Published in final edited form as:

Vaccine. 2009 February 11; 27(7): 1093–1100. doi:10.1016/j.vaccine.2008.11.112.

DEVELOPMENT OF EFFECTIVE VACCINES FOR OLD MICE IN A TUMOR MODEL

David N Posnett^{*,†}, Manuel E. Engelhorn[†], Yun Lin[†], Taha Merghoub[†], Fei Duan[†], Jedd D Wolchok[†], and Alan N Houghton[†]

* Division of Hematology/Oncology, Dep. of Medicine, Weill Medical College of Cornell University

† The Swim Across America Laboratory, Memorial Sloan Kettering Cancer Center

Abstract

Vaccines are often inefficient in old people and old mice. Few studies have focused on testing vaccines in old populations. Here we used DNA tumor antigen vaccines against melanoma and showed that old mice were not protected. Vaccines incorporating fusions of the tumor antigen with microbial adjuvant proteins OmpA (*E. Coli*) or Vp22 (*Herpes simplex virus-1*) dramatically improved protection of old mice. The mechanisms by which these adjuvant proteins act are distinct. TLR2 was not required for either OmpA or Vp22. Antigen processing and presentation were not boosted by these fusion constructs. However, fusion constructs with Vp22 gave a strong CD4 response to B16 melanoma and the OmpA response is MHC-II dependent. Both adjuvant fusion constructs stimulated CD4 and CD8 responses otherwise diminished in old mice.

Keywords

Tumor Immunity; Vaccination; Antigen Presentation/Processing; Rodent; Immunodeficiency of aging

Introduction

Vaccines are indicated for the very young, the infirm and the aged populations. However, these very same populations are frequently immunodeficient and fail to respond effectively to vaccination. Curiously there are few studies attempting to optimize vaccines for aged human populations and remarkably few studies in old mice. A need for such studies has been prominently discussed[1].

Cancer is predominantly a disease of old people (<http://seer.cancer.gov/publications/raterisk/rates22.html>). Exciting new studies have shown efficacy of vaccines for cancers in experimental murine systems, even in therapeutic situations where vaccination is started after experimental tumors have grown in vivo[2]. Efficacy has also been demonstrated in

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

dogs with naturally arising melanomas[3] and an approved vaccine for dogs is now available. Clinical trials in humans with melanoma and other cancers are in progress[4,5].

Immune responses to tumor antigens and vaccine responses in the aged are both deficient. The bar is therefore set high in attempting to surmount both of these problems. Our goal was to test the efficacy of melanoma vaccines in old mice and to improve these vaccines such that they could be as efficacious as in young mice. Moreover, we expected that an understanding of the mechanism by which improved vaccines overcome the immunodeficiency of aging, would lead to some basic insights in to dysfunction of the immune system in the elderly.

To approach these questions we used established DNA vaccines for melanoma tumor antigens, such as tyrosinase related protein-1, Tyrp-1. Such vaccines have been administered by “gene gun” to young mice with excellent results. To break tolerance these vaccines must contain heterologous epitopes (f.ex. human Tyrp-1 administered to mice), or random mutations[2], or engineered heteroclitic epitopes[6], referred to as “epitope enriched”, for example Tyrp-1ee.

We now show that old mice, in contrast to young mice, are not protected by these vaccines. We also show that improved vaccine constructs containing microbial adjuvant proteins are equally protective for both young and old mice. The mechanisms by which these microbial adjuvant proteins may boost the immune response of old mice are investigated.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TLR-2 knock out mice on a C57BL/6 background were obtained from Dr. Eric Pamer at MSKCC and bred at MSKCC. They were backcrossed for 9 generations. Young mice were 1–3 months old. Old mice were 18 – 22 months old. For experiments comparing young and old mice, both were purchased from the colonies of the National Institute of Aging. Care of mice was in accordance with MSKCC institutional guidelines and these studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Peptides

Peptides were synthesized and purified by HPLC to >80% purity by GeneMed Synthesis, Inc. (San Francisco, CA). The peptides used were both wild type(wt) and mutated (mut) versions of known immunodominant peptides of Tyrp-1: Tyrp-1_{455–463} = 455wt (TAPDNLGYA, D^b-restricted; mutated variant 455mut TAPDNLGYM); Tyrp-1_{522–529} = 522wt (YAEDYEEL, K^b-restricted; 522mut YAYDYEEL); Tyrp-1_{521–535} = 521wt (RYAEDYEELPNPNHS, I-A^b restricted; 521mut RYAYDYEELPNPNHS).

Plasmid constructs

mTyrp-1 (autologous sequence of C57BL/6 mice), Tyrp-1ee[6], Tyrp-1ee-OmpA, Tyrp-1ee-Vp22, Tyrp-1ee-Exo (*Pseudomonas aeruginosa* exotoxin A) and Tyrp-1ee-Ent (subunit B of the heat-labile enterotoxin from *E.coli*) have each been described[7]. Each of these

constructs was generated using the pCRAN multiple cloning site variant of pCR3 (Invitrogen, Carlsbad, CA) which served as the parental backbone for all Tyrp-1ee fusion constructs. All fusion partners were positioned N-terminal to the Tyrp-1ee antigen coding sequence.

Immunizations

Genetic immunization using DNA-coated gold particles was performed using a gene gun provided by PowderMed, Inc. (Oxford, UK), as previously described [8]. Four (or six for experiments in Figures 4,5) shots at 400 pounds/inch² were delivered to the depilated abdominal skin for a total of 4–6 µg plasmid DNA/mouse/vaccination under anesthesia. Vaccinations were given 4 times at weekly intervals and tumor challenge was 5 days after the last vaccination. For isolation of antigen exposed dendritic cells (DC), vaccination was given once and mice were sacrificed 1–6 days later.

Tumor challenge

B16 melanoma cells were injected IV at 50,000 cells per mouse. Sentinel mice were sacrificed beginning 2–3 weeks after challenge to determine the optimal time point for sacrifice. This was usually about 30 days after tumor challenge. Lungs were dissected and washed in 5% H₂O₂. Counts of visible tumor metastases were then obtained.

IFN-γ ELISpot assay

IP-Multiscreen plates (Millipore, Burlington, MA) were coated with 100 µl anti-mouse IFN-γ antibody (10 µg/ml; clone AN18, MabTech, Mariemont, OH) in PBS for 2 hours at 37°C, then washed with PBS and blocked with RPMI containing 7% fetal bovine serum for 2 hours at 37°C. T cells were G8 hybridoma cells specific for peptide 455. They recognize both 455wt peptide and 455mut present in Tyrp-1ee and in all fusion constructs. T cells were plated at 30,000 or 50,000 cells/well. Dendritic cells (DC) were obtained from 3–10 pooled mice from draining LNs. These mice were sacrificed at varied time points after DNA vaccination (D1 to D6). DC were isolated by MACS sorting with CD11c as per the manufacturer protocol. 30,000–330,000 DC were plated/well. To determine the maximal degree of stimulation of the G8 T cells, exogenous Tyrp-1 455-A463M peptide was added at 1 µg/ml to wells. After incubation for 24–48 hours at 37°C, plates were washed with PBS +0.05% Tween and incubated with 100 µl/well of biotinylated-antibody against mouse IFN-γ (2 µg/ml; clone R4-6A2, MabTech). Plates were incubated for an additional 2 hours at 37°C and spot development was performed as described [9]. Spots were counted with an Automated ELISpot Reader System with KS 4.3 software (Carl Zeiss, Thornwood, NY). For CD4 or CD8 ELISpots, the T cells were enriched by magnetic bead sorting (MACS) and plated at 100,000 cells/well with either peptide pulsed EL4 cells, splenocytes or B16 melanoma cells (20,000 to 100,000 cells/well). Generally, the peptides used were mutated peptides corresponding to the immunogen sequence, or wild type peptide sequences, thus testing the ability of the vaccines to generate anti-self immune responses. Peptides were used at 1 µg/ml (CD8 ELISpot assay) or 100 µg/ml (CD4 ELISpot assay).

Statistics

T tests were used to compare groups.

Results

Old mice are known to be immunodeficient and lack efficient vaccine responses. Previous reports on antitumor immunity in aging mice have described profound impairments with age[10–15]. However these studies did not examine the B16 melanoma system in C57Bl/6 mice. Therefore, we vaccinated young and old mice with the tumor antigen Tyrp-1ee, previously shown to be a powerful vaccine for both protection against subsequent tumor challenge and for therapy of established tumors[6]. As indicated in Fig. 1 old mice were poorly protected compared with young mice.

In early experiments, vaccinated mice were challenged ID or SQ with B16, rather than IV. Untreated old mice had inherently slower tumor growth in the intradermal or subcutaneous tissues, as previously reported[16] and presumably related to poor vascularization of nascent tumors in the skin of old mice. This was less evident with tumor metastases to the lung, a highly vascular organ, after IV challenge. However, lesser numbers of metastases in naïve unvaccinated old mice compared with naïve young mice were noted. Thus, it is possible that neovascularization of fast growing tumor metastases is relatively deficient in old mice, even in the lungs.

The poor protection in old mice following vaccination with Tyrp-1ee, an otherwise very potent vaccine, was overcome when this antigen was fused to microbial adjuvants (Fig. 1). Both OmpA and specially Vp22 were effective.

We next asked what mechanisms might be underlying the greater efficiency of OmpA and Vp22 containing vaccines in old mice. Was this in fact due to greater numbers of tumor antigen specific CD8 cells? CD8 splenocytes from vaccinated mice were isolated and tested in vitro in an ELISpot assay for production of IFN- γ . As shown in Fig. 2 the Tyrp-1ee-Vp22 vaccine increased the number of IFN- γ spots compared with the usual Tyrp-1ee vaccine. This increase occurred most strikingly in old mice. CD8 cells responding to both major Tyrp-1 Kb-restricted wild type epitopes (455 and 522), and CD8 cells responding to self Tyrp-1 antigen expressed by B16 melanoma cells were all increased in old mice vaccinated with Tyrp-1ee-Vp22. These results clearly show that Vp22 containing vaccines result in increased numbers of antigen specific CD8 cells, a finding that correlates with in vivo control of metastases (Fig. 1). The results imply that the effect of Vp22 was upstream of the generation and maturation of effector CD8 cells. This could include a) direct effects on CD8 cells, b) effects on CD4 helper T cells, c) effects on maturation, processing or migration of APCs. or any combination of these three major categories.

In the case of OmpA, prior work had suggested that this protein shared by many Gram negative bacteria, bound to TLR2 on antigen presenting cells (APC) and resulted in their activation, f.ex. IL8 production. However key experiments implicating TLR2 were performed in vitro with transfected fibroblasts, expressing one of several transfected TLR molecules[17]. Following this lead we reasoned that TLR2^{-/-} mice would not show

enhanced vaccine responses when using OmpA vaccines. However, as shown in Fig. 3, CD8 cell responses to Tyrp-1ee-OmpA, Tyrp-1ee-Vp22, and other fusion constructs were similar, regardless of whether TLR2 was present or not. This in vivo experiment calls in to question the proposed TLR2 dependent mechanism proposed for OmpA[17]. One caveat however, is that our constructs were designed using OmpA derived from *E. Coli* while the prior report examined the effects of OmpA derived from *Klebsiella*.

Prior work had indicated that Vp22 facilitated cell to cell transfer of genetic material which might be beneficial after gene gun transfection, for example by promoting plasmid DNA or RNA transfer from keratinocytes to cutaneous dendritic cells (DC) [18–21]. However, other results using GFP encoding plasmids, with or without Vp22, were not supportive of this hypothesis[22,23]. If Vp22 did in fact facilitate spread of antigen encoding DNA from keratinocytes to DC, within the dermis or epidermis, after gene gun immunization, one expected outcome would be an increase in antigen loaded dendritic cells arriving in the draining LNs. We surmised that an assay for biologically competent DC, with endogenously processed antigen in the MHC-I groove, would best detect numeric differences or altered maturation of these cells. Assays have been described in which isolated DC from draining LNs have been used to present endogenously processed antigen, such as OVA, to homogeneous TCR transgenic T cells [24]. In addition to detecting effects of Vp22 on DC, such an assay should be able to confirm whether OmpA has the ability to accelerate migration of antigen loaded DC from the skin to the draining LNs as suggested by others[25].

A T cell hybridoma specific for the immunodominant epitope of Tyrp-1 (peptide 455) presented by H2 Kb was prepared (unpublished data, Jun Lin et al). This hybridoma, called G8, reacts specifically with both the non-mutated self peptide and with the altered epitope, 455mut contained within the immunogenic constructs such as Tyrp-1ee. DC were magnetically sorted based on expression of CD11c from draining LNs at various indicated time points after gene gun vaccination. DC were then incubated with G8 hybridoma cells and IFN- γ production was assessed by ELISpot assay.

Endogenous processing of Tyrp-1ee derived peptide 455 in mice vaccinated with either Tyrp-1ee, Tyrp-1ee-OmpA or Tyrp-1ee-Vp22 was examined. Small responses assessed on days 1 or 4 after vaccination were detectable with DC from Tyrp-1ee and Tyrp-1-Vp22 immunized mice: about 2–4 fold greater than background levels with naïve DC (not shown). When exogenous 455mut peptide was added to the coculture of DC with T hybridoma cells, IFN- γ spots were 40–80 times higher indicating that the DC were functionally capable of presenting peptide and that the T cells were capable of responding.

As assessed by the influx of FITC+ DC, after painting skin with FITC and after gene gun immunization, greater than 10% of CD11c+ DC in the LN are FITC+ at 24 – 48 hours (not shown). These numbers were no different when using OmpA or Vp22 containing vectors. Others have calculated that 25–30% of CD11c+ DC in the draining LN are recent immigrants from the skin even without gene gun vaccination[26]. Thus, there are large numbers of dermal DC and epidermal LC that leave the skin after gene gun immunization [24] and there are large numbers of immigrating DC that enter the draining LN after gene

gun immunization. To address the efficiency of antigen processing, which is clearly sufficient for in vivo tumor protection (Fig. 1), one can compare the relatively small numbers of antigen loaded DC in the draining LN with the large numbers of migrating DC after gene gun immunization.

Therefore we further assessed the numbers of immigrating DC in the draining LNs after gene gun immunization (Fig. 4) by comparing dose response curves of varying numbers of DC either a) loaded in vivo 5 or 7 days post gene gun immunization, or b) loaded in vivo 7 days post foot pad immunization with 455mut peptide in Titermax adjuvant, or c) loaded ex vivo with 455mut peptide and diluted with unpulsed DCs to obtain known percentages of pulsed DCs. Variable numbers of DCs were then incubated with a constant number of G8 hybridoma cells. The plateau of ELISpot values obtained with each curve (Fig. 4) was proportional to the known percentage of pulsed DCs in the preparation. By extrapolation, the curve obtained with DCs from peptide 455 + Titermax immunized mice approximates 1% pulsed DC. This indicates that 6 days after immunization with peptide 455 + Titermax, approximately 1% of CD11c+ DC from the draining LNs were loaded with 455 antigen.

One might expect lower percentages of in vivo loaded DCs, after using an intact immunogen requiring processing of peptide 455. In addition, relative inefficiency of transfection of skin DCs after gene gun immunization might further decrease the fraction of DC loaded with 455mut in the draining LN. In deed, 1–6 days after gene gun immunization, we measured no more than 40 ELISpots/20,000 G8 cells regardless of the ratio of DC:T cells (Fig. 4). This suggests that less than 0.01% of CD11c+ DC from the draining LNs were loaded with endogenous antigen after gene gun immunization. Similar results were obtained with all constructs tested: Tyrp-1ee, Tyrp-1ee-OmpA, and Tyrp-1ee-Vp22, in both young and old mice. Therefore, Vp22 and OmpA do not amplify DC migration or DC antigen processing as measured in vivo by this reverse ELISpot assay.

Microbial adjuvant proteins might enhance vaccine responses by providing epitopes presented by MHC-II to CD4 helper T cells. IFN- γ ELISpot responses of isolated CD8 cells obtained from vaccinated mice, were examined in both wt and MHC-II knock out mice[7]. While all the responses were muted in MHC-II^{-/-} mice, those that received Tyrp-1ee-Vp22 vaccines still had a CD8 response suggesting at least partial independence from CD4 help[7]. By contrast, mice vaccinated with Tyrp-1ee-OmpA lacked any CD8 response in MHC-II^{-/-} mice[7]. Therefore, this response was completely dependent on a response to an MHC-II restricted antigen, most likely provided by the OmpA protein itself (see below).

In additional experiments we determined that Vp22, but not OmpA, needed to be covalently linked to the Tyrp-1ee antigen for maximal immuno-enhancing effects (not shown). This further suggested that Vp22 acts via mechanisms that differ from OmpA.

To further assess the role of CD4 responses after immunization with fusion constructs, we performed CD4 ELISpot assays for IFN- γ . CD4 cells from mice immunized 3 times at weekly intervals were isolated from spleen or LNs. Responses were tested to B16 melanoma cells, or to splenocytes. Mice Immunized with Tyrp-1ee-Vp22 had a significant response to B16 melanoma cells (Fig. 5,6), primarily in LNs rather than spleen (not shown). This

response exceeded the response observed after Tyrp-1ee-OmpA or Tyrp-1ee immunization (Fig. 5). Pulsing the B16 cells with 521wt immunodominant peptide did not alter the response of CD4 cells to B16 (not shown). These data suggested that CD4 cells from Tyrp-1ee-Vp22 immunized mice were seeing an unknown epitope expressed by B16 melanoma cells.

B16 normally express low levels of MHC-II. A variant of B16 (B16 Gabi) expressing high levels of MHC-II, and another variant expressing transfected Tyrp-1ee, were examined (Fig. 6). The previously noted CD4 response was observed with all variants of B16. In order to determine whether a Vp22 derived peptide might be generating this response we immunized with a plasmid encoding just Vp22 and also with a mixture of two plasmid DNAs (Vp22 alone + Tyrp-1ee) bound to the same gold particles. Fig. 6 shows that Vp22 needed to be expressed as a fusion construct with Tyrp-1ee to observe the full CD4 response to B16. This result suggests that an epitope of Tyrp-1ee is processed from the fusion protein only. Alternatively, an immunogenic epitope of Vp22 may be uniquely processed from the fusion protein, generating a CD4 response to a cross-reactive but still unknown B16 antigen.

To simultaneously compare both CD4 and CD8 responses in old and young mice we immunized 4 times at weekly intervals with various constructs and isolated both CD4 and CD8 T cells from draining LNs and spleen. As expected (see Fig. 2), CD8 responses to the immunodominant peptide 455 of Tyrp1 were markedly reduced in old mice compared with young mice immunized with standard Tyrp-1ee vaccine (Fig. 7A). This deficiency of old mice affected the response to 455mut, the heteroclitic response to 455wt peptide, and the response to B16 melanoma. In each case the response was rescued in old mice immunized with fusion constructs, specially with Tyrp-1ee-Vp22 (Fig. 7A). In the same mice, CD4 responses to immunodominant 521mut and 521wt peptide and the response to B16 described above (in Fig 5,6), were deficient in old mice immunized with Tyrp-1ee (Fig. 7B,C). Immunization with the fusion constructs minimally enhanced the responses to 521 peptides (Fig. 7B), but enhanced the response to the unknown epitope of B16 more significantly (Fig. 7C). These trends were similar for both LN and spleen derived CD4 cells. Thus, both deficient CD8 and CD4 responses of old mice to a tumor vaccine were rescued by using microbial fusion constructs such as Tyrp-1ee-OmpA and Tyrp-1ee-Vp22.

Discussion

This paper shows that vaccine responses in old mice can be augmented using microbial “adjuvant” genes incorporated in to DNA vaccines. These adjuvants augment not only T cell responses to the dominant and subdominant epitopes of the vaccine antigen (Tyrp-1ee), but also augment cross-reactive or heteroclitic responses to the self-antigen expressed by B16 melanoma cells. This likely results in improved in vivo control of the tumor as shown in Fig. 1.

These data lend support to the notion that vaccines can be optimized for usage in old animals and elderly humans. Others have reported on different approaches to the same problem. Although early antigen responses of naïve CD8 cells are normal in ageing[27], CD4 cell responses are often impaired. CD4 priming and effector function can be rescued

with added IL2 [28–30] or with cocktails of inflammatory cytokines, namely TNF α , IL1 and IL6, or with added LPS[31]. Pro-inflammatory cytokines may promote a Th1 response known to be weak in old subjects receiving influenza vaccines. Additional co-stimulation of T cells with antibody to 41BB may have a similar effect [32]. A vector prime/protein boost vaccine has been shown to partially overcome the deficient vaccine response in old mice and this was associated with 2-fold lesser percentages of FoxP3+ cells in the tumor tissues[33]. Finally CpG-containing oligodeoxynucleotides, which stimulate TLR9 on antigen presenting cells, have been shown to boost antibody and CD4 T cell responses to a pneumococcal vaccine [34] and to enhance hepatitis B vaccines [35] in old mice. In sum, vaccines can be optimized for the elderly through various methods that either boost antigen presentation, CD4 responses, and/or CD8 responses, perhaps by decreasing Treg suppression or by providing otherwise deficient pro-inflammatory cytokines and correcting deficient responses to these cytokines.

The microbial adjuvants described in this paper have not yet been tested in old animals. As these proteins derive from commensal organisms that everyone is exposed to, there is little a priori concern that immunization with such compounds should cause serious side effects in humans or lead to neutralizing antibody formation that might reverse their beneficial effects. We wanted to understand the mechanisms by which these proteins augment the efficacy of vaccines so dramatically in old mice. Conceivably, understanding these mechanisms might shed light on how the immune system of old mice is deficient in its response to a vaccine.

For OmpA the reported mechanism, namely binding and stimulation of TLR2, was based on an in vitro experiment with transfected 293 cells[17]. When transfected with TLR2, but not other TLR molecules, these cells secreted IL8 after exposure to OmpA. This experiment however did not address the in vivo mechanism(s) of OmpA in augmenting vaccine responses. Herein we show that TLR2^{-/-} mice show normally augmented vaccine responses to OmpA containing vaccine. Thus, TLR2 activation is not required for the in vivo mechanism. Moreover, we noted that OmpA responses were critically dependent on the presence of MHC-II[7]. Thus, a likely explanation is that OmpA provides a strong CD4 helper response due to, as yet unidentified, helper epitopes within the OmpA protein that are presented to CD4 cells by MHC-II molecules. Augmenting CD4 help is certainly compatible with current notions of a deficient CD4 response in aged mice and humans [36,37].

Regarding the proposed mechanism for Vp22, this protein is normally expressed in HSV-I infected cells and contained in virions where it may bind to viral RNA. In Hep2 cells transduced with a viral GFP-fusion protein, the presence of co-transfected Vp22 resulted in transfer of GFP to co-cultured Vero cells [18]. These virologic data have spawned a literature on Vp22 suggesting that Vp22 traffics between cells along with attached moieties [38], as in the case of HSV-I, where this might facilitate early spread of viral infection from cell to cell. This was suggested to be due to a positively charged transduction domain causing cell surface adherence and endocytosis[20] and to facilitate intercellular antigen spread after DNA vaccination [21]. However, fixation procedures often used for cells prior to microscopic examination of the subcellular localization of Vp22 or Vp22-GFP have been shown to induce seepage of Vp22 from the nucleus[39]. Several authors have been unable to reproduce intercellular trafficking of Vp22 as assessed with GFP constructs[22,23].

After DNA vaccination plasmid DNA must be expressed in skin derived DC. Either these cells are directly transfected (probably a rare event) or they phagocytose dying keratinocytes and acquire antigen by “cross-priming”. At any rate, the DNA encoding the antigen must be transcribed and translated. The relevant peptide must be processed in endosomes or in the cytoplasm and loaded on nascent MHC-I molecules, which must transport the antigen to the cell surface. In addition, antigen-presenting cells must have undergone appropriate maturation. Our reverse ELISpot assay tests all these requisite steps prior to Ag presentation to specific T cells. When DCs from draining LNs are co-cultured with a pure population of peptide specific T cells, such as the G8 hybridoma, the response should be proportional to the frequency of mature DC containing endogenously processed antigen. In vivo dose response curves with footpad administered peptide (Fig. 4) showed that 1% of the mature DC in the draining LN are loaded with peptide, when measured 7 days after peptide immunization. However, after gene gun immunization less than 0.01% were loaded. Therefore, the combined efficiency of gene gun transfection, maturation and antigen processing of skin derived DC appears to be quite low.

The data show no advantage of Tyrp-1ee-Vp22 or Tyrp-1ee-OmpA over Tyrp-1ee vaccine in this assay in old or young mice. This indicates that neither Vp22 or OmpA have a major effect on immigration, maturation and antigen processing of skin derived DC in the draining LNs, at least not in the 1–6 day period after a single vaccination, or 6 days after 4 consecutive weekly vaccinations.

Although the response to Tyrp-1ee-Vp22 was still present in MHCII^{-/-} mice, it was muted[7]. Thus, it is possible that Vp22, like OmpA, provides helper epitopes for a CD4 response. In support of this possibility, CD4 responses to Vp22 occur in humans [40,41]. Thus, the most straightforward explanation for the beneficial effect of OmpA and Vp22 in reversing the deficient immune responses in old mice is that both vaccines provide powerful CD4 help. In the case of OmpA, this CD4 helper response is required as there was no observable CD8 response in MHC-II^{-/-} mice [7]. In the case of Vp22, the CD4 response is not essential, as a CD8 response is seen in MHC-II^{-/-} mice [7], but it may still contribute towards an optimal CD8 response. We show that Tryp-1ee-Vp22 immunization results in a strong CD4 response to B16 melanoma cells directed at a yet unidentified antigenic epitope. The response is directed to untreated B16 melanoma cells regardless of expression levels of MHC-II or of wildtype Tyrp-1 in these cells. In view of the CD4 deficiency associated with aging, it is plausible that the CD4 response to B16 is critical in overcoming the general lack of a response to Tyrp-1ee in old mice.

In summary, OmpA and Vp22 are useful additions to DNA vaccines for old mice. They need to be tested in other mouse models using other DNA vaccines both for tumor antigens and antigens of infectious agents, such as the flu virus. If their usefulness for vaccination in the aged is generally valid, they should then be tested in vaccines for humans.

Acknowledgments

Grant from Swim Across America, PHS grants PO1 CA33049, RO-1 CA56821.

References

1. Aspinall R, Henson SM, Pido-Lopez J. My T's gone cold, I'm wondering why. *Nat Immunol.* 2003; 4(3):203–5. [PubMed: 12605224]
2. Engelhorn ME, Guevara-Patino JA, Noffz G, Hooper AT, Lou O, Gold JS, Kappel BJ, Houghton AN. Autoimmunity and tumor immunity induced by immune responses to mutations in self. *Nat Med.* 2006; 12(2):198–206. [PubMed: 16444264]
3. Bergman PJ, McKnight J, Novosad A, Charney S, Farrelly J, Craft D, Wulderk M, Jeffers Y, Sadelain M, Hohenhaus AE, Segal N, Gregor P, Engelhorn M, Riviere I, Houghton AN, Wolchok JD. Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. *Clin Cancer Res.* 2003; 9(4):1284–90. [PubMed: 12684396]
4. Conry RM, Curiel DT, Strong TV, Moore SE, Allen KO, Barlow DL, Shaw DR, LoBuglio AF. Safety and immunogenicity of a DNA vaccine encoding carcinoembryonic antigen and hepatitis B surface antigen in colorectal carcinoma patients. *Clin Cancer Res.* 2002; 8(9):2782–7. [PubMed: 12231517]
5. Stan R, Wolchok JD, Cohen AD. DNA vaccines against cancer. *Hematol Oncol Clin North Am.* 2006; 20(3):613–36. [PubMed: 16762727]
6. Guevara-Patino JA, Engelhorn ME, Turk MJ, Liu C, Duan F, Rizzuto G, Cohen AD, Merghoub T, Wolchok JD, Houghton AN. Optimization of a self antigen for presentation of multiple epitopes in cancer immunity. *J Clin Invest.* 2006; 116(5):1382–90. [PubMed: 16614758]
7. Engelhorn ME, Guevara-Patiño JA, Liu C, Ferrone CR, Merghoub T, Posnett DN, Houghton AN. Chimerical Antigen Strategies for Immunization against Melanoma. 2006 submitted.
8. Dyall R, Bowne WB, Weber LW, LeMaout J, Szabo P, Moroi Y, Piskun G, Lewis JJ, Houghton AN, Nikolic-Zugic J. Heteroclitic immunization induces tumor immunity. *J Exp Med.* 1998; 188(9): 1553–61. [PubMed: 9802967]
9. Scheibenbogen C, Lee KH, Mayer S, Stevanovic S, Moebius U, Herr W, Rammensee HG, Keilholz U. A sensitive ELISPOT assay for detection of CD8+ T lymphocytes specific for HLA class I-binding peptide epitopes derived from influenza proteins in the blood of healthy donors and melanoma patients. *Clin Cancer Res.* 1997; 3(2):221–6. [PubMed: 9815676]
10. Flood PM, Urban JL, Kripke ML, Schreiber H. Loss of tumor-specific and idiotypic-specific immunity with age. *J Exp Med.* 1981; 154(2):275–90. [PubMed: 6973599]
11. Flood PM, Liu X, Alexander R, Schreiber H, Haque S. Loss of resistance to a highly immunogenic tumor with age corresponds to the decline of CD8 T cell activity. *J Immunother.* 1998; 21(4):307–16. [PubMed: 9672852]
12. Urban JL, Schreiber H. Rescue of the tumor-specific immune response of aged mice in vitro. *J Immunol.* 1984; 133(1):527–34. [PubMed: 6609995]
13. Win S, Uenaka A, Nakayama E. Immune responses against allogeneic and syngeneic tumors in aged C57BL/6 mice. *Microbiol Immunol.* 2002; 46(7):513–9. [PubMed: 12222940]
14. Provinciali M, Argentati K, Tibaldi A. Efficacy of cancer gene therapy in aging: adenocarcinoma cells engineered to release IL-2 are rejected but do not induce tumor specific immune memory in old mice. *Gene Ther.* 2000; 7(7):624–32. [PubMed: 10819579]
15. Provinciali M, Smorlesi A, Donnini A, Bartozzi B, Amici A. Low effectiveness of DNA vaccination against HER-2/neu in ageing. *Vaccine.* 2003; 21(9–10):843–8. [PubMed: 12547592]
16. Kaptzan T, Skutelsky E, Itzhaki O, Sinai J, Michowitz M, Yossipov Y, Schiby G, Leibovici J. Age-dependent differences in the efficacy of cancer immunotherapy in C57BL and AKR mouse strains. *Exp Gerontol.* 2004; 39(7):1035–48. [PubMed: 15236763]
17. Jeannin P, Renno T, Goetsch L, Miconnet I, Aubry JP, Delneste Y, Herbault N, Baussant T, Magistrelli G, Soulas C, Romero P, Cerottini JC, Bonnefoy JY. OmpA targets dendritic cells, induces their maturation and delivers antigen into the MHC class I presentation pathway. *Nat Immunol.* 2000; 1(6):502–9. [PubMed: 11101872]
18. Sciortino MT, Taddeo B, Poon AP, Mastino A, Roizman B. Of the three tegument proteins that package mRNA in herpes simplex virions, one (VP22) transports the mRNA to uninfected cells

- for expression prior to viral infection. *Proc Natl Acad Sci U S A*. 2002; 99(12):8318–23. [PubMed: 12060774]
19. Bennett RP, Dalby B, Guy PM. Protein delivery using VP22. *Nat Biotechnol*. 2002; 20(1):20. [PubMed: 11753353]
 20. Lundberg M, Wikstrom S, Johansson M. Cell surface adherence and endocytosis of protein transduction domains. *Mol Ther*. 2003; 8(1):143–50. [PubMed: 12842437]
 21. Chhabra A, Mehrotra S, Chakraborty NG, Mukherji B, Dorsky DI. Cross-presentation of a human tumor antigen delivered to dendritic cells by HSV VP22-mediated protein translocation. *Eur J Immunol*. 2004; 34(10):2824–33. [PubMed: 15368298]
 22. Roy V, Qiao J, de Campos-Lima P, Caruso M. Direct evidence for the absence of intercellular trafficking of VP22 fused to GFP or to the herpes simplex virus thymidine kinase. *Gene Ther*. 2005; 12(2):169–76. [PubMed: 15483667]
 23. Perkins SD, Hartley MG, Lukaszewski RA, Phillpotts RJ, Stevenson FK, Bennett AM. VP22 enhances antibody responses from DNA vaccines but not by intercellular spread. *Vaccine*. 2005; 23(16):1931–40. [PubMed: 15734065]
 24. Garg S, Oran A, Wajchman J, Sasaki S, Maris CH, Kapp JA, Jacob J. Genetic tagging shows increased frequency and longevity of antigen-presenting, skin-derived dendritic cells in vivo. *Nat Immunol*. 2003; 4(9):907–12. [PubMed: 12910266]
 25. Jeannin P, Magistrelli G, Herbault N, Goetsch L, Godefroy S, Charbonnier P, Gonzalez A, Delneste Y. Outer membrane protein A renders dendritic cells and macrophages responsive to CCL21 and triggers dendritic cell migration to secondary lymphoid organs. *Eur J Immunol*. 2003; 33(2):326–33. [PubMed: 12548563]
 26. Kissenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, Romani N, Tripp CH, Douillard P, Leserman L, Kaiserlian D, Saeland S, Davoust J, Malissen B. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity*. 2005; 22(5):643–54. [PubMed: 15894281]
 27. Li SP, Cai Z, Shi W, Brunmark A, Jackson M, Linton PJ. Early antigen-specific response by naive CD8 T cells is not altered with aging. *J Immunol*. 2002; 168(12):6120–7. [PubMed: 12055223]
 28. Linton PJ, Haynes L, Klinman NR, Swain SL. Antigen-independent changes in naive CD4 T cells with aging. *Journal of Experimental Medicine*. 1996; 184:1891–900. [PubMed: 8920876]
 29. Linton PJ, Haynes L, Tsui L, Zhang X, Swain S. From naive to effector--alterations with aging. *Immunol Rev*. 1997; 160:9–18. [PubMed: 9476661]
 30. Haynes L, Linton PJ, Eaton SM, Tonkonogy SL, Swain SL. Interleukin 2, but not other common gamma chain-binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice. *J Exp Med*. 1999; 190(7):1013–24. [PubMed: 10510091]
 31. Haynes L, Eaton SM, Burns EM, Rincon M, Swain SL. Inflammatory cytokines overcome age-related defects in CD4 T cell responses in vivo. *J Immunol*. 2004; 172(9):5194–9. [PubMed: 15100256]
 32. Bansal-Pakala PC, Croft M. Defective T cell priming associated with aging can be rescued by signaling through 4-1BB (CD137). *J Immunol*. 2002; 169(9):5005–9. [PubMed: 12391215]
 33. Tang Y, Akbulut H, Maynard J, Petersen L, Fang X, Zhang WW, Xia X, Koziol J, Linton PJ, Deisseroth A. Vector prime/protein boost vaccine that overcomes defects acquired during aging and cancer. *J Immunol*. 2006; 177(8):5697–707. [PubMed: 17015759]
 34. Sen G, Chen Q, Snapper CM. Immunization of aged mice with a pneumococcal conjugate vaccine combined with an unmethylated CpG-containing oligodeoxynucleotide restores defective immunoglobulin G antipolysaccharide responses and specific CD4+-T-cell priming to young adult levels. *Infect Immun*. 2006; 74(4):2177–86. [PubMed: 16552048]
 35. Qin W, Jiang J, Chen Q, Yang N, Wang Y, Wei X, Ou R. CpG ODN enhances immunization effects of hepatitis B vaccine in aged mice. *Cell Mol Immunol*. 2004; 1(2):148–52. [PubMed: 16212903]
 36. Weng NP. Aging of the immune system: how much can the adaptive immune system adapt? *Immunity*. 2006; 24(5):495–9. [PubMed: 16713964]
 37. Haynes L, Swain SL. Why aging T cells fail: implications for vaccination. *Immunity*. 2006; 24(6):663–6. [PubMed: 16782020]

38. Kueltzo LA, Middaugh CR. Nonclassical transport proteins and peptides: an alternative to classical macromolecule delivery systems. *J Pharm Sci.* 2003; 92(9):1754–72. [PubMed: 12949995]
39. Blouin A, Blaho JA. Assessment of the subcellular localization of the herpes simplex virus structural protein VP22 in the absence of other viral gene products. *Virus Res.* 2001; 81(1–2):57–68. [PubMed: 11682125]
40. Koelle DM, Frank JM, Johnson ML, Kwok WW. Recognition of herpes simplex virus type 2 tegument proteins by CD4 T cells infiltrating human genital herpes lesions. *J Virol.* 1998; 72(9): 7476–83. [PubMed: 9696844]
41. Koelle DM, Schomogyi M, McClurkan C, Reymond SN, Chen HB. CD4 T-cell responses to herpes simplex virus type 2 major capsid protein VP5: comparison with responses to tegument and envelope glycoproteins. *J Virol.* 2000; 74(23):11422–5. [PubMed: 11070045]

Figure 1. Deficiency of DNA vaccine responses in old can be reversed with fusion vaccines incorporating a microbial adjuvant

Numbers of lung metastases 30 days after IV challenge with 50,000 B16 tumor cells in groups of mice (15 mice/group) **A** Tyrp-1ee vaccination provides no significant protection in old mice (yellow), but Tyrp-1-OmpA does provide protection even in old mice. **B** Both Tyrp-1ee-OmpA and Tyrp-1ee-Vp22 provide better protection in old mice compared with Tyrp-1ee. **C** Tyrp-1ee-Vp22, but not Tyrp-1ee, provides significant protection in old mice. Note the consistent lower number of metastases in unvaccinated old versus unvaccinated

young mice. T test comparisons with the corresponding naïve group gave significant p values of * $p < 0.0000003$, ** $p < 0.00002$ in panel A, * $p < 0.000001$, ** $p < 0.01$ in B, * $p < 0.0003$, ** $p < 0.007$ in C.

Figure 2. In vitro response of CD8 cells from vaccinated old mice is greatly enhanced using the Vp22 construct

IFN- γ ELISPOT with EL4 target cells alone, or pulsed with peptides 455wt or 522wt, or with B16 target cells. 10,000 target cells/well and 100,000 effector CD8 cells/well (pooled from 5 mice per group) were plated in triplicates and cultured for 24 h. Vp22 has a dramatic effect in old mice, increasing specific T cell responses to both Tyrp-1 peptides and to the B16 tumor cells.

Figure 3. The effects of OmpA, Vp22 and other fusion vaccines do not require TLR2

Pooled CD8 spleen cells were isolated from mice vaccinated 3 times (3 mice/group) and stimulated with either EL4 pulsed with 455wt, or control peptide (SSI), or with B16 melanoma in triplicate cultures. IFN- γ spots were measured by ELISpot assay 24 h later. Responses with CD8 cells derived from either young TLR2^{-/-} mice or heterozygote littermates were compared. In addition to OmpA and Vp22 constructs, additional microbial fusion constructs were used: exotoxin A from *P. aeruginosa* (Exo) and the mature form of heat-labile enterotoxin's b subunit from *E. coli* (Ent). Results represent means of triplicates $\pm$ standard deviation. Reproducible results were obtained in a total of 2 experiments.

Figure 4. Estimate of loaded DCs in the draining LNs after immunization

DC from young naïve mice were pulsed with 455mut peptide and then diluted with unpulsed DCs (as indicated in the legend) to create preparations containing 10%, 1%, 0.1% and 0.01% loaded DCs. DCs were incubated with 20,000 G8 hybridoma cells at different numbers ranging from 0 to 266,000 DCs. All cultures were done in triplicates. The titration curves were then compared with a curve created from data using *in vivo* loaded DCs (day 7, after foot pad immunization with 455mut plus Titermax). 100,000 or 200,000 DC from gene gun immunized mice yielded only 7 – 24 ELISpots/20,000 G8 cells regardless of which vaccine construct was used: Tyrp1-ee, Tyrp1-ee-Vp22 or Tyrp1-ee-OmpA, and regardless of the time point examined (5 or 7 days post immunization). Thus, estimates for peptide loaded DCs are ca. 1% 7 days after 455mut + Titermax, and less than 0.01% 5–7 days after Tyrp1-ee vaccination. Results represent means of triplicates. Standard deviations were less than 10% of experimental values. Reproducible results were obtained in a total of 3 experiments.

Figure 5. CD4 response to B16 melanoma cells after vaccination with Tyrp-1ee-Vp22

CD4 cells were isolated from total pooled lymph nodes of 5 mice/group and cocultured with splenocytes or B16 melanoma cells in triplicate cultures. Young mice were immunized 3x at weekly intervals with the indicated constructs. Results represent means of triplicates $\pm$ standard deviation. Reproducible results were obtained in a total of $\pm$ similar experiments.

Figure 6. The immunogenic CD4 epitope leading to a response to B16 melanoma (Fig. 5) is provided by the fusion construct Tyrp-1ee-Vp22. Young mice (5/group) were immunized by gene gun with the indicated fusion constructs on a weekly schedule and sacrificed 5 days after the 3rd immunization. Purified CD4 cells pooled from total LNs (or from the spleen, not shown) were co-cultured with 3 different B16 cell lines. Results represent means of triplicates $\pm$ standard deviation. Reproducible results were obtained in a total of 2 similar experiments.

Figure 7. Deficient CD8 and CD4 responses in old mice are improved after immunization with fusion constructs

Groups (N=5) of young and old mice were immunized weekly times four with the constructs indicated in the legend. Splenic (SPL) CD8 (A) and splenic or lymph node (LN) CD4 cells (B,C), pooled from groups of 5 mice, were used as effector cells. IFN- γ spots were measured after 1–2 day incubations with target cells, either irradiated splenocytes pulsed with known immunodominant epitopes of Tyrp1 as indicated under the X axis, or B16 melanoma target cells. Immunization with Tyrp1-ee-OmpA and Tyrp1-ee-Vp22 constructs (T-OmpA, T-Vp22) completely reversed the non-responsiveness of CD8 cells from old mice (A) and

partially reversed the poor responsiveness of CD4 cells (B,C), specially when tested against B16 target cells (C). Results represent means of triplicates $\pm$ standard deviation.