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Elevated Tumor-Associated Antigen Expression Suppresses Variant Peptide Vaccine Responses

Charles B. Kemmler, Eric T. Clambey, Ross M. Kedl, and Jill E. Slansky

Variant peptide vaccines are used clinically to expand T cells that cross-react with tumor-associated Ags (TAA). To investigate the effects of elevated endogenous TAA expression on variant peptide-induced responses, we used the GP70 TAA model. Although young BALB/c mice display T cell tolerance to the TAA GP70\textsubscript{423-431} (AH1), expression of GP70 and suppression of AH1-specific responses increases with age. We hypothesized that as TAA expression increases, the AH1 cross-reactivity of variant peptide-elicited T cell responses diminishes. Controlling for immunosenescence, we showed that elevated GP70 expression suppressed AH1 cross-reactive responses elicited by two AH1 peptide variants. A variant that elicited almost exclusively AH1 cross-reactive T cells in young mice elicited few or no T cells in aging mice with Ab-detectable GP70 expression. In contrast, a variant that elicited a less AH1 cross-reactive T cell response in young mice successfully expanded AH1 cross-reactive T cells in all aging mice tested. However, these T cells bound the AH1/MHC complex with a relatively short half-life and responded poorly to ex vivo stimulation with the AH1 peptide. Variant peptide vaccine responses were also suppressed when AH1 peptide is administered tolerogenically to young mice before vaccination. Analyses of variant-specific precursor T cells from naive mice with Ab-detectable GP70 expression determined that these T cells expressed PD-1 and had downregulated IL-7R to a degree that suggested they were anergic or undergoing deletion. Although variant peptide vaccines were less effective as TAA expression increases, data presented in this article also suggest that complementary immunotherapies may induce the expansion of T cells with functional TAA recognition. The Journal of Immunology, 2011, 187: 4431–4439.
Materials and Methods

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee of National Health. BALB/cByJ mice >11 mo of age were purchased from the National Institute on Aging. Two- to 4-mo-old BALB/cAnNcr mice were purchased from Charles River Laboratories. Similar results were obtained using 2- to 4-mo-old BALB/cByJ mice (data not shown). Mice deficient for the functional locus of endogenous ecotropic MuLV, BALB/cByJ env<sup>-/-</sup> or gp70<sup>-/-</sup> mice, were produced by selective breeding as previously described (19). Mice sufficient for the BALB/c MuLV locus, gp70<sup>-/-</sup> mice, were also selected in these crosses. gp70<sup>-/-</sup> and gp70<sup>-/-/-</sup> mice were further backcrossed to BALB/cAnNcr mice for 22 generations, intercrossed, and screened as previously described (19).

Immunizations

S99 insect cells (Invitrogen) were infected with recombinant baculovirus encoding the indicated peptides: AH1 (SPSYVVYHQF), A5 (SPSYAYHQF), 39 (MNKYAYHML), or ßgalactosidase (ßgal; TPHPARIGL), and cultured as previously described (20). Unless otherwise noted, mice were given two injections of 5 million baculovirus-expressing insect cells separated by 1 wk.

H-2L<sup>d</sup> tetramer staining

R-PE-conjugated, H-2L<sup>d</sup> tetramers were produced as previously described and incubated in >200-fold molar excess of the indicated peptide overnight (6). Blood lymphocytes were isolated using Ficoll Paque PLUS (GE Healthcare). One million splenocytes were incubated at room temperature for 1.5 h with peptide-loaded tetramer, FcR-blocking Ab (clone 2.4.G2), viability-discriminating agent 7-aminoactinomycin D (7-AAD; Sigma), and fluorochrome-conjugated Abs (BioLegend) against CD8 (allophycocyanin-Cy7), CD11a (allophycocyanin), CD4 (PerCP), B220 (PerCP), and I-A<sup>i</sup>-E<sup>h</sup> (PerCP) molecules in PBS containing 2% FBS, 10 mM HEPES buffer, and 0.1% sodium azide (FACS buffer). Cells were analyzed on a FACSCalibur flow cytometer (Beckman Coulter), and data were processed using FlowJo software (Tree Star). The ratio of tet+ (CD8<sup>+</sup>CD11a<sup>+</sup>CD<sub>4<sup>d</sup>B220<sup>-</sup>I-A<sup>i</sup>-E<sup>h</sup>7-AAD<sup>-</sup>) cells to total cells in the forward × side scatter lymphocyte gate was multiplied by the total number of splenocytes to determine the total number of tet<sup>+</sup> cells per spleen.

GP70 staining

Two million splenocytes were incubated in Perm/Wash buffer (BD Pharmingen) with FcR-blocking Ab for 30 min at 4°C. Protein G-purified, GP70-specific Ab (clone 35/299) (23) was added at 25 µg/ml for 1.5 h. Cells were washed twice and stained in Perm/Wash buffer with PE-conjugated Ab specific for rat IgG2a (clone R2a-21B2; E Bioscience) for 45 min. Cells were washed three times and stained in FACS buffer with fluorochrome-conjugated Abs (BioLegend) against B220 (allophycocyanin), CD11c (FITC), CD4 (PerCP), CD8 (allophycocyanin-Cy7), and CD11b (Pacific blue) molecules for 30 min. Cells were analyzed as described earlier.

Intracellular cytokine staining

One million splenocytes were cultured for 5 h with the indicated amount of peptide in the presence of GolgiStop (BD Pharmingen). Cells were surface stained with Abs specific for CD8 (allophycocyanin-Cy7), CD4 (PerCP), B220 (PerCP), and I-A<sup>i</sup>-E<sup>h</sup> (PerCP) molecules in the presence of FcR-blocking Ab. Cells were then stained intracellularly for IFN-γ expression (Cytofix/Cytoperm Plus; BD Pharmingen). The frequency of cytokine-producing cells within the CD8<sup>+</sup> CD4<sup>+</sup> B220<sup>-</sup>I-A<sup>i</sup>-E<sup>h</sup> population was determined. For the analysis of IFN-γ expression within AH1-tet<sup>+</sup> cells, splenocytes were stained with AH1-tet, anti-CD8, and FcR-blocking Ab for 1.5 h in culture medium at room temperature. Cells were washed three times with culture medium and incubated with clone 30.5.7S, an Ld/Lq-specific Ab (24, 25), for 15 min at 4°C. Cells were washed three times with culture medium, stimulated with peptide, stained, and analyzed as described earlier. In analyses, frequencies of AH1-tet<sup>+</sup> cells in samples from ßgal-vaccinated mice were subtracted as background.

Tetramer dissociation assay

Lymphocytes were enriched from homogenized splenocytes using Ficoll-Paque PLUS, stained with AH1-tet as described earlier, and washed twice with FACS buffer. An aliquot of cells was removed as the non-dissociated, zero-time point control and the remaining cells were resuspended at room temperature with 10 µM L<sub>5</sub>-Specific Ab Fab fragment (clone 28.14.8S) (26), containing 2% FBS, 0.1% sodium azide, and 10 mM HEPES buffer. Aliquots of cells were removed at 1, 2, 3, 4, 6, 10, 20, 45, and 90 min and immediately placed in PBS containing 2% paraformaldehyde. Cells were analyzed by flow cytometry. The total fluorescence was determined by multiplying the total number of tet<sup>+</sup> cells by their mean fluorescence intensity and dividing by the total number of CD8<sup>+</sup> T cells in the sample (27). The total fluorescence was normalized to percent of the total fluorescence at the zero time point and converted to the natural logarithm. An exponential decay curve was applied to each data set using Prism software. The half-life of this curve was used as the half-life of tetramer binding, AH1-tet<sup>+</sup> cells from 2-mo-old BALB/c and >11 mo-old BALB/c and gp70<sup>-/-</sup> mice demonstrated similar TCRβ cell surface expression by AB staining (clone H57-597).

Peptide-induced tolerance

AH1 or ßgal peptides (CHI Scientific; 25–95% purity) were solubilized in HBSS at 10, 1, or 0.1 mg/ml. Equal parts peptide and IFA (Sigma) were emulsified. Two-month-old mice received 100 µl i.p. injections on days 0, 3, and 6. The same peptide concentration was used for all three injections of a given mouse. On day 11, mice were given a single immunization as described earlier. Splenocytes were analyzed on day 18.

Identification of H-2L<sup>d</sup> tetramer<sup>+</sup> cells in naive mice

Similar protocols have been used previously to enrich, quantify, and analyze tetramer-specific T cells from naive mice (28–30). The spleen and inguinal, cervical, axillary, brachial, and mesenteric lymph nodes of naive mice were macerated and treated with ACK lysis buffer. Cells were resuspended in 250 µl FACS buffer and 250 µl FcR-blocking Ab hybridoma culture supernatant. HEPES buffer (15 mM final concentration), sodium azide (0.2% final concentration), Ab specific for the CD8 molecule (Pacific blue), A5-loaded R-phycocerythrin tetramer, and allophycocyanin-conjugated tetramer containing the A5 peptide bound via a linker to the B2<sub>2</sub>-microglobulin molecule (7, 31) were added and incubated for 1.5 h at room temperature. Cells were washed in culture medium containing 0.2% sodium azide and incubated for 30 min in 500 µl MACS buffer (PBS containing 0.5% BSA and 2 mM EDTA) containing 0.2% sodium azide and 50 µl PE-specific magnetic beads (Miltenyi). Cells were washed with MACS buffer, and bead<sup>+</sup> cells were enriched using an LS column (Miltenyi). Bead<sup>+</sup> cells were incubated with 7-AAD and Abs (BioLegend) specific for CD4 (PerCP), B220 (PerCP), I-A<sup>i</sup>-E<sup>h</sup> (PerCP), CD127 (IL-7Rα, FITC), and PD-1 (PE-Cy7) molecules. Cells were analyzed by flow cytometry, and the total number of PE-tet<sup>+</sup> allophycocyanin-tet<sup>+</sup> cells was determined for each mouse.

Results

Variant 39, but not variant A5, uniformly induces AH1-specific T cell responses in aging mice

In young BALB/c mice, vaccination with the AH1 tumor Ag elicits few AH1-specific T cells and induces little or no AH1-specific tumor protection (5, 7). However, vaccination of young mice with either of two peptide variants of AH1, A5 or 39, elicits robust AH1 cross-reactive T cell responses and AH1-specific tumor protection (5–7). Although variant A5 deviates from the AH1 epitope by only a valine-to-alanine substitution at position 5, variant 39, identified in a positional scanning library, varies at six of nine residues from the AH1 epitope. The robust induction of AH1 cross-reactive responses by these variants in young mice led us to hypothesize that, despite increased AH1-specific tolerance in aging mice (19), immunizations using these variants may also elicit AH1 cross-reactive responses in aging mice. Therefore, we immunized young and aging mice with variant A5 or 39, or an irrelevant control peptide, ßgal (Fig. 1A, 1B). AH1 cross-reactive T cells were detected using H-2L<sup>d</sup> tetramer loaded with AH1 peptide, AH1-tet (Fig. 1A). Immunization with variant 39 successfully induced AH1-tet<sup>+</sup> responses in young mice and all of the aging mice tested (Fig. 1B). Although variant A5 also induced robust AH1-tet<sup>+</sup> responses in young mice, it failed to uniformly induce AH1-tet<sup>+</sup> responses in the aging mice tested (Fig. 1B).
FIGURE 1. Variant 39, but not variant A5, uniformly induces AH1-specific T cell responses in aging mice. A–D, Two- or >11-mo-old mice were immunized twice, 1 wk apart, with insect cells expressing the indicated peptide (as described in Materials and Methods). A, One week after the second immunization, spleen cells were analyzed by flow cytometry, gating on CD8$^+$ T cells. B, The total number of AH1-tet$^+$ CD11ahi T cells per spleen was determined at 1 (day 14) or 2 (day 21) wk after the second immunization. Each symbol (black circle, gray circle, open circle, or black triangle) denotes mice from a unique experiment. C, Variant A5-immunized mice were bled 5 d after the second immunization, and cells were analyzed by flow cytometry to determine the frequency of CD8$^+$ T cells that bound AH1-tet or A5-tet. Dotted lines connect the AH1-tet$^+$ and A5-tet$^+$ frequency in the same mouse. The graph depicts five >11-mo-old mice. D, Variant 39-immunized mice were analyzed as in C using AH1-tet and 39-tet. E, Two- or >11-mo-old BALB/c and gp70$^-/$ mice were immunized with A5 as described earlier. Mice were bled 5 d after the second immunization and the frequency of AH1-tet$^+$ cells was determined. Each symbol color (black, gray, or open) denotes mice from a unique experiment. *$p$ ≤ 0.05, **$p$ ≤ 0.01, ***$p$ ≤ 0.001, as determined by the Student t test. ns, not significant.

Reduced response to immunization with variant A5 is associated with elevated splenic GP70 expression

Previous reports show that, although GP70 expression is detectable in aging mice, it varies between mice and between organs within individual mice (19, 22). We examined GP70 expression in splenocytes of immunized mice to determine whether increased GP70 expression inversely correlated with the response to immunization with variant A5. Although aging gp70-deficient and young gp70-sufficient splenocytes did not stain with GP70-specific Ab (clone 35/299) (23), positive staining was detectable in some aging gp70-sufficient splenocytes (Fig. 2A, 2B). We refer to these aging mice with Ab-detectable splenic expression of GP70 as GP70$^+$ mice. The majority of splenic GP70$^+$ cells were B220$^+$, although some GP70$^+$ cells stained positive for CD4, CD8, CD11b, and CD11c cell surface markers. Comparing GP70 expression in individual mice with the number of AH1-specific T cells elicited by variant A5 immunization, we found that increased GP70 expression was associated with a failure to respond to A5 immunization (Fig. 2B). These data suggest that elevated GP70 expression suppresses T cell responses to variant A5 immunization.

To determine whether the poor response of aging mice to variant A5 was the result of tolerance to the AH1 Ag or was caused by immuno senescence, we immunized mice deficient for the gp70 locus (19). Although AH1-tet$^+$ responses induced by A5 in aging gp70$^-/$ mice were less robust than those induced in young gp70$^-/$ mice, all of the aging gp70$^-/$ mice tested produced AH1-tet$^+$ responses (Fig. 1E). These data suggest that, although immunosenescence diminishes the A5-elicited response, the lack of a response in some aging BALB/c mice is not entirely due to age and is likely the result of an increased endogenous expression of the AH1 Ag.

Similar results were also observed in variant A5-immunized aging BALB/cAnNCr mice (data not shown).

Because the amino acid sequences of variant 39 and variant A5 differ at five residues, it is likely that the T cell repertoires they stimulate vary (32). Analysis of variant A5-elicited responses with either A5- or AH1-loaded tetramer demonstrated that the majority of vaccine-elicited cells cross-reacted with the AH1 epitope (Fig. 1C). The frequency of A5-tet$^+$ cells in each mouse was similar to the frequency of AH1 cross-reactive (AH1-tet$^+$) cells. As further evidence of this cross-reactivity, A5-tet$^+$ cells were not detected in mice in which an AH1-tet$^+$ response was absent (Fig. 1C). However, analysis of the variant 39-elicited response showed ~50% of the 39-elicited T cell response cross-reacted with AH1-tet$^+$ (Fig. 1D). These data are consistent with previously obtained data derived by costaining splenocytes from variant 39- and A5-immunized mice with AH1- and variant-loaded tetramers conjugated to different fluorophores (7). Collectively, these data demonstrate that the 39-elicited response is less AH1 cross-reactive than the A5-elicited response.

To determine whether the poor response of aging mice to variant A5 was the result of tolerance to the AH1 Ag or was caused by immunosenescence, we immunized mice deficient for the gp70 locus (19). Although AH1-tet$^+$ responses induced by A5 in aging gp70$^-/$ mice were less robust than those induced in young gp70$^-/$ mice, all of the aging gp70$^-/$ mice tested produced AH1-tet$^+$ responses (Fig. 1E). These data suggest that, although immunosenescence diminishes the A5-elicited response, the lack of a response in some aging BALB/c mice is not entirely due to age and is likely the result of an increased endogenous expression of the AH1 Ag.
Similar to observations in the indirect assays, AH1-tet+ cells from young mice produced IFN-γ in response to 39 peptide, but not AH1 peptide. These data suggest that the unresponsiveness of AH1-tet+ T cells elicited by each variant is similar to that observed in aging GP70hi mice (Fig. 1D). These AH1-specific, and these T cells would not provide AH1-specific tumor protection.

In young mice, a fraction of the AH1-tet+ repertoire elicited by variant 39 immunization does not respond to AH1 stimulation

Variant 39 immunization of young mice expands AH1-tet+ T cells that respond to AH1 stimulation (Fig. 3D, 3E) (6, 7). To determine whether this response in young mice also contains T cells that bind AH1-tet, but respond poorly to AH1 peptide stimulation, similar to those observed in GP70hi aging mice, we immunized young mice with variant 39 and monitored IFN-γ production in AH1-tet+ T cells (Fig. 5A, 5B). We compared this response with that elicited by variant A5 immunization. Similar frequencies of AH1 cross-reactive (AH1-tet+) T cells elicited by each variant produced IFN-γ after stimulation with the immunizing variant peptide (Fig. 5B). However, a significantly lower frequency of 39-elicited AH1-tet+ T cells produced IFN-γ in response to AH1 stimulation than A5-elicited AH1-tet+ T cells. These data suggest that variant 39 immunization of young mice elicits AH1-tet+ T cells that fail to functionally respond to the AH1 epitope. In aging GP70hi mice, tolerance may preclude a response from T cells with functional AH1 recognition, leaving only a repertoire of functionally AH1-unresponsive AH1-tet+ cells available to respond to 39 immunization.

Tolerogenic administration of AH1 peptide in young mice suppresses variant A5- and 39-induced AH1 cross-reactive responses

We sought to determine whether our findings in aging GP70hi mice could be recapitated in another model of elevated AH1-specific tolerance. Repeated injections of peptide emulsified in IFA tolerize peptide-specific T cell responses (15, 36, 37). Therefore, we used repeated injections of AH1 peptide in IFA to enhance AH1-specific tolerance in young mice. Five days after the final IFA/AH1 peptide injection, mice were immunized with A5, 39, or βgal peptide. The provision of systemic AH1 peptide was sufficient to suppress variant A5- and 39-induced AH1-specific responses, as detected by AH1-tet staining (Fig. 6A, 6B) and ex vivo IFN-γ production in response to AH1 peptide stimulation (Fig. 6C). Interestingly, at a particular dose of tolerizing AH1 peptide (5 μg injection), the AH1-tet+ response induced by variant 39 immunization was significantly less suppressed than the AH1-tet+ response induced by variant A5 immunization (Fig. 6B). This phenotype is similar to that observed in aging mice (Fig. 1B). These data support a model in which elevated GP70/AH1 expression suppresses variant peptide-induced responses. In addition, AH1 cross-reactive T cells elicited by variant A5 immunization are more sensitive to the induction of AH1-specific tolerance than those elicited by variant 39 immunization.

A5-tet+ T cells are present in naive GP70hi mice

Given that the A5-elicited T cell response is almost entirely AH1 cross-reactive (Fig. 1C), the robust expression of GP70 by splenocytes in GP70hi aging mice may be responsible for deletion
of the T cell precursors that respond to A5 immunization. To test this hypothesis, we determined the number of T cells in naive GP70hi mice that recognize the A5 Ag. Using magnetic beads to enrich the A5-tet+ T cells from the spleen and lymph nodes of individual mice, we found that similar numbers of A5-specific T cells were present in GP70hi, young BALB/c, and aging gp70−/− mice (Fig. 7B). These data suggest that the failure of GP70hi mice to respond to A5 immunization is not because of an absence of A5-reactive T cells.

Alternatively, A5-specific T cells in naive GP70hi mice may be anergic or exhausted because of the peripheral expression of GP70, and subsequently unresponsive to the A5 peptide during immunization. To test this possibility, we determined whether the A5-tet+ cells enriched from GP70hi mice express PD-1, a surface molecule expressed by anergic (38) and exhausted (39) T cells. In contrast with the A5-tet+ cells enriched from young BALB/c and aging gp70−/− mice, A5-tet+ cells enriched from GP70hi mice expressed PD-1 (Fig. 7C). A5-tet+ PD-1+ T cells also displayed diminished surface IL-7Rα expression (Fig. 7C), a phenotype associated with T cells responding to cognate Ag, including T cells undergoing peripheral deletion (3, 40). These data suggest that the failure of GP70hi mice to respond to A5 immunization may be, in part, caused by anergy in, or exhaustion or deletion of, A5-specific precursors in response to peripheral AH1 presentation.

Discussion

Variant peptide vaccines are used clinically to induce T cell responses against tumor Ags (4, 41–44). However, in many patients, these vaccines are ineffective at inducing clinical tumor regression or T cell responses with high TAA recognition efficiency (4, 35, 45–48). Although the amount of TAA expressed by similar tumors in different patients varies (49–53), this factor has been given little attention as it relates to variant peptide vaccinations. Previous studies have not determined whether the amount of TAA expression affects the quality of the TAA cross-reactive response induced by variant peptide vaccinations. We examined this issue by taking advantage of an increase in expression of the TAA GP70 that occurs with age in BALB/c mice. Young mice demonstrate T cell tolerance to GP70423–431 (AH1). However, GP70 expression increases with age, and AH1-specific T cell responses are further diminished in aging mice (19, 22). Vaccination of young mice with either of two peptide variants of AH1, A5 or 39, has previously been shown to elicit robust AH1 cross-reactive T cell responses and AH1-specific tumor protection (5–7). In this study, we determined whether the increase in GP70 expression that occurs with age alters the AH1 cross-reactive responses elicited by these two peptide variants.

Interestingly, the responses induced by variants A5 or 39 were affected differently by increased AH1-specific tolerance. Although immunization with variant A5 induced a robust response of A5-specific and AH1 cross-reactive T cells in mice with undetectable splenic GP70 expression, they are absent in nearly all aging mice that have elevated splenic GP70 (Fig. 1B, 2B). Conversely, immunization with variant 39 induced a response containing 39-specific and AH1 cross-reactive T cells in all aging mice regardless of the presence of detectable GP70 expression (Fig. 1B, data not shown). However, the AH1 cross-reactive T cells elicited by variant 39 immunization of aging mice do not respond to stimulation with the AH1 epitope (Fig. 3).

The extent and quality of AH1 cross-reactivity in the T cell populations induced by each of these variants suggests a mechanism by which they might be affected differently by AH1-specific
tolerance. First, nearly all of the variant A5-elicited T cells bind AH1-tet (Fig. 1C), whereas only half of the variant 39-elicited T cells bind AH1-tet (Fig. 1D). The 39-specific cells that do not cross-react with the AH1 peptide should not be susceptible to tolerance induced by the increased expression of GP70. Further, the AH1-tet+ T cells expanded by variant A5 in young mice respond significantly better to AH1 stimulation than the AH1-tet+ population expanded by variant 39 (Fig. 5). Thus, responses with greater AH1 reactivity, as detected by AH1-tet staining and AH1 stimulation, are more susceptible to the induction of tolerance by AH1 presentation.

These data suggest two reasons that T cell responses to variant 39 are maintained in AH1-tolerant mice. First, part of the 39-elicited response does not cross-react with the AH1 Ag (Figs. 1D, 3A–C). The lack of AH1 recognition by these T cells suggests precursors of these cells would be ignorant to AH1-specific tolerance. Second, a portion of the 39-elicited population recognizes AH1-tet but does not respond to stimulation with AH1 peptide (Fig. 3C–E). These cells do, however, respond to stimulation with 39 peptide (Fig. 3C–E). The short half-life of AH1-tet binding with TCR suggests that insufficient TCR recognition may be responsible for poor AH1 functional recognition (33–35). Tetramer binding, but failure to respond to peptide stimulation, has been observed in T cells previously (54, 55). Thus, it seems unlikely that precursors of either of these 39-elicited T cell populations, both lacking functional recognition of AH1 (Fig. 3C), would be susceptible to AH1-specific tolerance. We propose that in aging GP70hi mice, T cells with no functional recognition of AH1 are the only cells available to respond to variant 39 vaccination, because the 39-reactive cells with functional AH1 recognition have been tolerated by anergy or deletion. The absence of functionally AH1-reactive cells in the response of GP70hi mice, present in the responses of both young BALB/c and aging gp70-deficient mice (Fig. 3C–E), demonstrates that variant-induced responses to TAA are increasingly suppressed by escalating TAA expression. The loss of A5-elicited responses in GP70hi mice also supports this conclusion (Figs. 1B, 1C, 2B).

To determine why immunization with variant A5 does not elicit cognate responses in GP70hi mice, we assessed the precursor frequency of T cells that bind A5-tet in naive GP70hi mice (Fig. 7). Similar numbers of A5-tet+ T cells were found in GP70hi, young BALB/c, and aging gp70-/- mice, suggesting that in GP70hi mice, these cells must be unresponsive to variant A5 immunization. Analysis of PD-1 and IL-7Rα surface expression suggests that some of the A5-tet+ T cells in GP70hi may be anergic, exhausted, or undergoing deletion (Fig. 7C) (3, 38–40, 56). However, not all of the A5-tet+ cells in GP70hi mice display a PD-1+ IL-7Rαlo phenotype. Immunoregulatory cells may suppress the response of these PD-1+ IL-7Rαlo cells. Others have shown that depletion or inhibition of Treg before immunization with the native AH1 Ag induces long-lasting and tumor-protective AH1-specific T cell responses, unlike immunization without Treg deletion or inhibition (57–59). Thus, Treg may suppress AH1-specific cells. Another group showed that Treg depletion enhanced the functional avidity of a TAA-specific response, suggesting that T cells with greater avidity for tolerizing Ag may be preferentially suppressed by Treg (60). Perhaps the increased GP70 expression in GP70hi mice makes A5-specific cells more susceptible to Treg-mediated suppression than those same cells in mice with less GP70 expression. Alternatively, the increased number and frequency of Treg in aging mice (61, 62) may result in the enhanced suppression of AH1-specific T cells in aging mice.
Further studies are needed to determine the mechanism and extent of suppression induced by Treg and anergy in this model. Although variant peptide immunizations often induce robust responses from TAA cross-reactive T cells, the functional avidity of these cells for the TAA may be relatively low (35, 47). Data presented in this article suggest that as endogenous TAA expression increases, these variant-elicited responses may become further biased toward a T cell repertoire with poor functional recognition of the TAA. This bias may result from the peripheral suppression of precursor T cells with functional TAA recognition. In the GP70 TAA model, Treg-mediated suppression has previously been demonstrated in young mice with low TAA expression (57, 58). The data presented in this article suggest that in mice with higher GP70 expression, anergy or deletion may also play a role in suppressing high-avidity T cells. We propose that T cells with poor TAA recognition escape peripheral tolerance because of this poor recognition and remain available for variant peptide vaccine-elicited expansion. However, these data also suggest that precursor T cells with high functional avidity for the TAA may remain in individuals with high TAA expression. The presence of these T cells suggests that treatments that block suppressive mechanisms, such as PD-1 (63, 64) and Treg (60, 65), may allow their expansion during vaccination. Thus, variant peptide vaccination in conjunction with one or more of these treatments may induce the proliferation of T cells with high TAA-specific avidity in patients bearing normal or transformed tissues with high TAA expression.

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