient to create clinical EAMG does not develop in mice immunized multiple times with α₁₄₆–₁₆₂ peptide plus CFA alone (42). This observation is likely in part related to absence of conformational B cell epitopes in α₁₄₆–₁₆₂ peptide needed for activation of pathogenic B cells (pathogenic anti-AChR Ab-producing B cells). Lymph node cells from control

**FIGURE 3.** Defective secondary anti-AChR IgM and IgG subclasses (IgG1, IgG2b, and IgG2c) Ab in Cat S⁻/⁻ mice. Affinity-purified mouse AChR 100 μl (1 μg/ml) was coated on ELISA plates. Sera dilution for IgM was 1/400, and for IgG, IgG2b, IgG2c, and IgG1 1/3000. The OD value from immunized mouse serum was subtracted from preimmunized serum value (OD). Cat S⁻/⁻ (n = 10) and wild-type (n = 10) are shown; *p < 0.05, **p < 0.01, ***p < 0.005, and ****p < 0.0001 (Student’s t test). Representative of two separate experiments.
Diminished lymph node B cell expansion in AChR-immunized Cat S−/− mice. Inguinal and axillary lymph nodes from AChR in CFA and PBS in CFA immunized mice were harvested on day 7 (A) (n = 3–5 of each Cat S−/− and wild-type mice), and day 90 (B) (n = 10 of each Cat S−/− and wild-type mice), and single cell suspension were counted for live cells by trypan blue staining. Expansion of B cells was analyzed by levels of B cell surface marker expression in AChR-immunized Cat S−/− mice and wild-type mice. The lymph node cells obtained after day 7 (C) and day 90 (D) post-AChR immunizations were analyzed with fluorescence-conjugated, B cell marker-specific mAbs and T cell-specific mAbs. The number of positive cells were quantitated by flow cytometry.

Cat S inhibition suppresses C57BL/6 and HLA-DR3 class II-restricted presentation of Torpedo or human AChR processed peptides to T cells

Myasthenia gravis is associated with HLA-DR3 polymorphism (44). HLA-DR3 transgenic mice, which express only the human HLA class II gene, are susceptible to clinical EAMG following immunizations with human AChR in CFA (14). We wanted to examine the effect on cellular immune responses by ex vivo inhibition of Cat S in human AChR immunized HLA-DR3 transgenic mice. We addressed this question by immunizing C57BL/6 and HLA-DR3 transgenic mice with T-AChR or human AChR in CFA, respectively. On day 7, lymph node cells were cultured in the presence of Cat S-specific inhibitor, LHVS, restimulated in vitro with PBS, T-AChR, human AChR, or their immunodominant peptide α146−162 (for T-AChR) or peptide α320−337 (for human AChR). The proliferation and cytokine (IFN-γ, IL-10, and IL-2) responses to T-AChR and human AChR, but not to α146−162 and α320−337 peptide, were significantly suppressed in presence of 10 nM LHVS indicating that specific inhibition of Cat S blocks the loading of human AChR in HLA-DR3 mice, or T-AChR-derived
peptides in C57BL/6 mice, onto newly synthesized class II molecules, thereby causing a severe defect in immune responses (Fig. 8). The 10 nM LHVS concentration used in this study was found to be optimal based on two preliminary experiments that tested doses of LHVS ranging from 5 to 80 nM. At the 10 nM concentration, active site labeling of all endosomal cysteine proteases indicates only Cat S is inhibited (20).

Discussion

Abs to AChR sufficient to cause myasthenia gravis, both clinically and experimentally as done in this report, require expansion of a B cell population capable of autoantibody production. Expansion of these B cells in turn requires MHC class II display of AChR peptides to initiate TCR signaling and cytokine elaboration, which synergizes with B cell receptor signals to promote B cell proliferation, Ig affinity maturation, and depending on the nature of the cytokine being elaborated by T cells, Ig isotype switching. There is no structural relationship between recognition motifs of the B cell receptor and the TCR, their immunodominant epitopes on AChR being mapped to different regions (8–10, 14). The data reported in this study indicate many key elements of the autoimmune response are defective in Cat S deficient mice; despite repeated immunization of Cat S null mice with AChR protein, there was little B cell expansion and markedly reduced levels of the key Ig isotype in EAMG, IgG2b (Figs. 3 and 4). These defects were accompanied by very weak in vitro T cell proliferative responses to either whole AChR protein or AChR peptides, indicating the supporting T cell repertoire in Cat S null mice never fully developed (Fig. 5, A and B). Accordingly, the Cat S null mice had almost complete attenuation of clinical EAMG (Fig. 1).

What is the mechanism(s) underlying defective AChR immune response in Cat S null mice? We considered several possibilities related to the fact that Cat S is primarily expressed in APC and not in T cells. The basic ability of DCs to migrate in vivo and to mature in response to LPS stimulation, as judged by the appearance of CD40 and other activation Ags on DCs, was not different between wild-type and Cat S null mice. Instead, Ag presentation was impaired. Purified splenocyte APC from Cat S null mice demonstrated impaired capacity to stimulate highly primed wild-type CD4+ T cells in vitro with AChR protein (Fig. 6). Proteolysis participates in generation of MHC class II-peptide complexes at two critical junctures. Proteolytic degradation of the Ii is important for efficient peptide binding to laa class II molecules, as intact αβIi trimers themselves are unable to bind peptides (45). However,
MHC class II molecules in Cat S null mice ultimately acquire peptides, with ~50% of surface class II molecules peptide loaded (23). This is sufficient for the apparently normal development of the immune system in Cat S null mice. In addition, proteolysis of large polypeptides within the endosomal compartment is required to generate the peptide Ag presented by class II molecules (24, 25). But there are several endoproteases in the endosomal compartment of B cells that could degrade internalized Ag. Our data favor independent restriction of peptide loading, and not Ag processing, as the key mechanism underlying the immune defects reported in this study. Compared with wild-type mice, Cat S null mice immunized with the T-AChR immunodominant peptide α146–162, which does not require further proteolytic processing for presentation, failed to develop normal T cell proliferative or cytokine (IL-2, IFN-γ) responses in response to Ag recall in vitro (Fig. 5A), indicating impaired capacity to present a key AChR peptide in the presence of accumulated Iip10-MHC class II complexes.

The strong dependence of the immune response to AChR on Cat S could be in part related to the Th1 nature of this autoimmune model. IFN-γ and IL-10 are required for anti-AChR Ab production in EAMG, especially for development of the critical complement binding Th1 isotype Ab, IgG2b (2, 11, 39–41). The requirement for IFN-γ and development of isotype switching to IgG2b may be related to Cat S in at least two ways. First, the presence of IFN-γ directly suppresses cathespin L expression and activity (46). As cathespin L is a second major li-degrading endosomal protease, expressed mainly in macrophages and nonprofessional APCs, IFN-γ production minimizes this alternative route of li degradation and MHC class II peptide display. Early Th1 polarization of the
immune response by the CFA adjuvant in C57BL/6 mice may lead to more complete dependence on Cat S for Ag presentation. Secondly, differentiation of naive T cells to IFN-γ-producing Th1 effector T cells appears to require higher strength of TCR signaling than Th2 differentiation (47). Because the density of antigenic peptide display in Cat S null C57BL/6 mice could be expected to be reduced, the resulting lower TCR signals may not support normal Th1 differentiation. Both of these mechanisms may explain prior findings that IgE responses and Th2 development are normal in OVA/alum immunized C57BL/6 or HLA-DR3 transgenic mice respectively were stimulated in vitro with PBS, T-AChR (0.5 μg/ml), α146–162 (for T-AChR) or α320–337 (for H-AChR) peptide (40 μg/ml) in the presence of PBS, 0.1% DMSO and 10 nM LHVS (Cat S inhibitor). Proliferation and cytokines following restimulation were measured as described in the text. Proliferation was assessed by measuring [3H] incorporation. Lymph node cell culture supernatants collected at 72 h following re-stimulation were examined by ELISA for IFN-γ, IL-2, and IL-10 content, respectively. Both C57BL/6 and HLA-DR3 mice showed a consistent 50% reduction of proliferative responses in presence of Cat S inhibitor, LHVS. The magnitude of defect was more marked for IFN-γ, IL-2, and IL-10 in the DR3 transgenic mice as well as C57BL/6. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 (in Student’s t test).

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