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*J Immunol* published online 30 April 2010
http://www.jimmunol.org/content/early/2010/04/30/jimmunol.0901618

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Maintenance of Immune Tolerance to a Neo-Self Acetylcholine Receptor Antigen with Aging: Implications for Late-Onset Autoimmunity

Sue Stacy,*† Earlanda L. Williams,* Nathan E. Standifer,‡,¹ Amanda Pasquali,* Keith A. Krolick,§ Anthony J. Infante,‡ and Ellen Kraig*†

Age-related changes in immune regulation are likely to account for the age-associated increase in serum autoantibody levels and in certain autoimmune disorders, such as myasthenia gravis (MG). To demonstrate directly a loss of immune tolerance in older individuals, responses to the acetylcholine receptor, the autoimmune target in MG, were assessed in transgenic mice expressing the *Torpedo californica* acetylcholine receptor (TACHR) ω-chain as a neo-self Ag. T cells from young transgenic mice had been shown to be tolerant to p146–162, the TACHR ω-chain peptide that dominated young nontransgenic T cell responses in vitro. The immunodominance of p146–162 was not lost with age; fine specificity was preserved. Moreover, T cell tolerance to p146–162, as well as to other epitopes of the TACHR ω-chain extracellular domain, was maintained in old transgenic mice. Even multiple TACHR immunizations coupled with the MG-enhancing cytokine, IL-12, did not break tolerance. In addition, T cells exhibiting CD4 upregulation, an early activation marker, were reduced in frequency equivalently in old and young transgenic animals, suggesting that immune regulation in this model was not impacted by aging. Moreover, B cell tolerance was also maintained with age. The persistence of immune tolerance was accompanied by an increase in the proportion of T regulatory cells; it is speculated that this may compensate for deficiencies in central tolerance that occur owing to thymic involution. In summary, our study reveals, for the first time, that some immune tolerance mechanisms do survive aging; this suggests that certain late-onset autoimmune disorders may be induced by a specific insult that disrupts immune homeostasis.

The *Journal of Immunology*, 2010, 184: 000–000.

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*Department of Cellular and Structural Biology, †Department of Pediatrics, ‡Department of Microbiology and Immunology, and †The Barshop Institute for Longevity and Aging Studies, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229

Current address: Amgen, Inc., Seattle, WA.

Received for publication May 22, 2009. Accepted for publication April 1, 2010.

This work was supported in part by an Executive Research Committee New Investigator award from The University of Texas Health Science Center at San Antonio (to S.S.); National Institute on Aging Training Grant T32AG021890; National Institutes of Health Grants R03 AG 14557 (to E.K.), R36 AG 029667 (to E.W.), and R03 AG 22675 (to S.S.); the Autoimmune Prevention Center (National Institute of Allergy and Infectious Diseases); the Semp Russ Foundation of the San Antonio Area Foundation; and the Nathan Shock Aging Center. The FACS facility is supported in part by the San Antonio Cancer Institute Cancer Center Support Grant P30 CA54174.

Address correspondence and reprint requests to Dr. Sue Stacy, Cellular and Structural Biology MC #7762, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900. E-mail address: stacy@uthscsa.edu

Abbreviations used in this paper: AChR, acetylcholine receptor; MG, myasthenia gravis; TACHR, *Torpedo californica* acetylcholine receptor; TAChR, Torpedo ω-chain acetylcholine receptor; Talpha, rTAChR ω-chain; Treg, regulatory T cell.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901618
cytokine IL-12 (21). Under these conditions, ~50% of young mice will develop symptoms of MG after the second or third immunization. However, this model has two serious limitations for studies of aging and tolerance. First, immunization of old mice does not result in MG, owing to the relatively low titers of Abs produced to the immunogen, TACHR; this limitation can be overcome by immunizing the animals when they are young and then recalling this memory response when they are old (22). The second limitation to this rodent model is that T cell tolerance cannot be assessed. Specifically, when C57BL/6 mice are immunized with TACHR, the T cell response is largely restricted to an immunodominant epitope, p146–162 of the TACHR α-chain. Because the homologous mouse α-chain lacks a critical lysine residue that is required for TCR binding to Torpedo p146–162, the responding TACHR-reactive T cells do not recognize “self” (mouse AChR) and would therefore not have been susceptible to tolerance (23, 24). To circumvent this limitation to the classical mouse MG model, we generated a transgenic mouse in which the TACHR α-chain is expressed as a neo-self Ag; transgene expression shows the appropriate physiological profile of muscle > brain > thymus (21). As expected, expression of TACHR α as a “self” protein did result in T cell tolerance (21). Moreover, when the transgenic mice were immunized with rTACHR-α-chain (Talpha) (aa 1–210) alone to eliminate by-stander help, B cell tolerance could also be demonstrated (21). Therefore, this transgenic model will allow us, for the first time, to examine the effects of aging on immune tolerance to this important autoantigen.

Materials and Methods

Ags

The three different immunogens used have previously been described, as follows. The intact TACHR was purified from the electric ray, T. californica (Pacific Biobrane, Venice, CA) by affinity chromatography on cobra-toxin conjugated to Sepharose 4B, as detailed previously (25). The AChR α-chain peptides were synthesized by the protein core facility of The University of Texas Health Science Center at San Antonio or by AnaSpec (San Jose, CA) from sequences previously described (23, 26, 27); they included Torpedo α-chain peptides, p111–126, p146–162, p182–198, and p360–378 and human α61–76 (negative control peptide). The Talpha (aa 1–210) was generated, purified, and refolded as previously described (21).

Mice

C57BL/6 mice were obtained from the National Institutes of Aging colony (Harlan Sprague Dawley, Indianapolis, IN; and Charles River, Stone Ridge, NY). The TACHR α transgenic animals (21) were bred and aged in our facility. All mice were housed in a specific pathogen-free animal facility and used as described in protocols approved by our Institutional Animal Care and Use Committee.

Immunizations

For T cell proliferation assays, mice were immunized s.c. at the base of the tail with 50 μl emulsion containing 25 μg TACHR or 3 μg p146–162 and 25 μl CFA (CFA, H37Raa; Difco Laboratories, Detroit, MI). To attempt to break tolerance, mice were immunized s.c. at five sites (above each shoulder, above each thigh, and at the base of the tail) with a total of 100 μl emulsion, containing 50 μg TACHR and 50 μl CFA. The immunizations were repeated twice at monthly intervals. IL-12 (Genetics Institute, Cambridge, MA) in 100 μl PBS with 1% normal mouse serum was given i.p. where indicated; the cytokine was administered for 5 consecutive days along with the primary and secondary TACHR immunizations, as described above. The dosage was 0.5 μg IL-12 for days 1 and 2 and 0.25 μg IL-12 on each of the next 3 d for the primary immunization and 0.25 μg/d for the 5 d prior to the secondary inoculation. To assess B and T cell responses to the α-chain, mice were immunized s.c. at five sites (as described above) with a total of 100 μl emulsion containing 10 μg Talpha (aa 1–210) and 50 μl CFA or, as a negative control, with 100 μl PBS/CFA. Secondary and tertiary immunizations were given at monthly intervals, using IFA as adjuvant.

Lymph node stimulation and proliferation

T cell proliferation was assayed in vitro as described previously (21). Briefly, draining lymph nodes were removed from mice 7 d after primary TACHR immunization or 7 d after tertiary TACHR α-chain immunization. Single-cell suspensions were prepared by mechanical disruption in RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 50 μM 2-ME, 50 μg/ml streptomycin, and 50 U/ml penicillin. The lymph node cells (2.5 × 10⁶ cells/0.2ml/well) were cultured in flat-bottom 96-well plates with indicated concentrations of individual synthetic peptides derived from the TACHR α-chain, the intact TACHR, or the recombinant T α-chain fragment (aa 1–210). Negative control wells received either no stimulus or a nonstimulating peptide, AChRα61–76, as indicated in individual figures. In the experiment showing the recombinant peptide, 2 μg polyclonal B was added to eliminate any LPS effects. As additional controls, cells were stimulated with either anti-CD3 (Ab supernatant from clone 145-2C11) to assess general T cell reactivity or heat-killed Mycobacterium tuberculosis (H37RA; Difco Laboratories), a CFA component. The plates were incubated for 96 h, pulsed with [3H]Thymidine (ICN Biomedicals, Irvine, CA) per well, and harvested 18 h later. [3H]Thymidine uptake was measured by liquid scintillation spectrometry, and the mean counts per minute of duplicate wells (or triplicate wells, when cell numbers were sufficient) was calculated. Results were expressed as average counts per minute ± SEM. with background subtracted. For Figs. 3B and 4, statistical differences in treatment groups were assessed by higher order ANOVA (p < 0.05), and the significance of differences was determined using a Tukey multiple range test. Prior to analysis, the data were squared to remove the confound of negative values and a log base ten transformation performed to satisfy the assumption of equality of variances (Mininab software).

Immunofluorescent staining and flow cytometry analysis

Lymph node cells were cultured in complete media (2.5 × 10⁶ cells/ml/well in a 12-well plate) and stimulated for 96 h with 20 nM p146–162 or p61–76, as previously described (28). Cells were washed and stained extracellularly with fluorescently labeled anti-CD4 (clone RM4-5), anti-CD25, 2 μg PC61, eBioscience, San Diego, CA) and anti-Vβ6 (clone RR4-7, BD Pharmingen, San Diego, CA). After washing, the cells were fixed, permeabilized, stained with either anti-Foxp3 (clone FJK-16s) or an isotype-matched control Ab (IgG2a) conjugated to PerCP-Cy5.5 (eBioscience). Multicolor flow cytometry analysis was performed on a BD Biosciences (San Jose, CA) FACSCalibur (The University of Texas Health Science Center at San Antonio Core Facility). The CD4<sup>ab</sup> gate was separated from CD4<sup>iso</sup> gate based upon the pattern of CD4 expression following in vitro culture with a nonstimulatory control peptide (p61–76). FACS data were analyzed using a higher order ANOVA protocol, as described above.

Measuring anti-TACHR Ab titers

Young and old mice were immunized twice (as described above) with Talpha (aa 1–210) and anti-Talpah Ab titers measured by ELISA. In short, 96-well plates (Nunc Maxisorp, Thermo Fisher Scientific, Rochester, NY) were coated with 50 μl/well of Talpha (aa 1–210) (20 μg/ml in PBS) and stored overnight at 4°C. The next day, after washing wells with 0.05% Tween 20 in PBS, the plates were blocked with 1% BSA/PBS, 200 μl per well, for 1 h at room temperature and washed again. Mouse serum diluted in 1% BSA/PBS was added in 5-fold dilutions from 1/25–1/78125 and tested in duplicate. The 1% BSA/PBS diluent also served as a background control. After incubation for 2 h at room temperature and washing, 50 μl/well of rabbit anti-mouse IgG (whole molecule) conjugated to HRP (Sigma-Aldrich, St. Louis, MO) was added and plates were incubated for 1 h. To develop color, 100 μl ABTS with 0.03% hydrogen peroxide was added per well and absorbance read after 15–30 min, using a Dynatech MRX ELISA plate reader (Dynatech Labs, Chantilly, VA). To determine the titer endpoints, the results were fitted to a linear absorbance measurement. The fourth order curve (GraphPad Prism 5), and the reciprocal dilution of serum required to obtain a 0.1 OD above background was determined by interpolation. For statistical analysis, the assumption of variance equivalence among transgenic and nontransgenic groups was satisfied by a log base ten transformation, and a one-way ANOVA (p ≥ 0.05) was performed on the transformed data for each age group.

Results

Maintenance of the fine specificity of the T cell response to TACHR with aging

The T cell response to TACHR in young C57BL/6 mice is dominated by a single epitope, p146–162, of the TACHR α-chain (23,
Effects of age on the fine specificity of the T cell response to TACHR. Female C57BL/6 mice 2, 12, and 20 mo of age were immunized with TACHR/CFA, and lymph nodes were extracted 7 d later. Single-cell suspensions were stimulated with individual α-chain peptides (p146–162, p111–126, p182–198, and p360–378). A peptide concentration toward the upper limit of the linear range for the p146–162 T cell responses (∼142 nM) was chosen to enhance evidence of epitope spreading. Proliferation was measured by [3H]thymidine incorporation and expressed as the mean cpm with peptide minus the mean cpm without peptide. The experiment was repeated twice and the results summarized graphically.

FIGURE 1.

Maintenance of T cell tolerance to the α-chain p146–162 with aging

Expression of the TACHR α transgene had been shown previously to result in tolerance of T cells specific for the immunodominant α-chain peptide, p146–162, in young mice (21). To assess whether tolerance in this model would be lost with increasing age, young (6 mo) and middle-aged (12–13 mo) mice were immunized with TACHR/CFA. The draining lymph node T cells were assayed for the ability to proliferate in vitro in response to the immunodominant peptide, p146–162. As expected, the T cells from the young TACHR α transgenic mice were tolerant, showing significantly lower responses to p146–162, compared with those from the nontransgenic animal (Fig. 2A). By middle age, no evidence was seen that the nontransgenic T cell response to the p146–162 α-chain peptide was waning or that the tolerance observed in T cell responses from transgenic mice was diminished (Fig. 2B). In addition, T cells from the transgenic and nontransgenic mice responded well to positive control stimuli, anti-CD3 Ab and M. tuberculosis, and to the intact TACHR (Fig. 2B). The response to TACHR in T cells from transgenic mice was possible because, even though the transgenic mice express the TACHR α-chain and would be tolerant to this polypeptide, they do not express the TACHR β, δ, or γ subunits. T cells from transgenic animals could therefore proliferate to intact TACHR, although reactivity would potentially be lower than that of nontransgenic responders, owing to lack of α-chain–specific T cells. Overall, the responses to positive control and p146–162 stimuli reveal that both T cell immunity and T cell regulation are maintained in middle-aged animals. It is notable that loss of humoral immunity to the TACHR had been seen in 10 mo-old mice (22). It could be that changes in T cell responses to this Ag occur at a later age, lagging behind B cell alterations.

Because it was possible that dysfunction of regulation would be seen with increasing age, tolerance was examined in mice of “early” old age (18–19 mo of age) and in mice that were truly old (23–33 mo of age). Draining lymph node T cells from TACHR-immunized mice were assessed for proliferation responses, as described above. Because it was critical to differentiate tolerance from lack of reactivity due to immunosenescence, based upon in vitro reactivity to the TACHR control, each old animal was judged as “responsive” with >2000 cpm in vitro (indicated with gray bars) or “minimally responsive” with <2000 cpm (lightly striped bars), as shown in Fig. 3A. In addition, in three mice, in vivo immunization elicited a low response, and few draining lymph node cells could be obtained; these animals were categorized as “senescent” (white bars in Fig. 3A). Lack of immune responsiveness was seen most markedly in the 23– to 33-mo-old age group, as expected. Nevertheless, mice with T cell responsiveness to TACHR were present in both of the old age groups, so the effects of age on tolerance in these mice could be addressed. T cells from the old “responsive” mice (Fig. 3A) were analyzed for responses to the α-chain p146–162 peptide, and the results for individual animals are shown graphically (indicated by the filled symbols in Fig. 3B). Proliferation readouts from young responders, tested in parallel with the old, are also shown. As expected, the T cell populations derived from young transgenic mice showed evidence of tolerance (lower proliferation, Fig. 3B). However, somewhat surprisingly, tolerance was also observed in the lymph node T cell populations from older (18–30 mo) transgenic mice. That is, the T cell responses from the old transgenic animals were, for the most part, lower than in age-matched nontransgenic
controls. Thus, tolerance to the α-chain p146–162 peptide was preserved with aging.

In an additional experiment, we asked whether tolerance to the AChR in old mice could easily be broken. A more stringent immunization protocol (used to induce MG in young animals) was applied. One nontransgenic and two transgenic mice (24 mo old) were given three immunizations with TAChR/CFA, 1 mo apart, and were further stimulated with the MG-enhancing cytokine IL-12 (21). When in vitro lymph node T cell responses to p146–162 were assessed, tolerance was well maintained in the transgenic animals (open square symbols in Fig. 3B). The lack of responsiveness was not due to immunosenescence because parallel in vitro stimulation with 1.9 nM TAChR invoked a vigorous response in T cells from the transgenic mice (data not shown). This suggests that tolerance to the immunodominant α-chain peptide is maintained in old animals even under conditions designed to promote a strong immune response.

**Maintenance of T cell tolerance to other α-chain epitopes with aging**

Despite the maintenance of tolerance to the p146–162 peptide in old transgenic mice (Fig. 3B), it was still possible that tolerance to other α-chain epitopes could be altered by aging. To address this possibility, young and old, transgenic and nontransgenic, mice were immunized with Talpha (aa 1–210) protein. The immunization was repeated twice 1 mo apart, allowing for further expansion of T cell clones directed against epitopes of the α-chain. Lymph node T cells were harvested 7 d after tertiary immunization, and the cells were stimulated in vitro with Talpha, the α-chain peptide, p146–162, or positive control stimuli (anti-CD3 Ab and M. tuberculosis). T cells from most of the young and old mice were responsive to anti-CD3 stimulation and to treatment with the M. tuberculosis-positive control, a component of the CFA used in the primary Talpha immunizations (data not shown). Mice that were unresponsive to M. tuberculosis (<5000 cpm) or that lacked sufficient draining lymph node cells to test for control responses were not included in this analysis. T cells from all other mice were stimulated with the rTAChR α-chain fragment or the immunodominant epitope, and proliferation was assessed. As shown in Fig. 4, T cells derived from nontransgenic mice, both young and old, were generally responsive when restimulated in vitro with either the Talpha immunogen (A) or p146–162 (B) following the tertiary immunization. In contrast, T cells from transgenic animals showed tolerance, as expected. The responses from old transgenic mice were essentially equivalent to the low levels of proliferation seen with younger transgenic individuals; this was true whether the in vitro stimulus was the α-chain fragment or the peptide. Taken together, these data suggest that T cell tolerance to other α-chain epitopes, as well as to p146–162, is maintained with age.

**Reduced Ag-specific T cell clonal expansion is maintained with age**

We next investigated potential mechanisms responsible for maintaining tolerance in old mice. T cell upregulation of CD4 is an early event following TCR activation, and it has been shown by us (28) and others (32, 33) that the CD4^high^ population contains the majority of Ag-specific T cells following challenge. In young
nontransgenic C57BL/6 mice immunized with p146–162, an expansion of CD4high cells expressing the canonical TCRBV6 gene segment is seen (10% of gated lymphocytes for the young nontransgenic mouse shown in Fig 5A). In contrast, a greatly diminished expansion of CD4high T cells is evident in young transgenic animals (1% in the representative plot, Fig 5A). To ask whether this decreased frequency of activated (Vβ6+CD4high) T cells, consistent with a tolerogenic phenotype, was maintained with age, old nontransgenic and transgenic mice were immunized with p146–162 and the draining lymph node cells expanded in vitro. As illustrated in Fig 5A, old nontransgenic mice were able to upregulate CD4 (5% of lymphocytes), whereas old transgenic animals maintained a clear imprint of tolerance very similar to that seen in young transgenic mice. The somewhat unexpected finding that T cell and B cell tolerance to neo-AChR/CFA immunizations and were also injected with the enhancing cytokine IL-12. The T cell responses to p146–162 for these animals are shown with □ (B).

B cell tolerance to the TACrR α-chain is not lost with age

It has been reported that B cell tolerance is compromised with aging, resulting in higher levels of circulating autoantibodies in the elderly (1). However, this possibility has not been interrogated directly for AChR or for any other muscle autoantigen; it is possible to do so in TACrR α transgenic mice by immunizing with the TACrR α-chain, which is seen as self (21). Toward this end, mice were immunized twice with Talpha (aa 1–210) and the resulting Ab titers were assessed by ELISA. As shown in Fig. 6, immunized young nontransgenic mice have significant titers to the immunogen, as expected. In contrast, young transgenic animals fail to respond to TACrR α immunization, consistent with B cell tolerance. In older mice it was anticipated that Ab titers would be lower, even after two immunizations (22, 34, 35). Nonetheless, detectable Ab responses were seen in ~50% of old nontransgenic mice, whereas older transgenic animals produced negligible levels of anti-TACrR α-chain Ab (Fig 6). Thus, B cell tolerance to neo-self AChR does appear to be maintained with aging.

Treg frequencies are elevated in the old transgenic mice

The somewhat unexpected finding that T cell and B cell tolerance to the muscle autoantigen AChR was not impacted by aging impelled us to examine other immune regulatory mechanisms in old mice. Specifically, it had been reported that the frequency of Tregs (CD4+CD25+Foxp3+) is increased with age in both rodents and humans, although their function is not always maintained (15–18). Thus, we asked whether Tregs in elderly transgenic mice could be responsible for continued tolerance to TACrR α-chain determinants. As expected, in immunized old transgenic mice, >24% of CD4+ lymph node T cells were CD25+Foxp3+ Tregs, compared with ~13% for young transgenic mice (Fig. 7A, 7B). This increase in Tregs with age was very similar to that seen in nontransgenic littermates (Fig. 7B). To determine the effects of aging on Treg levels following Ag-specific expansion, young and old transgenic mice were immunized with the TACrR p146–162, and the draining lymph node T cells were stimulated in vitro with p146–162. The Foxp3+ T cells in this population expressed high levels of CD25, and the Tregs were gated accordingly. The percentages of

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**FIGURE 3.** Effects of increasing age on T cell tolerance to TACrR p146–162. Old mice were immunized once with TACrR, and draining lymph node T cells were stimulated in vitro and [3H]thyridine incorporation measured. In vitro responses to TACrR were judged as “responsive” (>2000 cpm) or “minimally responsive” (<2000 cpm) (A). The TACrR stimulus used (1.9 nM for experiments B and C or 1.2 nM for experiments A and D) was within the linear range for the responses from most old mice. In three old mice, low numbers of draining lymph node cells prohibited complete in vitro analysis; these mice were labeled “senescent” (A). T cell populations from individual “responsive” old mice were used to assess reactivity to the α-chain peptide, p146–162. T cells from young immunized mice were tested in parallel (B). Proliferation to 19 nM p146–162 was graphed for all animals using , , and ; this peptide concentration was within the linear range for responses from nontransgenic mice. The upper 95% confidence limit for the mean of young transgenic responses (dashed line) and the mean value for each experiment group (solid lines) are indicated. Several mice (numbers B61, 140, and 216) had enlarged spleens (>7 mg/g body weight); they were included in A for completeness. In an additional experiment (Old/IL-12 in B), a more strenuous immunization protocol was used, as described in Materials and Methods. Briefly, 24-mo-old mice were given three TACrR/CFA immunizations and were also injected with the enhancing cytokine IL-12. The T cell responses to p146–162 for these animals are shown with (B).

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**TABLE 1.** Increasing immunosenescence in old mice

<table>
<thead>
<tr>
<th>Mouse ID (Exp)</th>
<th>18-19 months</th>
<th>23-33 months</th>
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<tr>
<td></td>
<td>Nontg</td>
<td>Transgenic</td>
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<td>217</td>
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**Response to TACrR**

- Responsive
- Minimally responsive
- Senescent

**FIGURE 4.** Tolerance to p146-162 in immunoresponsive old mice

In vitro: 19 nM 146-162

Young

- 3 - 5 mo

Old

- 18 - 19 mo

- 23 - 30 mo

Old/IL-12

- 24 mo

**TABLE 2.** Tolerance to p146-162 in immunoresponsive old mice

<table>
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<th>T-cell proliferation (%)</th>
<th>Nontg</th>
<th>Transgenic</th>
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<td>[3H]thymidine incorporation</td>
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<td>Young</td>
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<td>Old</td>
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<td>Old/IL-12</td>
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**TABLE 3.** Tolerance to p146-162 in immunoresponsive old mice

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<td>Old/IL-12</td>
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CD25<sup>high</sup> Tregs in the cultures derived from old transgenic mice were still higher on average (9%) than those of the young transgenics (4.4%) (Fig. 7C, 7D). By comparison, the Treg differential between old and young immunized nontransgenic animals was smaller, probably owing to the expansion of Ag-specific cells in the nontransgenics (Fig. 7D). Thus, Treg frequency was elevated in old transgenic mice, and this differential was maintained after exposure to the p146–162 TAChR Ag.

**Discussion**

For the first time, the effects of age on immune tolerance have been examined in a well-defined model, a transgenic mouse in which the TAChR α-chain is expressed primarily in muscle. This model is ideal because it can be used to assess both B cell and T cell tolerance. In addition, the T cell fine specificity of the TAChR response has been mapped to a single determinant, and tolerance to this T cell epitope in the young transgenic model was previously demonstrated (21). However, it was possible that this fine specificity would be altered with age. We reasoned that a reduced frequency of dominantly responding clones due to a decline in receptor diversity (10) might reveal reactivities against other less dominant TAChR epitopes. Alternatively, mechanisms that regulate the fine specificity of T cell responses in young animals might be altered in old ones. For example, epitope spreading, reported in induced T cell tolerance to specific Ags (36) and in the progression of T cell responses (37), could conceivably be increased with age. However, this was not observed in the AChR response; the immunodominance of p146–162 was well maintained with age in TAChR-immunized nontransgenic mice (Fig. 1). This result is in agreement with a study of T cell responses to hen egg lysozyme in 2- and 20-mo-old mice; no change in the fine specificity of the peptide response was seen when measured as the frequency of IFN-γ producing T cells (38). Our results suggest that factors controlling T cell fine specificity of the TAChR response are still intact in 20-mo-old mice and verify the importance of addressing tolerance to p146–162 in old transgenic mice.

**FIGURE 4.** Effects of age on tolerance to other TAChR α-chain epitopes. Young (5 mo of age) and old (23–25 mo of age) mice were immunized s.c. in multiple sites with the Talpha (aa 1–210), as described in Materials and Methods. The immunizations were repeated twice at monthly intervals. The tertiary immunization was given at staggered time points (experiments A, B, and C), and draining lymph nodes were harvested 7 d later. T cells were stimulated with incremental concentrations of Talpha (aa 1–210) or the TAChR peptide, p146–162, and [3H]thymidine incorporation was measured. The responses to 19 nM Talpha (A) or 19 nM p146–162 (B) are shown graphically for individual mice. One old nontransgenic and two old transgenic mice had enlarged spleens (>7 mg/g body weight) but responded to positive controls and thus were included in the tolerance study. Two mice with insufficient draining lymph node cells or with T cells that were not responsive to the M. tuberculosis control were not included; these mice also had enlarged spleens. The upper 95% confidence limit for the mean of young transgenic responses (dashed line) and the mean values for each group (solid line) are indicated.

**FIGURE 5.** Effects of age on CD4 upregulation in p146–162-responsive Vβ6<sup>+</sup>CD4<sup>high</sup> T cells. Young (2–4 mo of age) and old (20–23 mo of age) transgenic and nontransgenic mice were immunized with p146–162; draining lymph nodes were extracted and expanded in vitro with the immunizing peptide. The lymph node cells were stained to detect CD4, CD25, and Vβ6, using Abs conjugated to FITC, APC, and PE, respectively. The cells were analyzed by FACS to determine whether CD4 had been upregulated in the Vβ6<sup>+</sup> population. The experiment was then repeated with an equivalent number of mice of the same age groups. Representative dot plots are shown (A). The frequency of Vβ6<sup>+</sup>CD4<sup>high</sup> T cells within the lymphocyte population is shown for the individual mice from the combined experiments (B). The solid line represents the mean value within each group.
In view of the reported age-associated alterations in T cell development and function (10, 12, 13), the maintenance of T cell tolerance to the AchR in old mice might not have been expected. Nonetheless, tolerance was evidenced by diminished T cell proliferative responses to p146–162 as well as other epitopes of the α-chain extracellular domain and by low Ab titers to the T AchR α-chain. Moreover, another measure of tolerance in young T AchR α-transgenic mice, the reduction in frequency of high-avidity CD4<sup>hi</sup> Ag-specific cells (28), was observed in old transgenic mice as well. Thus, in seeming contradiction to the paradigm that reactions to self-Ags increase with age (1, 39, 40), we found that both T cell and B cell tolerance to the AchR neo-self Ag was maintained.

Given this persistence of tolerance in the old transgenic mice, it appears that mechanisms thought to promote the loss of T cell tolerance with age are not applicable in this model. For example, it has been proposed that thymic involution would compromise mechanisms thought to promote the loss of T cell tolerance to the AchR neo-self Ag was maintained.

Significantly, the effects of age on Treg profiles in healthy humans and mice have been addressed and could have implications for late-onset MG. Although age-related alterations in certain Treg functions were seen in some studies (15, 44), suppressive capabilities were generally maintained (15–18). In addition, the frequencies of Tregs are widely reported to be increased in old individuals (15–18). In support of this finding, we found that the Treg frequency within the CD4<sup>+</sup> T cell population from old unimmunized T AchR α-transgenic mice was significantly elevated over that of young transgenic mice, and this enhanced frequency was also evident in CD4<sup>+</sup> T cells derived from p146–162-immunized lymph node cells. Although it is far from clear whether the increased proportion of Tregs in old transgenic mice helps to maintain tolerance to the T AchR studied in this paper, this is an important area for future investigation.

Our results indicating that tolerance to the neo-self AchR is not broken in the old mice raise a more basic question. In the face of this tolerance maintenance, how is tolerance disturbed in autoimmune diseases in which symptoms are first seen in old age? One possible explanation for loss of tolerance in late-onset MG is that in some individuals, deficiencies in T cell regulation are present at a young age but are not sufficient to result in clinical manifestations of autoimmunity. Thus, autoimmune challenges may be held in check although autoimmune memory T cells are generated. Upon
re-exposure to the self Ag in old age, caused by age-related tissue damage or exposure to mimicking foreign Ags, “recall” autoimmune memory cells established at a young age would respond vigorously and rapidly. T cell tolerance, already weak in these individuals, would be “broken” by the strong memory response. In support of this hypothesis, in B cell studies, Nobrega et al. (45) have demonstrated that although autoantibody levels were higher in old C57BL/6 mice, the autoantigen recognition profile was the same in young and old. Moreover, our own work has shown that autoreactive B cells first established by immunization of young mice could be recalled in old age to give a vigorous Ab response and produce autoimmune MG (22, 46). Thus, similar to autoreactive B cells, it may be that certain self-reactive T cells in the elderly reflect specificities first established in youth.

Acknowledgments

We thank Crystal Davenport and Bruce Gelb for technical help, Irma Gonzales for isolating the AChR from electroplaque tissues, Peggy Rifleman for We thank Crystal Davenport and Bruce Gelb for technical help, Irma Gon- zales for isolating the AChR from electroplaque tissues, Peggy Rifleman for We thank Crystal Davenport and Bruce Gelb for technical help, Irma Gon- zales for isolating the AChR from electroplaque tissues, Peggy Rifleman for We thank Crystal Davenport and Bruce Gelb for technical help, Irma Gon- zales for isolating the AChR from electroplaque tissues, Peggy Rifleman for We thank Crystal Davenport and Bruce Gelb for technical help, Irma Gon- zales for isolating the AChR from electroplaque tissues, Peggy Rifleman for We thank Crystal Davenport and Bruce Gelb for technical help, Irma Gon- zales for isolating the AChR from electroplaque tissues, Peggy Rifleman for We thank Crystal Davenport and Bruce Gelb for technical help, Irma Gon- zales for isolating the AChR from 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