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Aged Mice Develop Protective Antitumor Immune Responses with Appropriate Costimulation

Joseph Lustgarten, Ana Lucia Dominguez, and Marilyn Thoman

There is a clear decrease in CD8+ T cell effector function with aging, a loss once thought to be intrinsic to the CD8+ T cells. Recent studies suggest, however, that this decline may be a consequence of altered stimulatory signals within the aged lymphoid microenvironment. In this study, we compared the immune responses of young and old mice against the BM-185 pre-B cell lymphoma expressing enhanced GFP (EGFP) as a surrogate tumor Ag. Young animals develop protective immune responses when immunized with BM-185-EGFP, but aged mice do not and ultimately succumb to the tumor. However, expression of CD80 (B7.1) on the BM-185-EGFP (BM-185-EGFP-CD80) results in rejection of the tumor by both young and old animals. Additionally, injection of BM-185-EGFP-CD80 cells in young mice promotes the development of long-lasting memory responses capable of rejecting BM-185 wild-type tumors. Aged mice similarly injected did not develop antitumor memory responses. Interestingly, old animals immunized with the BM-185-EGFP-CD80 cells plus injections of the agonist anti-OX40 mAb did develop long-lasting memory responses capable of rejecting the BM-185 wild-type tumors with the same vigor as the young animals. We show that old mice have the capacity to develop strong antitumor responses and protective memory responses as long as they are provided with efficient costimulation. These results have important implications for the development of vaccination strategies in the elderly, indicating that the aged T cell repertoire can be exploited for the induction of tumor immunity. The Journal of Immunology, 2004, 173: 4510–4515.
Materials and Methods

Mice and cell lines

Young (2- to 3-mo-old) and old (18- to 22-mo-old) BALB/c mice were purchased from the National Institute of Aging (Bethesda, MD) and housed under specific pathogen-free conditions. The cell lines BM-185 wild-type (w.t.), BM-185 expressing enhanced GFP (EGFP) (BM-185-EGFP), and BM-185 expressing EGFP and CD80 (BM-185-EGFP-CD80) were kindly provided by D. Kohn at the University of Southern California (Los Angeles, CA). All three forms of the tumor cell line are H2-K10 positive, but do not express CD80, CD86, or OX40L, with the exception of the BM-185-EGFP-CD80, which, of course, expresses CD80. Anti-OX40 (OX86) mAb-producing cell line was obtained from the European Cell Culture Collection (Wiltshire, U.K.). Anti-OX40 mAb was purified by passage of culture supernatant through a protein G matrix affinity column. All cell lines were maintained in complete RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 5 × 10–5 M 2-ME, and 50 μg/ml gentamycin.

Tumor formation

Animals were injected s.c. with 10⁵ BM-185-w.t., BM-185-EGFP, or BM-185-EGFP-CD80 cells, and monitored for tumor growth. To evaluate whether young and old mice developed a memory response after inoculation of the BM-185-EGFP or BM-185-EGFP-CD80 cells, animals were challenged 30 or 90 days later with an s.c. injection of 10⁵ BM-185-w.t. cells. To evaluate the antitumor effect of anti-OX40 mAb, old mice were injected on day 0 with 10⁵ BM-185-EGFP or BM-185-EGFP-CD80 cells, and animals received four weekly injections of anti-OX40 mAb (100 μg/injection). Animals were challenged 90 days later with an s.c. injection of 10⁵ BM-185-w.t. cells to assess memory generation. Mice were examined twice per week for tumor development and survival. Survival analysis used the Breslow modification of the Kaplan-Meier test.

In vivo depletion of T lymphocytes

Anti-CD4 (GK1.5) and anti-CD8 (56-6.37) mAbs were used for in vivo depletion of T cell subsets. Hybridoma cell lines were purchased from the American Type Culture Collection (Manassas, VA), and supernatants were passed through a protein G matrix (Sigma-Aldrich, St. Louis, MO) to purify the Abs. Animals were injected i.p. with 300 μg of the respective Ab twice per week, starting 1 wk before immunization with the tumor cells and continuing for the duration of the experiment. Depletion of T cells was confirmed by FACS analysis of the lymph nodes and spleen (data not shown).

Stimulation of T cell responses

Young and old BALB/c mice were immunized with an s.c. injection of 10⁵ BM-185-EGFP or BM-185-EGFP-CD80 cells. Two weeks later, spleens from inoculated animals were removed and spleen cells were restimulated in vitro with BM-185-EGFP-CD80 cells. After 5 days, CTLs were assayed for lytic activity. The BM-185-w.t., BM-185-w.t. pulsed with the H2-K10-HYLSTQSL peptide (a K10-EGFP-derived immunodominant epitope), BM-185-EGFP, and BM-185-EGFP-CD80 cells were incubated with 150 μCi of ⁵¹Cr sodium chromate for 1 h at 37°C. Cells were washed three times and resuspended in complete RPMI 1640 medium. For the cytotoxic assay, ¹⁵Cr-labeled target cells (10⁴) were incubated with varying concentrations of effector cells in a final volume of 200 μl in U-bottom 96-well microtiter plates. Supernatants were recovered after 5 h of incubation at 37°C, and the percentage of lysis was determined by the formula: percent specific lysis = 100 × (experimental release – spontaneous release)/ (maximal release – spontaneous release).

Tetramer staining

Young and old mice were immunized with 10⁵ BM-185-EGFP or BM-185-EGFP-CD80 in the absence or presence of anti-OX40 mAb (single injection of 100 μg). Two weeks later, animals were sacrificed and spleen cells were stained with the H2-K10-EGFP-200–208-PE tetramer and anti-CD8 FITC. The H2-K10-EGFP-200–208-PE tetramer was obtained from the National Institute of Allergy and Infectious Diseases Tetramer Core Facility. Samples were stained with the tetramer (1 μg/sample) for 1 h at room temperature and then with anti-CD8 FITC for an additional 30 min at 4°C. Samples were analyzed in a FACSCalibur (BD Biosciences, San Jose, CA). Fifty thousand events were collected per sample, and data analysis was performed using CellQuest software.

Results

Analysis of antitumor responses in old and young animals

To evaluate the ability of old mice to reject tumors, we used BM-185, a pre-B cell line that causes 100% mortality in BALB/c mice (20). This cell line was transduced with the EGFP gene (BM-185-EGFP) or with the EGFP and CD80 genes (BM-185-EGFP-CD80) (20). It has been established that EGFP is an antigenic molecule and tumor cells expressing this protein are rejected. As shown in Fig. 1, 100% of young and old animals implanted with BM-185-w.t. cells developed tumors. When BM-185-EGFP cells were implanted in young and old mice, these cells were rejected by young animals (Fig. 1A), but not by old animals (Fig. 1B). Although not rejected by aged mice, the BM-185-EGFP tumor grew more slowly than did tumors induced by the BM-185-w.t., behavior suggesting that a weak immune response was generated, which was ineffective tumor rejection.

It has been demonstrated that tumor immunity can be enhanced by the enforced expression of costimulatory molecules on the tumor cell surface. Therefore, the effect of expressing the CD80 molecule on the BM-185-EGFP cells (BM-185-EGFP-CD80) was evaluated. As expected, young animals rejected the tumor cells. Aged mice also rejected the tumors (Fig. 1). These experiments led us to hypothesize that old mice maintain a functional T cell repertoire that can generate effective antitumor activity if the T cells are properly stimulated.

We next evaluated whether initial immunization with the BM-185-EGFP-CD80 would induce a memory response against the BM-185-w.t. cells. Young and old mice were injected with the BM-185-EGFP-CD80 cells on day 0, and on day 30 animals were challenged with the BM-185-w.t. cells. As shown in Fig. 2, young animals reject the BM-185-w.t. cells. This memory response is long lasting, as 90 days postimmunization animals are still able to reject a challenge with BM-185-w.t. cells. In contrast, old mice challenged with the BM-185-w.t. cells at any time after immunization with BM-185-CD80 tumor cells developed tumors, indicating no protective memory response was established.

Effect of anti-OX40 in the antitumor immune responses

The preceding results demonstrate that expression of CD80 on the tumor stimulates an immune response in old mice that results in the rejection of the tumor, but not in the generation of memory responses. We hypothesized that additional costimulatory signals might be necessary for the aged immune system to develop protective antitumor and memory responses. Recently, OX40 has gained importance as a costimulatory molecule capable of expanding the clonal immune response, enhancing cytokine secretion and longevity of stimulated T cells (9–11). Therefore, anti-OX40 mAb was evaluated for its ability to augment the antitumor response in old animals. Animals were injected on day 0 with the BM-185-EGFP or BM-185-EGFP-CD80 cells and received four weekly injections of anti-OX40 mAb (100 μg/injection) starting the second day after primary tumor inoculation. The results are shown in Fig. 3. The addition of anti-OX40 mAb injections markedly improved the ability of aged animals to respond, as 80% of animals injected with BM-185-EGFP and anti-OX40 mAb cleared the tumor (Fig. 3). More importantly, the anti-OX40 mAb-treated animals displayed long-lived antitumor memory in animals originally immunized with BM-185-EGFP or those immunized with BM-185-EGFP-CD80. Forty percent of the animals originally injected with the BM-185-EGFP rejected BM-185-w.t. tumor cells when challenged 90 days postprimary immunization (Fig. 3), while 100% of the mice immunized with BM-185-EGFP-CD80 cells developed a
protective memory response (Fig. 3). Taken together, these findings indicate that the aged immune system does indeed retain the capacity to mount an effective primary and memory response to tumor Ags.

Antitumor responses are dependent on CD8 T cells, but not on CD4 T cells

To confirm whether tumor rejection was mediated by T cells and to evaluate the role of CD4+ and CD8+ T cell subsets, animals were depleted of CD4+ and CD8+ T cells with anti-CD4 and anti-CD8 mAb, respectively. Young mice were treated with anti-CD4 and anti-CD8 mAbs and inoculated with BM-185-EGFP-CD80 cells. As shown in Fig. 4A, depletion of CD8+ T cells abrogates the antitumor response. In contrast, animals depleted of CD4+ T cells were still able to reject the tumors (Fig. 4A). These results demonstrate that the antitumor response against the EGFP-positive tumors is dependent on CD8+ T cells. Experiments performed in young hosts depleted of CD4+ T cells revealed that these animals reject the primary tumor, but they are not able to develop memory responses (Fig. 4B). These results indicate that the generation of memory CD8 T cell responses depends on the help provided by CD4 T cells.

Analysis of the antitumor T cell responses

To dissect and understand the effects of anti-OX40 mAb administration and CD80 expression on the immune response in the aged, an in vitro system was used to follow the Ag-specific CD8 T cells identified by multimer binding. Soluble MHC multimers
(tetramers) have provided an important tool to isolate and enrich T cell populations with a defined specificity (21, 22), as well as allowing direct quantification of Ag-specific T cells by flow cytometry. For analysis of specific CD8 T cell responses, we took advantage of the recently identified EGFP-H2-K\(^d\)-restricted peptide (HYLSTQSAL) (23). Young and old mice were immunized with the BM-185-EGFP and BM-185-EGFP-CD80 cells in the presence or absence of anti-OX40 mAb. Fourteen days later, the binding activity of total spleen cells from the immunized animals with the EGFP-H2-K\(^d\) tetramer (Fig. 5) was quantified. As a control for specificity for the tetramer-binding assay, we used animals immunized with an immunogenic irrelevant tumor cell line. No tetramer binding was detected in animals immunized with the control tumors (0.17%), demonstrating that the binding to the EGFP-H2-K\(^d\) tetramer was specific. In young animals immunized with BM-185-EGFP cells, 3.1% of the CD8\(^d\) population bound the tetramer. We detected almost the same number of tetramer-binding cells in the spleens of young animals immunized with BM-185-EGFP as in those immunized with BM-185-EGFP-CD80 cells (data not shown). In contrast, the number of tetramer-binding cells in the spleens of aged mice immunized with BM-185-EGFP cells fell below the detectable range (0.3%). However, in old mice immunized with BM-185-EGFP-CD80 cells, tetramer-binding cells were detected (1.6%), albeit at lower levels than those from young spleen cells. Treatment with anti-OX40 mAb increased the number of tetramer-binding cells in both young and old mice. The aged animals displayed a much greater increase in tetramer-binding cells than did the young mice. We could detect 5.1% tetramer-binding cells from young animals immunized with BM-185-EGFP plus anti-OX40 mAb, while in old mice immunized with BM-185-EGFP plus anti-OX40 mAb we detected 2.0% tetramer-binding cells. Old mice that received BM-185-EGFP-CD80 cells plus anti-OX40 mAb (3.4%) showed an even higher increase in tetramer-binding cells.

Spleen cells from animals immunized as described above were stimulated in vitro with BM-185-EGFP-CD80 cells for 5 days and

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**FIGURE 4.** Antitumor responses are dependent on CD8\(^{+}\) T cells. A, Young animals were depleted of CD4 and CD8 T cells with anti-CD4 and anti-CD8 (300 \(\mu\)g/injection, i.p. twice per week starting 1 wk prior to tumor implantation and throughout the experiment). Animals were s.c. implanted with \(10^6\) BM-185-EGFP cells and monitored for the development of tumors. B, Young animals were depleted of CD4 T cells with anti-CD4, as described above, immunized by s.c. implantation of \(10^6\) BM-185-EGFP cells. Sixty days later, animals were challenged with \(10^7\) BM-185-w.t. cells and monitored for the development of tumors. Five animals were included per group. Percentage of survival was determined. Data are representative of two experiments.

**FIGURE 5.** Treatment with anti-OX40 mAb enhances T cell responses in old mice. BALB/c young and old mice were immunized with \(10^6\) BM-185-EGFP or BM-185-EGFP-CD80 cells in the presence or absence of anti-OX40 mAb (100 \(\mu\)g). Two weeks after immunization, animals were sacrificed and splenocytes were stimulated with BM-185-EGFP-CD80 for 5 days in complete medium. In vitro stimulated splenocytes were stained with the EGFP-H2-K\(^d\)-tetramer-PE and anti-CD8 FITC. Viable CD8\(^{+}\) cells were gated, and the fraction of cells stained with tetramers was analyzed (A). Cytotoxic activity of restimulated cultures was measured against the BM-185-w.t., BM185-EGFP, and BM-185-EGFP-CD80 (B) in standard 5-h \(^{51}\)Cr release assay at the indicated E:T ratios. The results shown are representative of three independent experiments.
assayed for both tetramer binding and cytotoxic activity against BM-185 cells. Following restimulation, the proportion of the tetramer-binding CD8 T cells recovered increased dramatically. Addition of anti-OX40 mAb increased tetramer binding in cultures from both young and aged animals, albeit to different degrees. The presence of tetramer-binding cells within the initial splenic T cell population (before culture; see Fig. 5) was associated with the ability to give rise to cytotoxic responses upon in vitro restimulation. As shown, young animals initially immunized with BM-185-EGFP (≥ anti-OX40 mAb) induced strong cytolytic responses upon restimulation. In contrast, old mice immunized with BM-185-EGFP cells could not be restimulated to cytotoxic activity unless anti-OX40 mAb had also been used in the original immunization. A stronger cytotoxic activity is observed in old animals immunized with BM-185-EGFP-CD80 plus anti-OX40 mAb. Taken together, these data support our results with the tumor model, suggesting that the presence of CD80/anti-OX40 mAb can activate and amplify the T cell responses in old mice, overcoming some of the defects that are intrinsic in the aged immune system.

Discussion

This study compared the generation of primary and memory antitumor immune responses in young and aged mice. The results demonstrate that aged animals are capable of mounting highly effective primary antitumor responses and generating protective memory when supplied with sufficient costimulatory signals. In this model, as in many others, the aged response to unmodified tumor cells was weak and did not protect against tumor growth. However, when additional CD80 and anti-OX40 signaling was provided, the aged animals effectively produced antitumor cytolytic CD8+ T cells and were protected from tumor growth when challenged with wild-type tumors. These in vivo effects were mirrored ex vivo by an increase in CD8 T cell cytolytic activity and Ag-specific tetramer-binding CD8+ cells.

These results show that aged CD8 T cells are able to become CTL effectors upon appropriate stimulation, indicating that deficiencies in aged CD8+ T cell function may be secondary to alterations in the costimulatory environment rather than intrinsic to the T cell. Others have also reported that aged naive CD8+ T cells are not intrinsically defective in their ability to become primary effectors, but may not be stimulated appropriately within the aged microenvironment. Using TCR-transgenic mice, Li et al. (24) showed that purified naive transgenic CD8+ T cells from aged mice produced cytokines and CTL effectors at levels equivalent to those from young mice when stimulated in vitro with artificial APC loaded with peptide. In contrast, when aged T cells are stimulated under conditions in which costimulatory signals and CD4 T cell help were provided by aged cells, aged CD8+ T cells proliferate less well and demonstrate a lower cytolytic activity and a skewed cytokine production profile when compared with similarly activated young T cells (25–27).

Despite having triggered an effective primary response by immunization with CD80-modified tumor cells, aged mice did not generate protective antitumor memory cells, while young mice were able to do so. Aged animals required additional costimulatory signals supplied by anti-OX40 Ab injection to successfully display antitumor memory. These results are consistent with those reported by Provinciali et al. (28), in which aged CD8 T cells were found to give vigorous primary responses, but upon challenge displayed weak or altered memory reactions. However, given the appropriate type or amount of costimulatory signals, aged CD8 T cells are capable of mounting both primary and memory responses.

The data presented in this work suggest that deficiencies in CD8 T cell function associated with aging may be secondary to ineffective cross-presentation of tumor Ags by endogenous APC. This deficiency is bypassed when tumor cells are made to express costimulatory molecules and thus become effective in self-presentation. The evidence regarding APC function in the aged is somewhat contradictory in that while there are some reports that dendritic cell function is unaltered in the aged (29), a number of reports indicate that splenic APC from aged mice are less effective in stimulating T cells (30–32). Although the level of costimulatory molecule expression may not be changed with age, the kinetics of Ag processing, mobilization, or the migratory properties of aged APC (33, 34) and/or T cells may be altered, thus limiting interaction between the two populations. Alternatively, while aged APC may be capable of activation and function upon stimulation in vitro, conditions in vivo may not be equally effective. Most reports of functional deficiencies in aged APC assessed mature splenic-resident cells.

Numerous studies point to deficiencies in CD4 function in aged mice (35). It is possible that these changes in CD4 T cell function with age impact CD8 activity in the model system that we have used. As demonstrated, depletion of CD4+ T cells results in animals that are capable of mounting primary antitumor responses, but not developing memory. This is reminiscent of the results of others (36–40) who demonstrated that CD8 memory development requires CD4 T cell function. It is possible that anti-OX40 mAb, shown in this study to restore the capacity for CD8 memory generation in aged mice, acts indirectly through CD4+ lymphocytes, rather than directly on the CD8 T cells. CD4+ T cells have more often been shown to be the target of anti-OX40 Abs, although De Smedt et al. (41) have identified a direct effect of anti-OX40 to enhance CD8 responses.

The mechanisms of action of the costimulatory signals in promoting the CD8 responses in aged mice are not known. The tetramer-binding studies suggest that a result of CD80 and anti-OX40 stimulation is to increase the number of Ag-specific CD8+ cells, and that the cytolytic activity is related to the number of Ag-specific CD8 T cells. Certainly, it has been demonstrated by others that anti-OX40 mAb is capable of inducing clonal expansion, enhancing cytokine secretion, and prolonging the survival of activated T cells.

The data presented in this study are particularly significant in light of the efforts to develop prophylactic antitumor therapies. The deficiencies in immunologic vigor of the elderly are well known, yet few, if any, strategies designed to overcome them have been used (42, 43). Adjuvant approaches with demonstrated promise in the young generally fail to have the same impact in the aged (28). Our results indicate that with appropriate costimulation, aged mice may indeed mount both a protective effector response to a primary tumor and a protective memory response to tumor challenge.

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