Implications of Aging and Self-Tolerance on the Generation of Immune and Antitumor Immune Responses

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Abstract

Cancer statistics show a disproportionately higher burden of tumors in the old. Most of the studies evaluating vaccination strategies have not taken into consideration the effect that aging has on the immune system. For the first time, we describe an animal tumor model in which self-tolerance and aging are present at the same time. FVB-Her-2/neu mice which are tolerant to neu antigens crossed with HLA-A2/Kb mice (A2xneu) develop spontaneous tumors when they are more than 22 months old. Analysis of CD8⁺ T-cell–specific responses in A2xneu mice indicated that the priming activity of old A2xneu mice to induce an immune response was diminished compared with young animals. Following intratumoral injections of CpG-ODN, ~30% of young A2xneu mice rejected the tumor; however, no antitumor effect was observed in old A2xneu mice. Analysis of T regulatory cells (Treg) indicated that there are significantly more Tregs in old animals. After CpG-ODN vaccination plus Treg depletion, 70% of young A2xneu mice rejected the tumor. The same treatment prolonged survival in old A2xneu mice, but none of the animals rejected the tumor. Even though CpG-ODN injections plus Treg depletion could rescue the antitumor responses against self-tumor antigens in young tolerant mice, the same therapy is not as effective in old tolerant hosts. Relevant tumor models such as the A2xneu mice in which self-tolerance and aging are present at the same time are critical to allow the optimization of vaccination strategies to effectively stimulate immune responses against self-tumor antigens in the young and the old. [Cancer Res 2008;68(13):5423–31]

Introduction

There is statistical evidence indicating that the incidence of cancer increases exponentially with advancing age (1, 2). The age-associated increase in cancer may be due in part to a global decrease in cell-mediated immunity (3, 4). Among the alterations that diminish the immune function in aged individuals are decreased proliferation of T cells (5), modifications in the production and secretion of cytokines (6, 7), reduced cytotoxic activity of CD8⁺ T-cells (8, 9), qualitative deficiency of B lymphocytes with a reduced response to exogenous antigens (10), and deficiencies in antigen-presenting cells (11, 12).

To understand the lack of immune responses against cancer and to achieve tumor immunity, awareness of the concept of self-antigens versus non–self-antigens is critical (13). The principle of the immune system is to tolerate self-antigens but develop vigorous responses against foreign antigens (14). Therefore, to optimize a vaccination strategy in order to stimulate an effective antitumor immune response, it is essential to consider factors such as T-cell tolerance (15) and expression of relevant tumor-associated antigens (16, 17). Over the past 20 years, many groups have described a multitude of immunotherapeutic strategies such as increasing the immunogenicity of the tumor antigen or manipulation of the immune system to promote or enhance antitumor responses. The majority of these studies have used young murine models to test the efficacy of these strategies. However, very little consideration has been paid to the effect that aging has on the immune system and neither has the immunotherapeutic potential of these strategies been investigated in elderly individuals. Considering that the number of cancer cases is rising mainly due to an increase in the elderly population, for any immune-based cancer therapy to succeed, it is vital to take into account the age-related changes in the activity of immune function.

The assessment of antitumor immune responses in animal models is critical for evaluating the basic paradigms of tumor immunology because these models provide all the immune-components which cannot be reproduced in vitro (18). We and others have shown that the immune system of the old could be manipulated and exploited for the induction of antitumor responses (19–23). However, these studies do not deal with the effect of self-tolerance or evaluate whether old hosts are able to generate antitumor responses against self-tumor antigens. For the first time, these studies describe an aged tumor model in which self-tolerance and tumor progression are present simultaneously.

For the past several years, we have used the A2.1/Kb transgenic mice (24) to identify A2.1/Her-2/neu epitopes (25) or to evaluate immune responses against self-antigens in tolerant hosts by crossing the A2.1/Kb transgenic mice with the FVB-neu transgenic mice (26). Our results show that A2xneu mice contain only a low-avidity repertoire to neu antigens indicating that the repertoire of A2xneu mice is tolerant to neu antigens (26–28). One of the consequences of crossing the FVB-neu mice with the A2.1/Kb mice is that spontaneous tumors appear in these animals when they are 22 to 27 months old. It is not well understood why these mice have a delay in the appearance of spontaneous tumors (29). Therefore, the A2xneu mouse model provides a unique opportunity to evaluate antitumor immune responses against a self-antigen in which aging and tolerance are present at the same time. In these studies, we compared the antitumor responses in young and old A2xneu mice. Our results indicate that the priming activity of old A2xneu and A2xFVB mice to induce a T-cell response is much weaker when compared with young animals. Interestingly, we observed that one of the effects of aging is that old A2xFVB mice lost their ability to reject tumors, in contrast with young A2xFVB mice which were able to reject N202.A2 tumors. Analysis of antitumor responses after CpG-ODN vaccination indicated that 100% of old A2xFVB mice rejected the N202.A2 cells, however, only...
~30% of young A2xneu mice rejected the tumor and no antitumor effect was observed in old A2xneu mice. We also observed that naïve old A2xFVB or A2xneu mice have a higher number of Tregs when compared with young A2xFVB or A2xneu mice. Depletion of Tregs plus CpG-ODN vaccination induced a strong antitumor response in which 75% of young A2xneu mice rejected the tumor. In contrast, the same therapy could not restore the antitumor responses in old A2xneu mice. These results indicate that there are drastic differences between young and old tolerant and nontolerant hosts in responding to adjuvant therapy. Taken together, these results indicate that the development of relevant tumor models, such as the A2xneu mice in which self-tolerance and aging are present at the same time, are critical because these models will allow for the optimization of vaccination strategies to effectively stimulate tumor immune responses against self-tumor antigens in the young and the old.

Materials and Methods

Mice. The FVB-neu transgenic mice (line 202) were commercially obtained from The Jackson Laboratory and maintained homozygously. The FVB mice were purchased from Harlan. The HLA-A2.1/Kb transgenic mice were kindly provided by Dr. Linda Sherman, from the Scripps Research Institute, La Jolla, CA. The FVB-neu and FVB mice were mated with the HLA-A2.1/Kb mice to generate A2xneu and A2xFVB mice.

The N202.A2 cell line was established from a spontaneous tumor from A2xneu mice (26). The T2-A2.1/Kb cell line was also provided by Dr. Linda Sherman. All cell lines were maintained in complete RPMI 1640 supplemented with 10% FCS, 2 mmol/L of L-glutamine, 5 × 10⁻⁵ m 2-ME, and 50 μg/mL of gentamicin.

Analysis of A2.1-neu restricted cytotoxic T lymphocyte responses in young and old mice. Young (2 months old) and old (18 months old) A2xFVB and A2xneu mice were immunized with the A2.1/Her-2-neu-p369–377 (KIFGSLAFL) or A2.1/Her-2-neu-p773–782 (VMAGVGSPTYV) peptides as previously described (25). Briefly, animals were immunized with a mixture of 100 μg of the peptide with 120 μg of the helper peptide (30) that induces a strong CD4 T-cell response in 100 μL of incomplete Freund's adjuvant. Ten days after immunization, animals were sacrificed and spleens were removed. For stimulation of cultures, T cells (10⁶ cells/well) were incubated with autologous irradiated (3,000 rad) lipopolysaccharide spleen blasts (2 × 10⁶ cells/well) that were pulsed with the p369–377 or p773–782 peptides in 24-well plates. After 5 days, cytotoxic T lymphocytes (CTL) were assayed for lytic activity. The T2-A2.1/Kb cells pulsed with the peptides were incubated with 150 μCi of ⁵¹Cr sodium chromate for 1 h at 37°C. Cells were washed thrice and resuspended in complete RPMI medium. For the cytotoxic assay, ⁵¹Cr-labeled target cells (10⁶) were incubated with varying concentrations of effector cells in a final volume of 200 μL in U-bottomed 96-well microtiter plates. Supernatants were recovered after 6 h of incubation. To evaluate whether CpG-ODN vaccination and Treg depletion enhances the T-cell's responses, animals received five daily s.c. injections of 30 μg CpG-ODN (1826, 5'-TCCATGACGGTTCAGCTT-3') or control-ODN (1982, 5'-TCCAGGACTTCTCTCAGGTT-3') or control-ODN (1982, 5'-TCCAGGACTTCTCTCAGGTT-3') on days −2, −1, 0, 1, and 2 prior to post–peptide vaccination. On day 0, mice were immunized with the peptide, as described above, near the site of the CpG-ODN injections. To deplete Tregs, animals were injected with anti-GITR i.p. twice a week prior to peptide immunization (300 μg/immunization).

ELISPOT analysis. For ELISPOT analysis of CTLs from young and old A2xFVB and A2xneu mice, we followed the same protocol as we previously reported (28).

Analysis of CD4⁺ Foxp3⁺ Tregs. The numbers of Tregs in young and old A2xFVB and A2xneu mice were determined by analysis of CD4 Foxp3⁺ in the spleen and lymph nodes. Spleen and lymph nodes from young and old animals were first surface-stained with anti-CD4 (FITC) monoclonal antibodies (ebioscience), Intracellular Foxp3 (PE) staining was performed using a commercially available kit (ebioscience) following the manufacturer's protocol.

In vivo tumor studies. Young and old A2xFVB and A2xneu mice were injected s.c. with 10⁶ N202.A2 cells and monitored for tumor growth. To evaluate the effect of vaccination with CpG-ODN, young A2xFVB mice and old A2xneu mice and A2xFVB mice were implanted with 10⁶ N202.A2 cells and tumors, and allowed to grow for 10 days before treatment was initiated. On day 10 after tumor challenge (tumor size ~ 200 mm³), animals were randomly divided into groups of five to eight mice. Animals received intratumoral (i.t.) injections of CpG-ODN and control-ODN. For the treatments, animals were injected thrice a week for 3 weeks in a final volume of 50 μL/injection. Animals injected with control-ODN served as controls. The 30 μg/injection dose was found to be an optimum dose because at higher doses, toxicity or side effects were observed (data not shown; ref. 31). To deplete Tregs, animals were injected with anti-GITR i.p. once a week for 3 days on days 9, 16, and 23 (300 μg/injection). To assess whether peptide vaccination in combination with CpG-ODN plus anti-GITR results in a stronger antitumor response, young and old A2xneu mice were implanted with 10⁶ N202.A2 cells. On day 9, animals began treatment with anti-GITR, and on day 10, they were immunized with a mixture p₃₆₉, p₇₇₃, and helper peptide as described above plus i.t. injections of CpG-ODN. Peptide immunization was repeated every 10 days for a total of three times. Tumor volume was expressed as (minor diameter)² × major diameter / 2. Statistical analysis was determined by Student's t test. Survival analysis used the Breslow modification of the Kaplan-Meier test.

Results

Analysis of tumor growth in young and old A2xneu and A2xFVB mice. We have observed that one of the consequences of crossing FVB-neu mice with A2.1/Kb transgenic mice (A2xneu) is that there is a delay in the appearance of spontaneous tumors in A2xneu mice. Typically, spontaneous tumors appear at an age of 45 to 52 weeks in old naive FVB-neu mice (Fig. 1A). However, spontaneous tumors appear in A2xneu mice when they are 104 to 116 weeks old (Fig. 1A). We have previously shown that N202.A2 cells grow in A2xneu as a consequence of immune-tolerance, but are rejected by young A2xFVB mice. In order to evaluate whether aging has an effect on the immune system altering the immune responses, we tested whether there was a difference between young and old mice in rejecting the N202.A2 cells. Young and old A2xFVB and A2xneu mice were implanted s.c. with 10⁶ N202.A2 cells and tumor growth was evaluated. As expected, young A2xFVB mice rejected the tumor; however, tumors grew in old A2xFVB mice (Fig. 1B). N202.A2 cells formed tumors in young and old A2xneu mice, as anticipated (Fig. 1B).

Analysis of T-cell responses in young and old A2xneu and A2xFVB mice. We previously identified two immunodominant A2.1/Her-2-neu epitopes using the A2.1/Kb transgenic mice (25). Additionally, we have shown that A2xneu mice only have low-affinity T-cells against the p₃₆₉–₇₇₇ and p₇₇₃–₇₈₂ epitopes, and that these animals do not respond with the same efficacy to peptide vaccination as A2xFVB mice (26–28). We evaluated the priming ability of young and old A2xneu and A2xFVB mice to induce a CTL response after immunization with the p₃₆₉–₇₇₇ and p₇₇₃–₇₈₂ peptides. Our results indicate that the CTL activity from young A2xFVB or A2xneu mice was stronger when compared with the CTL activity of A2xFVB or A2xneu old mice (Fig. 2). Interestingly, the CTL from old A2xFVB mice (nontolerant) had a similar cytotoxic activity as the CTL from young A2xneu mice (tolerant). A very weak CTL activity was detected in old A2xneu mice. No cytotoxic activity against T2-A2/Kb cells pulsed with an irrelevant peptide (HLA-A2.1/HIV-POL; ref. 25) was observed, indicating that recognition of the p₃₆₉ and p₇₇₃ peptides is specific (Fig. 2B and C). Taken together, these results clearly...
showed that aging severely compromises the immune system and that old A2xneu mice did not have the same capacity to prime a T-cell response as young mice.

**Evaluation of antitumor responses in young and A2xFVB and A2xneu mice.** We have recently shown that CpG-ODN, but not other TLR-ligands, induce an immune response in old BALB/c mice (31). Here, we evaluated the effect of i.t. injections of CpG-ODN in inducing an antitumor response in young A2xneu and old A2xFVB and A2xneu mice. I.t. injections of CpG-ODN induced an antitumor response resulting in tumor rejection in 100% of old A2xFVB mice (Fig. 3A). Only ~30% of young A2xneu mice treated with i.t. injections of CpG-ODN rejected the tumor (Fig. 3A) and those animals that did not reject the tumor significantly prolonged the survival of the animals when compared with control groups. In contrast, no tumor rejection was observed in old A2xneu mice and only a subtle delay in survival was observed in CpG-ODN-treated old A2xneu mice when compared with control groups (Fig. 3A). These results indicate that there are drastic differences between tolerant (A2xneu) and nontolerant (A2xFVB) young and old hosts as to how they respond to adjuvant stimulation and activate an immune response. Old A2xFVB mice that rejected the tumor after CpG-ODN treatment established a protective memory response capable of rejecting the tumor challenge (Fig. 3B). In contrast, young A2xneu mice that rejected the tumor after CpG-ODN treatment failed to further reject the tumor when challenged 70 days later (Fig. 3B), indicating the incapacity of tolerant mice to develop memory responses against self tumor antigens.

**Analysis of Tregs in young and old hosts.** There is extensive evidence indicating that Tregs can control and/or suppress primary and memory immune responses (32–34). Very little is known about Tregs in old mice. We evaluated whether there were differences in the number of CD4+Foxp3+ from spleens in young and old naïve A2xFVB and A2xneu mice. We observed that the number of Tregs in spleens from naïve old A2xFVB and A2xneu mice were almost double when compared with the number of CD4+Foxp3+ cells in spleens from naïve young A2xFVB and A2xneu mice (Fig. 4). These results suggest that the excess of Tregs might control the activation of immune responses in old and old tolerant hosts in a more stringent manner (35).

**Analysis of peptide-specific T-cell responses after CpG-ODN treatment and Treg depletion.** Our results indicate that CpG-ODN enhances the immune responses in old mice. Next, we evaluated whether the priming of T-cell responses could be enhanced in the presence of CpG-ODN and depletion of Tregs in old mice. Young and old A2xFVB and A2xneu mice were immunized with the p773–782 (Fig. 5) peptide in combination.
with CpG-ODN and in the presence or absence of anti-GITR. Our data indicated that CTL responses were not significantly altered in young A2xFVB mice in the presence of CpG-ODN or after depletion of Tregs with anti-GITR (Fig. 5A). However, an additive effect was observed when old A2xFVB and young and old A2xneu mice were treated with CpG-ODN plus anti-GITR resulting in an enhanced cytotoxic activity (Fig. 5A). Our results also indicate that the killing activity of CTLs from young and old A2xFVB (Fig. 5B) and A2xneu (Fig. 5C) against the N202.A2 cells was enhanced in the presence of CpG-ODN and anti-GITR. No killing was observed against N202 cells (Her-2/neu+/A2.1–). Overall, these results indicate that the CTL responses were restored in old A2xFVB mice almost to similar levels as the young A2xFVB mice and the CTL responses were significantly enhanced in young and old A2xneu mice. Next, we evaluated whether the enhanced cytotoxic activity after CpG-ODN plus anti-GITR treatment correlated with an increase in peptidespecific CTL frequency. The frequency of p773 peptide-specific CTLs was evaluated by ELISPOT assay. As shown in Fig. 5D, old A2xFVB and young A2xneu mice immunized with p773 in the presence of CpG-ODN plus anti-GITR showed a ~3-fold increase in the frequency of p773 peptide–specific CD8 T-cells when compared with p773 peptide–immunized mice. Animals immunized with p773 peptide plus CpG-ODN or peptide plus anti-GITR showed only a slight increase in the frequency of p773 peptide–specific CD8 T-cells, indicating that there is a cooperative effect when CpG-ODN and anti-GITR is used at the same time. In the presence of anti-Rat antibody, no improvements in the immune responses were observed. These data suggest that the improvement of the immune response in the presence of CpG-ODN plus anti-GITR is due to a greater number of peptide-specific T cells that were expanded after immunization. Taken together, these results indicate that the incorporation of an adjuvant that induces a strong proinflammatory response like CpG-ODN plus Treg depletion into the immunization protocols could be critical to effectively prime a T-cell response in young and old tolerant hosts.

Evaluation of CpG-ODN treatment and Treg depletion in young and old A2xneu mice. Having shown that CpG-ODN treatment and depletion of Tregs with anti-GITR has an additive effect significantly enhancing the CTL responses, we evaluated whether this treatment could induce an immune response capable of controlling tumor growth in young and old A2xneu mice. The combination of CpG-ODN plus anti-GITR significantly delayed the tumor growth in old A2xneu mice (Fig. 6A) and prolonged the survival of the animals (Fig. 6C) when compared with control animals. However, none of the animals could reject or control the tumor growth. Young A2xneu mice that rejected the primary tumor were challenged with a second tumor and two of five animals rejected the tumor challenge indicating that these animals could develop a memory response. To assess whether the antitumor responses could be enhanced, young and old A2xneu mice were immunized with a mixture of p369, p773, and helper peptide in combination with i.t. injections of CpG-ODN plus anti-GITR. As shown in Fig. 5B, seven of seven young A2xneu mice rejected the primary tumor and six of seven rejected the tumor challenge. In old A2xneu mice, we observed that two of seven animals rejected the primary tumor and those mice which did not reject the tumor, had a significant delay in tumor growth (Fig. 5B) and further prolonged the survival of the animals when compared with animals treated with CpG-ODN and anti-GITR alone (Fig. 5C). However, old A2xneu mice that rejected the tumor did not develop memory responses (Fig. 5B). Peptide immunization

Figure 2. Analysis of peptide-specific CTL responses in young and old A2xFVB and A2xneu mice. Young and old A2xFVB and A2xneu mice were immunized with the p369–377 (A and B) and p773–783 (C and D) peptides as described in Materials and Methods. Animals were sacrificed 10 d later and spleen cells were restimulated in vitro. After 5 d, stimulated spleen cells were assayed at different E/T ratios for cytotoxicity against T2-A2/Kb–pulsed target cells. A, p369–377–CTLs plus T2-A2/Kb cells pulsed with p369–377 peptide. B, p369–377–CTLs plus T2-A2/Kb cells pulsed with HIV-POL peptide. C, p773–783–CTLs plus T2-A2/Kb cells pulsed with HIV-POL peptide. D, p773–783–CTLs plus T2-A2/Kb cells pulsed with HIV-POL peptide. Points, mean of three individually analyzed mice per group representative of two independent experiments; bars, SE.
alone has a minimal effect in preventing tumor growth in young A2xneu mice (26). These results indicate that although it was possible to restore the primary and secondary antitumor immune responses in young tolerant hosts, the same therapy was not as effective in old tolerant hosts.

Discussion

Although many laboratories are evaluating a variety of vaccination strategies to induce antitumor immune responses, none of these laboratories have taken into consideration the effect that aging has on the immune responses. Because the immune system of the aged is different to that of the young, and is in a state of hyporesponsiveness, the conclusions drawn from studies on young animals cannot be extrapolated to represent the events taking place in aged individuals. Studies from our group (19, 20) and other groups (21–23) indicate that the antitumor responses can be exploited in old mice. However, all these studies rely on tumor models that are immunogenic. As such, it will be very difficult to translate the results from these immunogenic tumor models into a clinical setting for the treatment of tumors in the old. There have been no reports, thus far, evaluating antitumor immune responses in aged tumor models in which tolerance and tumor progression are present simultaneously. Therefore, it is clear that there is a need for relevant animal tumor models in which it would be possible to evaluate immune and antitumor responses in aged tolerant hosts.

We have previously shown that A2xneu mice are tolerant to neu antigens (26). Our data indicate that one of the consequences of crossing FVB-neu mice with A2.1/Kb mice (A2xneu) is that spontaneous tumors appear in these animals when they are 22 to 27 months old. Therefore, the A2xneu mouse model represents a unique model in which aging, tolerance, and tumor progression are present simultaneously. The A2xneu mouse model closely reflects human disease wherein the testing of immune responses, vaccination, or immunologic strategies against self-tumor antigens will have a higher chance of being relevant in the human situation. Previous studies from our group indicate that N202.A2 cells are rejected by young A2xFVB (nontolerant host) mice but are able to grow in young A2xneu mice (tolerant host) due to tolerance.

Figure 3. Analysis of antitumor effect of CpG-ODN in young and old mice. A, young A2xneu and old A2xFVB and A2xneu mice were implanted with 10^6 N202.A2 cells on day 0. On day 10, animals received i.t. injections of CpG-ODN (30 μg/injection) thrice a week for 3 wk. Survival was monitored and the percentage of survival was determined. Seven animals were included per group. Animals injected with control-ODN serve as a control for the induction of antitumor responses by CpG-ODN. Points, mean of two experiments. A significant P < 0.001 and P < 0.02 difference was found between control mice and old A2xFVB and young A2xneu mice, respectively, injected with CpG-ODN. B, old A2xFVB and young A2xneu mice that rejected the tumor were challenged 70 d later with a 10^6 dose of N202.A2 cells. Survival was monitored and the percentage of survival was determined. Points, mean of two experiments.
considerations (26). Following the injection of N202.A2 cells into old A2xFVB mice, these animals lost the ability to reject the tumor cells. This is an indication of decay in immune function in the old. Also, the priming ability and cytotoxic responses in old A2xFVB mice were significantly lower than young A2xFVB mice. The CTL activity from old A2xFVB (nontolerant) mice was similar to the cytotoxic activity of CTL from young A2xneu (tolerant) mice. Old A2xneu mice generated a very weak CTL response when compared with young A2xneu mice. Taken together, these results indicate that aging compromises the ability to promote T-cell responses and it is more difficult to prime an immune response against self-tumor antigens in old tolerant hosts. These results have important clinical implication considering that the majority of tumor antigens are self-proteins (36) and that more than 50% of newly diagnosed cancers are from the elderly (37).

Our results indicated that i.t. injections of CpG-ODN could rescue the immune response and promote antitumor responses in A2xFVB old mice. One hundred percent of old A2xFVB survived and developed a protective memory response. However, only 30% of young A2xneu survived but none of these animals were able to develop a memory response. Young A2xneu mice that did not reject tumors significantly survived for longer periods of time when compared with control animals. In contrast, none of the old A2xneu mice survived after CpG-ODN treatment, and a very subtle delay in survival was observed when compared with the control group. A possible explanation for why old A2xFVB mice rejected the tumor whereas young or old A2xneu did not is in the type of T-cell repertoire that exists against the self–tumor antigens present in each host (38, 39). We have shown that A2xneu mice only contain a low-affinity repertoire for neu antigens and it is more difficult to activate and expand this population (26–28). Even though the immune system of the old is diminished, old A2xFVB mice might have high-affinity T-cells for neu antigens, and under proper conditions, these cells could be activated and expanded resulting in the activation of an effective antitumor response (20, 21). There is strong evidence indicating that Tregs continuously suppress self-reactive T cells (40). We analyzed whether young and old mice differ at the level of Tregs. Our results indicate that old A2xFVB or A2xneu mice have double the amount of Tregs when compared with young A2xFVB or A2xneu mice (35). This could be an indication that the accumulation of Tregs might influence or inhibit the activation of immune responses in the old (41). Next, we evaluate the effect of CpG-ODN or CpG-ODN plus Treg depletion with anti-GITR monoclonal antibody in enhancing CTL responses. Our results show that treatment with CpG-ODN or anti-GITR monoclonal antibody alone slightly enhances the peptide-specific CTL responses in old A2xFVB or in young and old A2xneu mice. However, when CpG-ODN and anti-GITR was combined, an additive effect was observed and the CTL responses were stronger in old A2xFVB or young and old A2xneu mice. The CTLs from young and old A2xneu mice were capable of recognizing N202.A2 targets, albeit at significantly lower levels than CTLs from young and old A2xFVB mice. The stronger cytotoxic activity against T2-A2/Kb peptide-pulsed cells or N202.A2 cells observed after CpG-ODN and anti-GITR treatment in old A2xFVB and young and old A2xneu mice correlate with the higher frequency of peptide-specific CTLs. These data suggest that the improvement of the immune response in the presence of CpG-ODN and anti-GITR is most probably due to the greater number of tumor-specific T cells that were expanded after immunization. Having demonstrated difficult to activate and expand this population (26–28). Even though the immune system of the old is diminished, old A2xFVB mice might have high-affinity T-cells for neu antigens, and under proper conditions, these cells could be activated and expanded resulting in the activation of an effective antitumor response (20, 21). There is strong evidence indicating that Tregs continuously suppress self-reactive T cells (40). We analyzed whether young and old mice differ at the level of Tregs. Our results indicate that old A2xFVB or A2xneu mice have double the amount of Tregs when compared with young A2xFVB or A2xneu mice (35). This could be an indication that the accumulation of Tregs might influence or inhibit the activation of immune responses in the old (41). Next, we evaluate the effect of CpG-ODN or CpG-ODN plus Treg depletion with anti-GITR monoclonal antibody in enhancing CTL responses. Our results show that treatment with CpG-ODN or anti-GITR monoclonal antibody alone slightly enhances the peptide-specific CTL responses in old A2xFVB or in young and old A2xneu mice. However, when CpG-ODN and anti-GITR was combined, an additive effect was observed and the CTL responses were stronger in old A2xFVB or young and old A2xneu mice. The CTLs from young and old A2xneu mice were capable of recognizing N202.A2 targets, albeit at significantly lower levels than CTLs from young and old A2xFVB mice. The stronger cytotoxic activity against T2-A2/Kb peptide-pulsed cells or N202.A2 cells observed after CpG-ODN and anti-GITR treatment in old A2xFVB and young and old A2xneu mice correlate with the higher frequency of peptide-specific CTLs. These data suggest that the improvement of the immune response in the presence of CpG-ODN and anti-GITR is most probably due to the greater number of tumor-specific T cells that were expanded after immunization. Having demonstrated

Figure 4. Analysis of Tregs in the spleens of young and old A2xneu mice. Spleen from naïve young and old A2xFVB and A2xneu mice were stained against CD4/Foxp3 as described in Materials and Methods. A, representative experiment on single animals analyzing the levels of CD4⁺Foxp3⁺ Tregs in the spleen of young and old A2xFVB mice. B, accumulative data of all animals analyzed for the levels of CD4⁺Foxp3⁺ Tregs. *, P < 0.05 in the number of Tregs between young and old mice.
that CpG-ODN and anti-GITR significantly enhance the CTL responses in young and old A2xneu mice, we then tested the effect of this combination therapy in activating an antitumor response. Our results indicate that five of seven young A2xneu mice treated with CpG-ODN plus anti-GITR induce the rejection of tumors and two of five of the animals that rejected the primary tumor developed a memory response. The same therapy delayed the tumor growth and prolonged the survival of old A2xneu mice, however, none of the animals rejected the tumor. The combination of specific-peptide vaccination with CpG-ODN plus anti-GITR resulted in 100% of the young A2xneu mice rejecting the primary response, and six of seven animals developing a memory response. Although this treatment significantly delayed tumor growth and prolonged the survival of old A2xneu mice, none of the animals developed a memory response. These results show that depletion of Tregs in combination with CpG-ODN and peptide vaccination induced an antitumor response capable of restoring the immune response in young tolerant hosts. Although the same therapy significantly enhanced the antitumor responses in old tolerant hosts, it was not capable of restoring the immune responses in these animals. These results raise the question as to why old A2xneu mice could not mount an immune response capable of rejecting the primary tumor or develop memory responses. New preliminary data from our laboratory indicate that (a) the T-cell receptor of old T cells is disengaged when compared with young T cells, (b) more indoleamine-2,3-dioxygenase is produced in old mice when compared with young mice, and (c) there is a higher accumulation of myeloid suppressor cells in old mice when compared with young mice. Therefore, it is possible that the accumulation of these defects further influence the immune response.

system, diminishing the activation of immune responses in old mice. As such, the depletion of Tregs alone might not be sufficient for the complete restoration of immune responses in old tolerant hosts and perhaps it is necessary to use alternative strategies to overcome an excess of immune-suppression and dysfunctional alterations. Future studies are required to better understand the immune function of old antigen-presenting cells and T cells, or answer why there is an accumulation of Tregs or other immune-suppressor mechanisms in old mice which impinge the activation of tumor-specific immune responses.

Although we might be able to develop immunotherapeutic protocols that are effective in controlling tumor growth in young A2xneu mice, we have to make sure that the same protocols are also effective in old tolerant animals because cancer is primarily a disease of aging individuals. Therefore, to optimize any immunotherapeutic therapy, it is important to consider the immune system of the aged because it is associated with a dramatic reduction in responsiveness as well as functional dysregulation (42, 43). The A2xneu mice represent the first animal model in which it is now possible to evaluate the antitumor immune responses in both old and self-antigen–tolerant hosts. This model is invaluable and is of great importance because the results derived from it will allow us to optimize antitumor immune responses in the old. Our group is currently evaluating the immune responses and strategies to further enhance antitumor responses in old A2xneu mice. For example, we have shown that there is a cooperative effect between antiangiogenic therapy and immunotherapy (44). Perhaps for complete tumor destruction in old tumor-bearing hosts, it might

Figure 6. Peptide vaccination in combination with CpG-ODN plus depletion of Tregs enhances antitumor responses in young and old A2xneu mice. A, young and old A2xneu mice were inoculated s.c. on day 0 with $10^6$ N202.A2 cells. On days 9, 16, and 23, animals were injected with anti-GITR (300 μg/injection). On day 10, animals started i.t. injections of CpG-ODN thrice a week for 3 wk (30 μg/injection). Young A2xneu mice that rejected the tumor were challenged 70 d later with a $10^6$ dose of N202.A2 cells. Tumor volume was measured every 3 to 5 d by caliper in two dimensions. Control points, mean within the group; bars, SE. Individual treated young and old A2xneu mice are indicated. B, young and old A2xneu mice were implanted with N202.A2 tumors as indicated above and immunized with a mixture of p369–377, p773–783, and helper peptide on day 10. Peptide immunizations were repeated on days 20 and 30. CpG-ODN and anti-GITR was applied as indicated above. Tumor volume was measured every 3 to 5 d. C, survival was monitored and the percentage of survival of all treated mice was determined. Seven animals were included per group. A significant $P < 0.001$ and $P < 0.05$ difference was found between control mice and young and old A2xneu mice, respectively.
be necessary to use two forms of therapies to increase the antitumor efficacy. The A2xneu mouse model will enable us to uncover some of the cellular basis for the decline in immune function in the elderly and determine the conditions and strategies to augment the antitumor activity against self-tumor antigens in the aged. The information generated from these animals will be more comparable to the aging environment and the results obtained from this animal tumor model would have a better chance to be translated for the treatment of cancer in the old.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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