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SYMPOSIUM IN WRITING

Cancer, aging and immunotherapy: lessons learned from animal models

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Abstract Aging of the immune system is associated with a dramatic reduction in responsiveness as well as functional dysregulation. This deterioration of immune function with advancing age is associated with an increased incidence of cancer. Although there is a plethora of reports evaluating the effect of immunotherapy in stimulating antitumor immune responses, the majority of these studies do not pay attention to the effect aging has on the immune system. Studies from our group and others indicate that immunotherapies could be effective in the young, are not necessarily effective in the old. To optimally stimulate an antitumor immune response in the old, it is necessary to (1) identify and understand the intrinsic defects of the old immune system and (2) use relevant models that closely reflect those of cancer patients, where self-tolerance and aging are present simultaneously. The present review summarizes some defects found in the old immune system affecting the activation of antitumor immune responses, the strategies used to activate stronger antitumor immune response in the old and the use of a tolerant animal tumor model to target a self-tumor antigen for the optimization of immunotherapeutic interventions in the old.

Keywords Aging · Immunotherapy · Cancer

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The immune system and aging

Aging involves morphological and functional changes in all organs, including the cellular and humoral immunological functions. It is well established that older individuals do not respond to vaccine therapy as well as younger adults [41, 57, 67, 68, 96]. The dysregulation of the immune function in the elderly population is referred to as immunosenescence [1]. There is no particular factor or cause that can be pointed as the mechanism for the age related changes in the immune function, rather it is an accumulation of events that deteriorate the immune responses. Among the alterations that diminish the immune function in aged individuals are: thymic involution resulting in the decreased number of T- and B cells [97], decreased proliferation of T cells [89], modifications in production and secretion of cytokines [14, 31], reduced cytotoxic activity of $CD8^+$ T cells [3, 99] and qualitative deficiency of B lymphocytes with a reduced response to exogenous antigens [66]. Another major significant burden for the activation of an effective antitumor immune response in aged hosts is the availability in the number of naive T cells capable of reacting to new antigens [98] and deficiencies in antigen presenting cells (APCs) [28, 83]. This is coupled with a decrease in the early events of signal transduction and an overall decrease in proliferation of T cells in response to various forms of stimulus [79]. Moreover, clonal outgrowths are common in both CD8 and CD4 T cells of the aged [9]. In addition, it is believed that a significant burden for the activation of an effective immune response in aged hosts is the availability in the number of naïve T cells capable of reacting to new antigens. In the aged host, there is a decrease in the naive T cell population and an increase of memory T cells. Such an imbalance in memory/naive T cell subpopulations may account in part for the hyporesponsive state of the aged. Recent studies

indicate that old mice have higher numbers of regulatory T cells (T-regs) [65, 87] or myeloid derived suppressor cells (MDSC) [27] inhibiting the activation of immune responses. The accumulation of these alterations is believed to lead to immune dysfunction, such that individuals are prone to the consequences of infectious disease, autoimmunity and cancer.

Aging, cancer and the immune system

The incidence of cancer increases exponentially with advancing age [58, 108]. The age-associated increase in cancer may be due in part to a global decrease in cell-mediated immunity [55, 88]. A variety of immune-based therapeutic approaches for the treatment of cancer have been proposed, all of which depend upon the interaction and mobilization of the patient's immune system to effectively combat the disease. For an immune-based cancer therapy to succeed in the aged, it is essential to consider factors such as T cell tolerance, expression of relevant tumor associated antigens (TAA), immune regulation/suppression and age-associated changes in immune function. The goal of immunotherapy is to enhance the immune responses that will effectively kill the tumor cells while sparing the normal cells. The knowledge acquired over the past few decades have launched a variety of immunotherapeutic strategies based on the activation of critical mediators of immune responses, such as cytotoxic and helper T cells, APCs, antibodies, use of cytokines, chemokines and costimulatory factors. More recently, it has become evident that tumors induce networks of immunosupression capable of inhibiting the activation or propagation of an antitumor immune response. For example, tumor cells can downregulate the expression of MHC molecules making it more difficult to be recognized by T cells [72]. Also tumor and stromal cells produce a variety of factors, such as vascular endothelial growth factor (VEGF), prostaglandin-E2 (PGE2), IL10, transforming growth factor-β (TGF-β) and indoleamine 2,3-dioxygenase (IDO), in which these pleotropic cytokines favor the tumor growth by inducing angiogenesis, stimulating tumor growth and inhibiting the function of APCs and T cells [18]. Though suppressor cells, such as regulatory T cells [59] and myeloid derived suppressor cells (MDSC) play a key role in the maintenance of self tolerance [56, 80], these cells are infiltrated in high numbers at the tumor site [19, 95] and elevated levels are found in the peripheral areas of tumor bearing hosts [43, 90] inhibiting T cell responses [70, 104]. It is becoming evident that by targeting these networks of immunosuppression, the activity of the vaccine therapy can be significantly augmented resulting in an enhance tumor immunity [49].

Cancer statistics show a disproportionately higher burden of tumors in the older population [107]. Furthermore, the numbers of older people diagnosed with cancer is expected to increase since the average life span within the elderly population has increased. There is a tremendous plethora of publications describing a variety of immunotherapeutic strategies to fight cancer. However, there is only a handful of publications dealing with effect of aging and tumor immunity. Most of these publications indicate that old hosts do not respond to immunotherapeutic interventions. For example, Provinciali et al. [73] demonstrated that young and old mice immunized with TS/A tumors secreting IL-2 and challenged with wild type cells resulted in 90% protection in young animals, while only 10% of old animals were protected. The group of Provinciali also demonstrated that old BALB/c mice transplanted with a tumor cell line derived from a spontaneous tumor from Her-2/neu mice and vaccinated with DNA-Her-2/neu plasmids had lower protective immunity, when compared to young mice [74]. Similarly, Gravekamp et al. [23] demonstrated that DNA-vaccination against Mage-b is more effective in reducing tumor metastases in young mice when compared to old mice. Our group had utilized a simple model to evaluate antitumor immune responses in the old. The BM-185 is a pre-B lymphoma cell line that causes 100% mortality in Balb/c mice [53]. It has been established that enhanced green fluorescent protein (EGFP) is an antigenic molecule capable of eliciting an immune response and tumor cells expressing this protein are rejected. This cell line was transduced with the EGFP gene (BM-185-EGFP) and we utilized the EGFP as a surrogate tumor antigen. We evaluated whether there were differences in the ability of young and old mice to mount an immune response against the BM-185-EGFP tumor. Our data indicate that young Balb/c animals were successful in eradicating the tumor and are additionally protected against subsequent challenges with either the EGFP-modified or wild type tumor [56]. In contrast, aged Balb/c mice were succumbed to the BM-185-EGFP tumor. Even though BM-185-EGFP cells are highly immunogenic that raises several important questions: (1) why the old cannot mount an immune response against an immunogenic tumor? (2) What aspects of the immune system (innate or adaptive) are dysfunctional or altered in the old, preventing the activation of an immune response? (3) Can the immune responses be restored in the old? (4) If immune responses against immunogenic tumors can be restored in the old, can immune responses capable of controlling tumor growth against self-tumor antigens be induced in the old? Below I briefly describe and discuss some alterations that we have identified, which contribute in the dysregulation and alterations of the innate and adaptive components of the immune system, which are essential for the activation of an antitumor response in the old.

Immune-costimulation, antitumor responses and aging

Often the addition of co-stimulatory signals is used to enhance and augment the immunogenicity of tumors [36, 71]. For example, expression of B7.1 or B7.2 by tumor cells enhances the antitumor response resulting in the rejection of the tumor [47, 75]. In addition, molecules of the TNF receptor family, such as CD27, CD30, CD40, 4-1BB and OX40 have gained importance as co-stimulatory molecules delivering signals that prolong and propagate T cell responses [60]. For example, the administration of monoclonal antibodies against OX40 or 4-1BB induce a vast amplification of T cell mediated immune responses [22], inhibit apoptotic cell death [78], stimulate long-lived T cell responses [105] and significantly enhances antitumor immune responses [106]. We tested whether the expression of CD80 enhance or restore the antitumor responses against the BM-185-EGFP cells in old mice. When BM-185-EGFP tumors express CD80 molecules (BM-185-EGFP–CD80), old mice were able to reject the primary tumors; however, old animals could not develop memory responses [53]. The results indicate that the addition of CD80 partially restore the immune responses in old. Next, we evaluated the effect of adding anti-OX40 or anti-4-1BB mAb. The addition of anti-OX40 or anti-4-1BB mAb injections, markedly improved the ability of aged animals to respond, as 80% of animals injected with BM-185-EGFP and injected anti-OX40 or anti-4-1BB mAb cleared the tumor [53]. However, only ~30–40% of the old animals developed a protective memory response. Only when BM-185-EGFP–CD80 tumors were given in combination with anti-OX40 or anti-4-1BB mAb, 100% of the old mice rejected the primary tumor developed long term protective memory responses capable of rejecting a challenge against wild type tumors [53]. Our results also indicate that EGPF-specific CTLs were significantly enhanced, which directly correlate with the antitumor response. Although the injection of anti-OX40 and anti-4-1BB mAbs enhances the T cell responses, it is also possible that these antibodies enhance the antitumor responses by activating macrophages or other APCs [12, 46]. In another study, we compared the efficacy of dendritic cell (DC)-vaccination in young and old mice. Young and old mice were immunized with young and old DCs pulsed with apoptotic TRAMP-C2 tumor cells. Our results showed that DC-vaccination in young animals induced an antitumor response resulting in ~60% tumor growth inhibition, while minimal protection was observed in old animals [86]. DC vaccination plus rIL-2 further enhanced the antitumor response in young animals (~70–75% tumor growth inhibition), while it was ineffective in old animals. The enhanced antitumor immune responses were generated only when DC-vaccination and anti-OX-40 or anti-4-1BB mAb were combined, inhibiting tumor growth in both young and old mice [86]. These results suggest that deficiencies in T cell function associated with aging may be due to insufficient or inappropriate costimulatory molecule display by the APC, or that the aged T cell requires a different level of help than do young to achieve an effective primary response and give rise to memory cells. These results have important implications for the development of vaccination strategies in the elderly, indicating that the aged immune repertoire can be exploited for the induction of tumor immunity and that it is possible to convert aged animals from non-responder to responder status with the inclusion of additional costimulation [2, 53, 86].

Innate immune system, induction of antitumor response and aging

The innate immune response relies on the recognition of the antigen by receptors that recognize specific structures found exclusively in microbial pathogens termed pathogen-associated molecular patterns (PAMPs) [38, 101]. The recognition of PAMPs by APCs is mediated by the Toll-like receptor (TLRs) family [39, 94]. There are more than ten known TLR family members capable of sensing bacterial components, such as Poly-I:C (TLR-3), LPS (TLR-4), flagellin (TLR-5), Imiquimod (TLR-7), CpG-ODN (TLR-9) and other microbial products [30]. The TLRs have distinct patterns and locations of cellular expression. A wide variety of TLRs are expressed in immature or mature DCs, macrophages (MΦ) and monocytes; and these receptors control the activation of those APCs. Now that, specific ligands have been identified for most of the TLRs, it is finally possible for immunotherapy to move away from the nonspecific effects of whole bacterial extracts and determine whether the same or even better therapeutic responses may be induced using synthetic TLR-ligands. There is accumulative evidence indicating that targeting APC with different types of TLR-ligands results in the induction of a strong antitumor immune response resulting in the rejection of tumors [6, 40, 81, 109]. With respect to aging and TLRs, Renshaw et al. [76] demonstrated that the expression of TLRs in splenic and thioglycollate-elicited macrophages is reduced in aged animals, which is associated with lower secretion of cytokines following stimulation with various TLR-ligands. Boehmer et al. [4] also showed that age-related deterioration of TLR-mediated signaling is due to the decreased expression of mitogen-activated protein kinases. We evaluated whether in vivo targeting of APCs with TLR-ligands results in the restoration of the immune responses and activation of antitumor responses in the old. We compared the antitumor potential of TLR-ligands, such as Poly-I:C, LPS, flagellin, Imiquimod and CpG-ODN in young and old tumor bearing mice. Our results indicated

that only intratumoral (i.t.) injections of CpG-ODN induced the complete rejection of tumors in young and old mice. Intratumoral injections of Poly-I:C also induced the rejection of tumors in the young, but not in the old. We observed significant differences in the activation of immune responses following CpG-ODN and Poly-I:C injections in the aged. The induction of an antitumor response by CpG-ODN correlates with the activation of a Th1 type pro-inflammatory response resulting in the generation of $CD4^+$, $CD8^+$ T and, NK cell responses, activation of APCs and significant reduction in the number of Tregs in the old [85]. These studies indicate that not all TLR-ligands have the same effector function and that the selection of an adjuvant (e.g. CpG-ODN) is critical to optimize a vaccination strategy for the young and the old. Taken together, these results indicate that there is a TLR age defect altering the function of the old innate immune system [103].

Networks of immune suppression in the old

Tumor progression is strongly associated with immunosuppression. T regulatory cells, myeloid derived suppressor cells, IDO and B7 family molecules are up-regulated in tumor bearing hosts and contribute to tumor escape, immune tolerance and immune suppression. Understanding how these cells, factors or molecules are expressed in the old is very important to improve the efficacy of cancer immunotherapy in the elderly.

T-regs, antitumor responses and aging

Although it is quite likely that central tolerance deletes the bulk of high affinity self-reactive T cells [20, 21], there is accumulating evidence that homeostatic balance of the immune system is maintained, not only by clonal deletion, but also by subpopulations of immune cells with immunoregulatory properties capable of preventing responses to self-antigens [92, 93]. The most common regulatory cells are the T regulatory cells (T-regs) [59, 80]. T-regs could be $CD4^+CD25^+$ or $CD8^+CD25^+$ T cells, which are characterized by the expression of the forkhead lineage specific transcription marker, Foxp3 [32]. T-regs maintain and induce immune cell tolerance [80] by directly inhibiting T cells, NK cells and DCs through direct cell-cell contact mechanisms [59, 80]. Depletion of T-regs leads to organ specific autoimmune disorder [24, 82]. We have recently demonstrated that old mice contained twice the amount of $CD4^+CD25^+Foxp3^+$ and $CD8^+CD25^+Foxp3^+$ populations in spleen and lymph nodes when compared to spleens and lymph nodes from young mice [87]. Furthermore, depletion of $CD25^+$ cells with anti-CD25 mAb in old mice resulted in the rejection of the immunogenic BM-185-EGFP tumor cells and restored antitumor T cell cytotoxic activity against the surrogate EGFP tumor antigen. These results indicate that the accumulation of T-regs in the old inhibit or prevent the activation of immune responses. We have recently also demonstrated that there is a higher accumulation of T-regs at the tumor in old tumor bearing host than in young tumor bearing hosts [85]. These results are in agreement with the findings of Gregg et al. [26] that show the numbers of $CD4^+CD25^{hi}$ T cells (T-regs) are increased in humans. Recently Lages et al. [44] showed a higher elevation of T-regs in humans and mice that alters immune responses against *Leishmania major* infections. The imbalance of T-reg homeostasis could then predispose the aged to immune dysfunction, resulting in a higher risk of immune-mediated diseases, cancer or infections. Additionally, the accumulation or increased numbers of Tregs will affect or disturb the activation of antitumor immune responses in the old [85, 87] and the depletion or reduction of T-regs might be critical to optimally activate an immune response in the aged.

Myeloid derived suppressor cells (MDSC)

Myeloid derived suppressor cells (MDSC) are a heterogeneous population comprised of macrophages, neutrophils, granulocytes and DCs. These cells are characterized by the expression of CD11b and Gr-1 cellular markers. MDSC can suppress the activation of $CD4^+$ and $CD8^+$ T cells inhibiting the generation of an antitumor responses [64, 77, 84] MDSC are thought to be induced by a variety of cytokines and growth factors (e.g. TGF-β and VEGF), which are produced within the tumor microenvironment [17, 110]. Though the biology and pathological functions of MDSC under non inflammatory conditions are not fully understood, we have evaluated whether there were differences in the level of $CD11b^+$ $Gr\text{-}1^+$ cells between young and old mice. Our results indicate that old mice have a higher accumulation of $CD11b^+$ $Gr\text{-}1^+$ cells in spleen and bone marrow of old mice when compared to young mice. Furthermore, tumor samples of old mice have a higher incidence of $CD11b^+Gr\text{-}1^+$ cells than young tumors (M.A.D. and J.L., unpublished results). These results are in agreement with the recent report by Grizzle et al. [19] that show that in 12-month-old BXD12 mice there is a higher accumulation of $CD11b^+Gr\text{-}1^+$ cells than in 2-month-old BXD12 mice. Furthermore, they demonstrated that old $CD11b^+Gr\text{-}1^+$ cells are more suppressive than young $CD11b^+Gr\text{-}1^+$ cells. This raises the question as to why there is an accumulation of $CD11b^+Gr\text{-}1^+$ cells in the old and why they are more suppressive. A simple explanation for these findings could be as follows: typically, freshly isolated $CD11b^+GR\text{-}1^+$ cells do not have the capacity to inhibit T cells [43]. Only $CD11b^+GR\text{-}1^+$ cells isolated from an inflammatory environment, such as a tumor have the capacity to inhibit T

cells [7, 34]. There is accumulative evidence indicating that the production of a number of inflammatory cytokines, such as IL-4, IL-10, TGF-β and others are elevated in the old [16, 35]. Perhaps this inflammatory condition of the old promotes the accumulation and activation of $CD11b^{+}GR\text{-}1^{+}$ cells that subsequently could inhibit the activation of immune responses.

Indoleamine-2,3-dioxygenase (IDO)

Indoleamine-2,3-dioxygenase (IDO) is an immunosuppressive molecule capable of inhibiting T cells and other immune cells [62]. IDO is primarily present in APCs. The expression IDO on tumor cells is implicated in suppression of immune responses and tumors become resistant to immunologic rejection [48, 63]. Expression of IDO is correlated with poor clinical prognosis in several types of cancer [5, 37]. 1-Methyl tryptophan (1MT) is an important competitive inhibitor of IDO [15, 33]. On the basis of these findings, 1MT is now tested in clinical trials. We compared whether there were differences in cell producing IDO between young and old tumor bearing mice by immunohistochemistry. Indeed our results indicate that the number of DCs producing IDO is higher in the old than in the young. We also observed that the intensity of staining is stronger in old DCs than in young DCs. Recently, Pertovaara et al. [69] show that IDO was significantly higher in nonagenarian compared to young. It is not clear yet to us as to why IDO is up-regulated in the old, but taken together these results suggest that elevation of IDO is another possible mechanism by which T cells responses are inhibited in the old.

The B7 family molecules

The B7 family molecules consist of activating and inhibitory co-stimulatory molecules that positively and negatively regulate immune responses [25, 111]. The B7 family molecules are involved in the regulation of T cell tolerance as well as in the activation of T cell responses [45, 50, 91, 102]. The B7 family is comprised of seven members, which are: CD80 (B7.1), CD86 (B7.2), B7-DC PD-L2 or (CD273), B7-H1 (PD-L1 or CD274), B7-H2 (ICOSL), B7-H3 (CD276) and B7-H4 (B7S1 or B7x). Although, initially it was thought that many of these molecules were exclusively expressed on APCs, recent evidence indicates that many of the B7 family of molecules could be expressed on T cells as well indicating that they can act as both ligand and receptor to execute immunoregulatory functions [8]. There is a plethora of evidence that tumors express B7-H1 and B7-H4 are capable of inhibiting immune responses [42, 100]. We have initiated to analyze the expression of B7 family molecules on T cells and APCs from young and old mice. Preliminary results indicate that many of these molecules are differentially expressed between young and old T cells and APCs (M.A.D. and J.L., unpublished results). We are currently evaluating how the expression of some of these molecules interferes or affects the regulation of the immune responses in the old. Again, these results indicate that molecules which are involved in immune regulation are dysregulated in the old, which could further influence the activation of antitumor immune responses.

Analysis of immune responses against self antigens in aging tumor model

Although the use of the BM-185-EGFP or TUBO tumor models provides valuable information on the behavior and how it might be possible to manipulate the old immune system, these tumor models and many other tumor models used by other investigators rely on immunogenic tumor. As such, it will be more difficult to translate the results from these immunogenic tumor models into a clinical setting for the treatment of tumors in the old. It is well established that the T cell component of the aged immune system is dramatically compromised, i.e., the immune response is impaired and the repertoire is constricted. To date, very little data exists on the immune responses against self-tumor antigens in the aging population. So far there are no reports evaluating antitumor immune responses in aged tumor models, where tolerance and spontaneous tumor progression are present simultaneously. The effect of aging on T cell tolerance remains to be elucidated. Therefore, it is clear that there is a need for relevant animal tumor models, which include aspects of self-tolerance and development of spontaneous primary and metastatic tumors in the elderly. Models like this are critical for the development and optimization of more accurate cancer-related immunotherapeutic strategies for the elderly.

The Her-2/neu transgenic mice over express the rat Her-2/neu oncogene under the control of the MMTV promoter [29, 61]. These animals develop spontaneous tumors and the clinical progression and pathogenesis of the disease closely resembles what is seen in human patients with breast cancer. Therefore, the neu mouse is a clinically relevant animal tumor model that can be used to (1) define the nature of the responsiveness to self-tumor antigens, (2) analyze the requirements for initiating and sustaining antitumor responses in tolerant hosts and (3) evaluate strategies for overcoming or circumventing tolerance to self-tumor antigens that can be effectively used as targets for immunotherapy. Over the past few years, my laboratory has studied the immune responses of Her-2/neu transgenic mice (neu mice). In order to be able to evaluate peptide specific immune responses in neu mice, we crossed the neu mice with the A2.1/Kb transgenic mouse (A2 $\times$ neu) [51, 52], so

that we could evaluate A2.1-Her-2/neu responses against the p369–377 and p773–782 peptides that we have identified [54]. Our results indicate that T cells obtained from A2 × neu mice were less efficient in recognizing target cells loaded with the peptides when compared to T cells derived from A2 × FVB mice (control animals, A2.1/Kb transgenic mice crossed with FVB mice) [51, 52]. A2 × neu mice contained a low avidity repertoire to neu antigens indicating that these animals are tolerant to neu antigens [51, 52]. Analysis of the antitumor responses indicate that multiple immunizations with DCs pulsed with the neu antigens is not effective to control the tumor growth in these animals [10, 11]. As described above, the use of intratumoral injections of CpG-ODN is an effective method for the induction of antitumor responses. Recent work from my laboratory indicates that intratumoral injection of CpG-ODN plus depletion of T-regs completely reject the primary tumor in A2 × neu mice and animals developed protective memory responses [85]. These results suggest that some immunotherapeutic strategies are able to overcome tolerance and are suitable for tumor elimination.

We have observed that one of the consequences of crossing FVB-Her-2/neu mice with HLA-A2 mice (A2 × neu) is that spontaneous tumors appear in these animals when they are 22–27 months old. Therefore, the A2 × neu mouse model represents a unique model, where aging, tolerance and spontaneous tumor progression are present simultaneously. There are several reasons as to why there is a lack of more studies evaluating antitumor responses in old mice and one critical factor is the extended time period, which is necessary to age these mice and the costs incurred towards this aging. To test the antitumor responses in the A2 × neu mice, we have developed a tumor model utilizing a tumor cell line derived from spontaneous tumors (N202.A2 cell line) that facilitates the rapid evaluation of the antitumor responses. We have previously demonstrated that N202.A2 cells grow in young A2 × neu as a consequence of immune-tolerance, but are rejected by young A2 × FVB mice [51]. When old A2 × FVB and A2 × neu mice were implanted s.c. with N202.A2 cells, surprisingly we observed that tumors formed in old A2 × FVB and as expected in A2 × neu mice [13]. Analysis of immune responses against the p773 indicates that the CTL activity from young A2 × FVB or A2 × neu mice was stronger when compared with the CTL activity of A2 × FVB or A2 × neu old mice. Interestingly, a very weak CTL activity was detected in old A2 × neu mice [13]. Taken together, these results clearly demonstrated that aging severely compromises the immune system and that old A2 × neu mice did not have the same capacity to prime a T cell response as young A2 × neu mice. In agreement with our previous reports [87], old A2 × FVB and A2 × neu mice have higher numbers of T-regs when compared to young A2 × FVB and A2 × neu mice. Our previous studies indicate that intratumoral injections of CpG-ODN could rescue the immune responses in the old and promote antitumor responses in young Her-2/neu mice. We tested the effect of intratumoral injections of CpG-ODN plus T-reg depletion in young and old A2 × neu mice. As we have observed previously, young A2 × neu mice rejected the tumor and developed protective memory responses. In contrast, this combination treatment only prolonged the survival of old A2 × neu mice, however, none of the animals could reject or control the tumor growth [13]. These results indicate that although it was possible to restore the antitumor responses in young tolerant hosts after targeting CpG-ODN to the tumor site plus T-reg depletion, the same therapy was not as effective in old tolerant hosts. These results raise an important question: why do old A2 × neu mice after CpG-ODN and T-reg depletion not mount an immune response capable of rejecting the tumor? Considering that old mice have an excess of networks of immune regulation (more T-regs, MDSC, IDO, etc.), we thought that if intratumoral injections of CpG-ODN were combined with T-reg depletion and inhibition of IDO, it could result in a stronger antitumor response. However, our results indicate that animals treated with this triple combination became very sick and 70% of the animals died. These results suggest that we should keep a balance between activating a safe and effective antitumor immune response and inducing an autoimmune reaction. Although, we might be able to develop immunotherapeutic strategies capable of overcoming tolerance and activating effective antitumor responses in young tolerant hosts, the same therapies may be not equally effective in the old.

The A2 × neu mice represent the first animal model through 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. The A2 × neu mouse model will enable us to uncover some of the cellular basis for the decline in immune function in the elderly and determine 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. Only models like this will allow the optimization of vaccination strategies to effectively stimulate tumor immune responses in both the young and the old.

Future directions

The ultimate goal in relation with aging and the immune system is to identify an optimal strategy capable of activating

an antitumor immune response that controls the tumor growth. Besides the effect that tolerance has on the immune responses against self-tumor antigens and the excess of immune regulation in the old, T cells and APCs from aged mice carry immune-alterations that also need to be overcome to optimally activate an immune response. Over the past 20 years, many groups had described the causative effects of why the immune responses are diminished in the old. However, we still do not have a clear understanding at the cellular, molecular or physiologic level what dictates the dysfunction and senescence of the old immune system. Until we do not clearly identify the alterations or defects of the aged immune system, we might be in a difficult position for optimizing the antitumor immune responses in the old. For example, it is very important to evaluate and understand how the function of old T cells and APCs are altered to effectively intervene and optimally stimulate an antitumor immune response. Our group has taken a genomic approach to evaluate the functional differences between young and old mice. We isolated naive and memory $CD4^+$ and $CD8^+$ T cells from young and old mice and cells were non stimulated or stimulated with anti-CD3 plus anti-CD28 for 4, 12, 24 and 72 h and evaluated by microarray analysis. Preliminary data from these studies revealed that many of the genes that regulate the function of T cells are differentially expressed in young and old naive and memory $CD4^+$ and $CD8^+$ T cells. For example, Fig. 1 shows differential expression of genes at time zero and under stimulation conditions. We expect that this gene expression analysis will provide valuable insight into the molecular pathways and processes that are different between young and old T cells. This information is critical in order to understand why these cells differ in their biological functions. The observation that many genes are differentially regulated between young and old T cells might provide a novel strategy to target some of these genes in old T cells to enhance or restore the aged immune responses. We hope that the analysis of these genomic profiles will provide important hints or clues as to why old T cells are dysregulated. We still do not understand the role of the differential expression of genes observed between young and old T cells and further studies will be necessary to unravel the precise role of these genes in T cell function.

We are taking a similar approach in order to evaluate the function of APCs. I strongly believe that until we understand the intrinsic defects or alterations of the old immune cells at the molecular level, it will be very difficult to optimize an antitumor immune response in the elderly. Currently we have the tools (e.g. genomic or proteomic analysis) to dissect the old immune system and the application of a system biology approach could provide valuable information as to why as well as, what is wrong with the aged immune system.

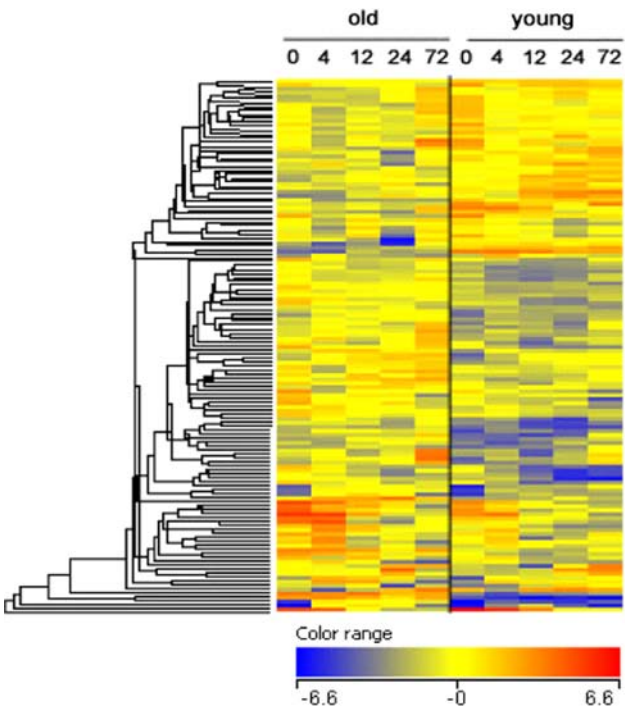

Fig. 1 Gene tree depicting the groups of expression patterns that differentiate the "old" expression profile from the "young" expression profile over time. A kinetic analysis of changes in gene expression after activation of naive CD4 cells derived from old mice and young mice reveals that 134 genes are differentially expressed in all five time points examined. These genes exhibited a greater than twofold change in expression levels between samples from old and young mice activated with α-CD3 and α-CD28 for the same time period, across all five time points. See *color scale* for intensity levels

Concluding remarks

There is undisputable evidence that the occurrence of cancer augments with aging. This could be attributed to a multitude of factors including the dysregulation of the immune system. The immune system of the elderly is very different from the young and it is difficult to extrapolate results obtained in the young, for use in the old. Most of the studies to evaluate the effect of immunotherapy on cancer have been conducted in the young without considering the effect of age-associated changes in immune function. Studies from my laboratory and others groups indicate that immunotherapeutic interventions could be effective in young animals, but that the same therapies are not as effective in old animals. Considering that the majority of cancers occur in the elderly and that the incidence of cancers is expected to increase due to the expansion of the aging population, it is imperative to pay attention to the effect that age imposes on the immune system to assure the effectiveness of immunotherapeutic interventions in the old. This raises the question as to whether it will be possible to do a type of immunological screening to evaluate if elderly patents will be responsive to the vaccine therapy. If this approach is

taken, it might be possible to identify that some elderly will have better T cell responses than others. Then the question becomes whether or not to treat those who show a weak or no response. Our job in the next few years is to figure out how to robust the old immune system, in order for all elderly patients to have a chance to generate an effective immune response. Critical for this is to better understand the intrinsic defects of the old immune system to properly stimulate primary and memory antitumor responses. Animal models like the A2 × neu mice, where tolerance and aging are present at the same time will enable us to uncover some of the cellular and molecular basis for the decline in immune function in the elderly and determine conditions and strategies to augment the antitumor activity against self-tumor antigens in the aged. Importantly, 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 in the clinical setting for the treatment of cancer in the old. Only with this knowledge, we will be able to successfully customize tumor vaccines to be effective for the treatment of tumors in the old.

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