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Using virally expressed melanoma cDNA libraries to identify tumor-associated antigens that cure melanoma

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Abstract

Multiple intravenous injections of a cDNA library, derived from human melanoma cell lines and expressed using the highly immunogenic vector vesicular stomatitis virus (VSV), cured mice with established melanoma tumors. Successful tumor eradication was associated with the ability of mouse lymphoid cells to mount a tumor-specific CD4⁺ interleukin (IL)-17 recall response *in vitro*. We used this characteristic IL-17 response to screen the VSV-cDNA library and identified three different VSV-cDNA virus clones that, when used in combination but not alone, achieved the same efficacy against tumors as the complete parental virus library. VSV-expressed cDNA libraries can therefore be used to identify tumor rejection antigens that can cooperate to induce anti-tumor responses. This technology should be applicable to antigen discovery for other cancers, as well as for other diseases in which immune reactivity against more than one target antigen contributes to disease pathology.

Melanoma is amenable to immunotherapy, in part because clearly defined tumor-associated antigens (TAA) can function as targets for both specific and nonspecific immunization therapies^{1–6}. However, tumors readily evolve to escape antigen-specific immune responses^{7,8}. Therefore, effective immunotherapies must target a broad repertoire of TAA to reduce the possibility of immune escape and development of resistance⁴. Melanomas arise from normal melanocytes and a strong correlation exists between successful immunotherapies for melanoma and autoimmune vitiligo^{2,3,9}. Therefore, we reasoned that it would be possible for the immune system to sample a wide range of self- antigens, some of

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AUTHOR CONTRIBUTIONS

J.P., T.K., J.T., F.G., P.W., R.M.D., D.R. and E.I. ran the experiments. J.P., T.K., L.P., H.P., K.H., P.S., A.M. and R.V. conceived the experimental approach and planned the experiments. J.P., T.K., H.P., K.H., P.S., A.M. and R.V. wrote the manuscript.

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which might serve as TAA against melanoma, by directly killing normal cells *in situ* in an inflammatory environment^{10–15}. This would, in theory, prime T-cell responses to those antigens against which tolerance could be broken. Consistent with this, gene-targeted killing of three different types of normal cells, with hsp70 as an adjuvant, led to effective rejection of associated tumor cells in melanoma, prostate and pancreatic cancer models^{10,12–15} by activating a wide repertoire of T-cell responses against multiple TAA. Although each T-cell response might have been individually relatively weak, cumulatively these responses imposed a strong selective pressure against immune escape. When this approach is used, TAA do not have to be identified and tumors do not have to be accessed by targeted vector delivery^{11,16}. In addition, in melanoma in particular, priming of autoimmune T-cell responses against normal melanocytes might be clinically manageable because vitiligo is not life threatening^{11,16}.

We recently expanded this approach for tackling prostate cancer by replacing *in situ* killing of normal cells with expression of a cDNA library of a normal tissue from the highly immunogenic vector VSV^{17,18}. In this approach, cDNA from normal human prostate tissue served as the source of the broad antigenic repertoire for *in vivo* immune selection of relevant TAA. The expression system itself—systemically delivered VSV—functioned as a strong adjuvant^{19–26} and induced the inflammatory signals that are required to activate anti-tumor T-cell responses *in vivo*. The prostate-specific, virally expressed, epitope library induced rejection of established mouse prostate tumors, without inducing detectable autoimmunity. To enhance the immunogenicity of relevant self-antigens, we used cDNA from normal human prostate tissue to vaccinate against mouse tumors as altered self-epitopes can be more immunogenic than the corresponding self-epitopes^{27–29}.

In this study we report that a VSV-expressed cDNA library constructed using melanoma cells is effective against established melanomas. Using an *in vitro* assay to screen the VSV-cDNA library from melanoma cells, we identified three VSV-cDNA viruses that, in combination, induced tumor rejection through priming of a CD4, T-helper cell 17 (T_H17), anti-tumor response. This technology can therefore be used to define repertoires of TAA that work in combination to induce anti-tumor immune responses. The identification of arrays of antigens that cooperate *in vivo* to cure established tumors will inform future strategies for the development of clinical vaccines through the use of virally expressed epitope libraries that are constructed with selected, relevant and defined epitopes that can be readily manufactured for clinical use. This technology should be directly applicable to the treatment of different cancer types, as well as to antigen discovery in diseases where pathology is induced by immune reactivity against multiple target antigens.

RESULTS

Altered self-melanoma-epitope VSV-cDNA library

cDNA from two human melanoma cell lines, Mel624 and Mel888, was cloned into VSV. This library was named ASMEL, because it expressed altered self-melanoma-epitopes (mouse) from the viral library. The ASMEL had a titer ranging from 5×10^6 to 1×10^7 plaque-forming units (pfu)/ml.

Cells infected with the ASMEL expressed the melanoma-specific genes *gp100* (also known as *PMEL*) and *TRP2* (also known as *TRNP2*), but not the prostate-specific gene *PSA* (also known as *KLK3*) (Fig. 1a). In addition, splenocytes from C57BL/6 mice infected with the ASMEL induced interferon (IFN)- γ from naive Pmel T cells (Fig. 1b,c), indicating that the ASMEL transferred expression of the KVPRNQDWL peptide epitope from the human melanocyte-specific gp100 antigen (hgp100_{25–33}), which is presented by both human HLA-A*0201 and mouse H-2K^b to T cells³⁰. Splenocytes infected with no virus, VSV-GFP, or a

VSV-cDNA library derived from normal human prostate cells¹⁸, did not activate Pmel (Fig. 1c) other than through nonspecific, VSV-mediated T-cell activation, as reported previously^{31–34}. In addition, splenocytes infected with the ASMEL could not activate OT-I T cells, which recognize the SIINFEKL epitope of the control ovalbumin (OVA) antigen³⁵ (Fig. 1c). Therefore, the human melanoma cDNA library in VSV transferred expression of T cell-activating epitopes to target cells.

Intravenous ASMEL cured mice with established B16 melanomas

Nine intravenous (i.v.) injections of the ASMEL cured 60% of mice ($n = 7$ or 8 per group) bearing established B16 tumors ($P < 0.0001$ compared with VSV-GFP) (Fig. 2a) but had no effect against TC2 tumors (data not shown)¹⁸ and no effect in tumor-bearing, immunodeficient mice. Depletion of CD4⁺ T cells significantly decreased the therapeutic effect ($P = 0.0005$, depleted compared with nondepleted) (Fig. 2a), although ASMEL treatment of CD4-depleted mice was still significantly better than i.v. VSV-GFP treatment ($P = 0.022$) (Fig. 2a). There was no significant difference between mock CD4⁺ T cell-depleted mice and CD8⁺ T cell-depleted mice after treatment with the ASMEL ($P = 0.11$) (Fig. 2a).

To test for the role of immune reactivity against xenogeneic, as opposed to tissue-specific, antigens in the ASMEL, we compared the therapeutic efficacy of a VSV-expressed cDNA library from B16 mouse melanoma¹⁸ (no xenogeneic, or altered self, antigens) (SMEL, self-epitope melanoma library) with that of the ASMEL. In two separate experiments, mice vaccinated with the ASMEL survived significantly longer than those treated with the SMEL ($P = 0.01$, data pooled from both experiments ($n = 9$)), whereas SMEL-treated mice survived significantly longer than mice treated with VSV-GFP ($P = 0.001$).

Mice cured by nine i.v. injections of the ASMEL did not develop detectable autoimmunity for 6 months following treatment. After 6 months, however, moderate symptoms of autoimmunity were observed in 100% of treated mice, namely, whitening of the whiskers (Fig. 2b) and tails (Fig. 2b,c). Up to 12 months after injection of the ASMEL no mice developed any hair depigmentation substantially different from that of control mice injected with PBS (Fig. 2b). The mild autoimmunity seen in mice at 6 months (Fig. 2b,c) progressed only marginally by 18 months after ASMEL infection (further whitening of tails and whiskers). We were unable to detect any reproducible abnormal immune infiltration, or tissue destruction, and there were no other clear signs of autoimmunity. Splenocytes of mice 18 months after ASMEL infection did not secrete IFN- γ in response to stimulation by B16 lysates or melanoma-associated epitopes.

The ASMEL primed a tumor-specific IL-17 response

Pooled lymph node cells and splenocytes (lymphoid cells) from ASMEL-cured mice did not secrete IFN- γ , IL-4, IL-6 or IL-10 upon restimulation with B16 cell lysates *in vitro* either with, or without, infection and/or restimulation with the ASMEL (data not shown). In contrast, lymphoid cells from ASMEL-cured mice secreted low levels of IL-17 upon restimulation with B16 lysates (Fig. 2d, lanes 2,3), but not lysates from normal mouse prostate, pancreas (data not shown) or TC2 prostate (Fig. 2d, lane 4) tumor lysates. Levels of IL-17 were significantly ($P < 0.01$) increased by restimulation with ASMEL at a multiplicity of infection (MOI) of 10 (Fig. 2d, lane 3). However, an MOI of 1 or less was ineffective at stimulating IL-17 secretion.

The ability of lymphoid cells from ASMEL-treated, immune subset-depleted mice to secrete IL-17 when infected with the ASMEL and restimulated with B16 lysates (Fig. 2d, lanes 5–7) correlated with their ability to secrete IL-17 after therapy of the ASMEL against B16 tumors (Fig. 2a).

Low MOI induction of recall IL-17 responses is mediated by hsp70

To detect individual VSV clones encoding specific cDNA that stimulates anti-tumor IL-17 responses, we wished to increase the sensitivity of the recall response from lymphoid cells when infected *in vitro* with the ASMEL at MOI of less than 10. Previously, we showed that hsp70 (also known as hsp1A/1B) is an effective adjuvant to induce anti-tumor immune responses^{10,12,13}. Consistent with this, hsp70 was induced within 24 h by infection of ASMEL-treated lymphoid cells with high, but not low (<10), MOI of VSV (Fig. 3a). Hsp70 induction was independent of whether the VSV transgene was GFP, or multiple different inserts (cDNA) (Fig. 3a). However, prolonged hsp70 expression was observed only with VSV clones expressing multiple cDNAs, as opposed to OVA or GFP (Fig. 3b). Therefore, (i) the MOI of VSV infection correlated with induction of hsp70 and (ii) detection of a tumor-specific recall IL-17 response (>200 pg/ml) (Figs. 2 and 3c) required both a range of VSV-expressed cDNA molecules and was associated with hsp70. Therefore, we reasoned that by supplying exogenous hsp70 to LN cell and splenocyte cultures it would be possible to detect recall IL-17 responses to the ASMEL containing viruses at reduced (>10) MOIs. Consistent with such a hypothesis, LN cell and splenocyte cultures from ASMEL-treated mice secreted IL-17 in response to restimulation with B16 (but not TC2) lysate when infected with the ASMEL (but not VSV-GFP) virus at an MOI of 1.0 or lower in the presence of recombinant hsp70 (Fig. 3c).

Induction of hsp70 after infection of LN cell and splenocyte cultures with ASMEL depended on TLR4 (Fig. 3d), but not MyD88 or TLR7, signaling (Fig. 3e). Although secretion of IL-17 required signaling by TLR4, TLR7 and MyD88 (Fig. 3e), it could be rescued by addition of recombinant hsp70 in the absence of MyD88 or TLR4 signaling.

Identifying VSV-cDNA viruses that induce IL-17 memory

We used the hsp70-enhanced *in vitro* assay to screen for individual viruses in the ASMEL stocks that induced tumor-specific IL-17 recall responses (Supplementary Fig. 1a). Individual VSV-cDNA viruses were cloned by limiting dilution of sequentially diluted, and screened, aliquots of the ASMEL stock that induced IL-17 at levels that were demonstrably increased over background (>100 pg/ml) (Supplementary Fig. 1a,b). Viruses recovered from 24 'single pfu'-containing wells (Supplementary Fig. 1a) were screened by PCR for their encoded cDNA inserts (Supplementary Fig. 1c). Seven viruses contained 5' coding sequences of the human oncogene *NRAS*; six were identical, containing 555 bp of the 5' coding region whereas one virus contained 444 bp of the same sequence. Codon 13 of all seven viruses contained a G-T mutation compared to the wild-type sequence but all the remaining sequences contained no mutations³⁶. Four viruses contained unmutated sequences of the 5' end of the human gene *TYRP1* (ref. 37); three of these contained 1,260 bp of human *TYRP1* sequence and a fourth virus contained 1,199 bp (ref. 37). Two identical viruses were recovered that contained part of the human cytochrome C1 gene *CYC1* with a G-A mutation at position 1 compared to the published sequence³⁸. A single virus encoded the 5' end of the human gene *TRIM33*. The remaining viruses had no interpretable sequences.

In vitro validation of VSV-encoded tumor antigens

To validate these four separately derived cDNA sequences as potential tumor-associated antigens, we expanded one virus from each group with the longest encoded sequence of each gene (VSV-NRAS clone no. 3, VSV-TYRP1 clone no. 7, VSV-CYC1 clone no. 11 or VSV-TRIM33 clone no. 12) in BHK cells. In addition, 12 single viruses were purified by limiting dilution from the parental, unselected ASMEL stock (ASMEL nos. 1–12). None of the randomly selected viruses ASMEL nos. 1–12 induced IL-17 after infection of LN cell and splenocyte cultures from ASMEL-vaccinated mice, even at high MOI (Fig. 4a). However, to our surprise, neither did any of the four selected viruses (VSV-NRAS, VSV-TYRP1, VSV-

CYC1 or VSV-TRIM33) (Fig. 4a). We previously reported that VSV induces potent suppressor activities *in vivo*³⁴. Consistent with those findings, infection of the LN cell and splenocyte cultures induced high levels of TGF- β , independent of the nature of the cDNA insert in the virus (Fig. 4b). When LN cell and splenocyte cultures were infected with either the VSV-NRAS, VSV-TYRP1 or VSV-CYC1 viruses, (but not the VSV-TRIM33 virus), in the presence of TGF- β blockade³⁴, significant levels of IL-17 were induced (Fig. 4c). In contrast, none of the randomly cloned viruses (ASMEL nos. 1–12) induced IL-17 even in the presence of TGF- β blockade (Fig. 4c).

***In vivo* validation of VSV-encoded tumor antigens**

A three-way combination of the VSV-NRAS, VSV-TYRP1 and VSV-CYC1 viruses were sufficient to overcome VSV-induced TGF- β -dependent suppression³⁴ and induced significant levels of IL-17 even without TGF- β blockade (Fig. 5a). Blockade of TGF- β further increased levels of IL-17 with the combination (Fig. 5b). The ability of VSV-cDNA viruses to induce IL-17 in combination depended on the cDNA insert as combining equal levels of three different nonselected viruses (ASMEL nos. 1, 2 and 3) or (ASMEL nos. 4, 5 and 6) did not induce IL-17, even if TGF- β was blocked (Fig. 5b).

We tested whether any viruses could prime an anti-B16 response in naive LN cell and splenocyte cultures, rather than stimulating a recall response from previously treated mice. Neither the randomly cloned VSV-cDNAs (data not shown), nor the selected viruses (Fig. 5c), induced either IFN- γ (data not shown) or IL-17 (Fig. 5c) from repeatedly infected lymphoid cells of naive C57BL/6 mice in the absence of TGF- β blockade (Fig. 5c). Two-way combinations were also ineffective (Fig. 5c). In contrast, a combination of the VSV-NRAS, VSV-TYRP1 and VSV-CYC1 viruses induced increased levels of IL-17 in response to B16 lysates (Fig. 5c), but not in response to TC2 prostate lysates (data not shown). The levels of IL-17 were ~60% of those induced by the unfractionated ASMEL stock (Fig. 5c). VSV-TRIM33 alone, in double combination, or along with any two of the other viruses, did not induce IL-17 (Fig. 5c) although inclusion of VSV-TRIM33 with the three other viruses did not inhibit IL-17 production (Fig. 5c).

Therefore, we tested the VSV-NRAS, VSV-TYRP1 and VSV-CYC1 viruses alone, in double or in triple combinations, to treat established B16 melanomas *in vivo*. VSV expressing the foreign, non-self, tumor-associated OVA antigen can induce tumor rejection in a proportion of mice bearing B16 ova tumors²³. However, VSV clones expressing previously defined self, or near-self, tumor antigens such as TRP-2 or gp100, did not induce significant therapy against B16 tumors (Fig. 5d), even though splenocytes from treated mice did secrete IFN- γ , but not IL-17, when restimulated with B16 tumor lysates or their cognate antigens *in vitro* (data not shown)³³. None of the individual VSV-NRAS, VSV-TYRP1 and VSV-CYC1 viruses cured mice with established B16 melanomas compared to PBS or VSV-GFP-treated controls (data not shown). Also consistent with the *in vitro* data, double combinations of these viruses had no effect on established tumors (Fig. 5e). However, using the same total dose of virus administration, a combination of the three different VSV-NRAS, VSV-TYRP1 and VSV-CYC1 viruses provided substantial efficacy compared with any of the control, or double-combination treatments, with up to 60% of mice cured for over 60 d (Fig. 5e). In addition, prophylactic vaccination (days 7, 6 and 5 before subcutaneous B16 challenge) with either the intact ASMEL (10^7 pfu/injection), or with the same total dose of virus containing the combination of VSV-cDNA clone no. 3 (NRAS) + clone no. 7 (TYRP1) + clone no. 11 (CYC1), completely prevented tumor growth in 6/7 and 8/8 mice (data not shown). In contrast, all mice vaccinated with VSV-GFP developed progressively growing tumors that reached a diameter of 1.0 cm by day 25 after tumor seeding.

DISCUSSION

Here we have extended our previous findings in a prostate model¹⁸ to show that virally expressed cDNA libraries are effective against melanoma and that an altered self-antigen library derived from tumor cells (as well as a library derived from normal cells)¹⁸ has efficacy against established tumors, and produces only limited autoimmunity (Figs. 1 and 2). Our results with the ASMEL and SMEL suggest that a cDNA library from a xenogeneic, altered self source (human in the mouse context) has additional adjuvant properties compared to a library from an autologous self-source (mouse in mouse). We believe that this adjuvant-like effect derives partly from altered self versions of self antigens in the xenogeneic library, which increases the immunogenicity of relevant epitopes³⁰. In addition, Figure 2a,d also shows that the immune reactivity generated is not simply directed against normal household protein xenoantigens.

To identify TAA, we developed an *in vitro* screen that was based on the ability of lymphoid cells from ASMEL-treated mice to present tumor-associated epitopes from the ASMEL upon *in vitro* infection and/or stimulation to memory IL-17 CD4⁺ T cells. These memory IL-17 CD4⁺ T cells were stimulated to secrete IL-17 in response to melanoma, but not prostate tumor, lysates (Fig. 2). Consistent with the importance of hsp70 as an adjuvant that enables tolerance to be broken^{10,12,13}, this response was mediated by a TLR-4-dependent, VSV-mediated induction of hsp70 with subsequent MyD88- and TLR-7-dependent signaling of IL-17 secretion by hsp70 (Fig. 3). Based on this, we showed that the tumor-specific IL-17 memory response was induced by infection at an MOI as low as ~1 by addition of exogenous hsp70 (Fig. 3). Using this *in vitro* assay, we isolated single VSV with cDNA inserts from coding sequences of NRAS, TYRP1, CYC1 and TRIM33 (Supplementary Fig. 1c). Surprisingly, none of these viruses alone induced B16-specific IL-17 recall responses *in vitro* (Fig. 4a) unless VSV-induced TGF-β³⁴ was blocked (Fig. 4c). However, combination of the NRAS, TYRP1 and CYC1 viruses overcame TGF-β-mediated suppression of the IL-17 recall response, even in the absence of a TGF-β blockade (Fig. 5a). Moreover, this combination of three viruses primed a *de novo*, tumor-specific, IL-17 response from lymphoid cells of naive C57BL/6 mice (Fig. 5a,c), although levels of IL-17 only reached ~60% of those induced by infection with the unfractionated ASMEL (Fig. 5c). Thus, it is possible that different epitopes signal either through different antigen-presenting cells (APCs), or with different strengths through the same APC, or a combination of both, so that multiple pathways of T-cell activation act synergistically to stimulate a threshold level of signaling that is required to prime and/or restimulate IL-17-producing cells. Alternatively, epitopes within these proteins and/or peptides may, cumulatively, selectively inhibit the activity of VSV-activated suppressor cells. In this respect, autoimmunity (Fig. 2) was very mild and, we assume, would progress very slowly, if at all, beyond 12 months. Therefore, infection with the ASMEL may induce anti-tumor effector responses that are suppressed in the long term by VSV-mediated induction of suppressor mechanisms³⁴, thereby limiting the development of autoimmunity.

Consistent with the *in vitro* data, therapy against B16 tumors was reconstituted *in vivo* only when VSV-NRAS, VSV-TYRP1 and VSV-CYC1 were used in a triple combination (Fig. 5e). These data therefore show that only specific self, or near-self, cDNAs can induce CD4-dependent, melanoma-specific, IL-17 responses which, cumulatively, translate into CD4-dependent anti-tumor therapy. cDNA-encoding parts of the NRAS, TYRP1 and CYC1 genes have this property, and can, therefore, be regarded as tumor antigens. One of these putative TAAs—TYRP1—represents a tissue-specific antigen, with known properties as a melanoma-rejection antigen³⁹, consistent with the observation that the ASMEL does not protect mice against non-melanoma tumors¹⁸. Because all the NRAS-encoding VSV-cDNAs encoded a RAS peptide with a mutated codon 13, it is possible that this insert represents a

class of tumor-specific epitopes. We are investigating whether our B16 cells express a similarly mutated RAS. VSV does not exclusively induce T_H17 responses, as VSV-OVA can induce a type 1, IFN- γ -dependent rejection response against B16 ova tumors²³. Therefore, we hypothesize that T_H17 polarization of the anti-tumor response by the ASMEL is due to properties of both the VSV and the wide range of cDNA-encoded antigens in the library.

Combining three VSV-cDNAs from the ASMEL library induced about 60% of the IL-17 recall response from LN cell and splenocyte cultures (Fig. 5c) and generated therapy similar to, or less than, that generated by the ASMEL (Fig. 5e). Therefore, it seems probable that the unselected ASMEL contains additional active components, lacking from the triple combination, that contribute to therapy. These may encode either additional antigenic cDNA or adjuvant properties.

An important issue for clinical translation of these studies is whether cDNA that is sourced from a xenogeneic, normal cell type is more, or less, efficacious against tumors than is VSV-cDNA derived from a xenogeneic, tumor source. From our studies in the prostate model¹⁸, it is clear that a cDNA library from either normal, or tumor, cells of the same histological type is very effective against established tumors. We hypothesize that a library from normal cells might incorporate a greater range of antigens against which tolerance could be broken for tumor rejection than a library from tumor cells, because key antigens might have been lost during *in vivo* evolution of the tumor cells in response to immune pressure. In addition, our data from the prostate model¹⁸ clearly show that a xenogeneic (human) library is immunologically superior (in terms of generating rejection of (mouse) tumors) to a fully self (mouse) library from the normal tissue when presented in the context of VSV. Therefore, in human clinical studies, we would predict that a VSV-expressed cDNA library derived from a xenogeneic melanocyte cell source would be at least as effective, if not more so, than a human tumor cDNA library, although further studies are required to confirm this hypothesis.

In summary, we describe here a technology to identify arrays of self, or near-self, TAA that act optimally in combination to signal T-cell activation and tumor rejection. This technology should be applicable for the treatment of a variety of cancer types. In addition, it should permit the cloning of antigens that in isolation might not be identified as TAA but that, when acting as one component of an array of different antigens, could play a role in generating optimal anti-tumor immunity. The success of VSV-expressed cDNA libraries in identifying self or near-self TAA is based on at least three properties. First, the immunogenicity of antigen presentation through viral-associated gene expression induces potent T-cell responses against the TAA, an effect which is augmented by the preparation of a cDNA library from normal, altered self tissue. Second, use of a cDNA library allows a large number of potential TAA to be surveyed by the immune system. Third, the broad tropism of VSV enables delivery *in vivo* of a large repertoire of potential TAA to a wide range of different APCs. Therefore, development of this technology should allow identification of a series of individually and clinically relevant epitopes that, when expressed combinatorially from an appropriate viral vector, can collectively be used to construct a translational pipeline of viral vaccines with a safe, but effective, therapeutic index.

ONLINE METHODS

Cells and viruses

B16 cells are derived from a mouse melanoma syngeneic to C57BL/6 mice (H2-K^b)⁴². TRAMP-C2 (TC2) cells are derived from a prostate tumor that arose in a TRAMP mouse and were characterized as described¹³.

VSV-cDNA libraries were generated as described previously¹⁸. Briefly, cDNA from two human melanoma cell lines, Mel624 and Mel888, was pooled, cloned into the pCMV.SPORT6 cloning vector (Invitrogen) and amplified by PCR. The PCR-amplified cDNA molecules were size fractionated to below 4 kbp for ligation into the parental VSV genomic plasmid pVSV-XN2 (ref. 43) between the G and L genes. The complexity of the ASMEL cDNA library cloned into the VSV backbone plasmid between the XhoI and NheI sites was 7.0×10^6 colony forming units. Virus was generated from BHK cells by co-transfection of pVSV-XN2-cDNA library DNA along with plasmids encoding viral genes as described⁴³. Virus was expanded by a single round of infection of BHK cells and purified by sucrose gradient centrifugation.

VSV-GFP was generated by cloning the cDNA for GFP into the plasmid pVSV-XN2, as described⁴³. Monoclonal VSV-GFP was obtained by plaque purification on BHK-21 cells. Concentration and purification were done by sucrose gradient centrifugation. Titers were measured by standard plaque assays on BHK-21 cells⁴³.

Preparation of Pmel, OT-I or C57BL/6 normal lymphocytes

OT-I mice and Pmel mice have been previously described^{30,35} and were bred at the Mayo Clinic. Pmel T cells express a transgenic T-cell receptor specific for the KVPRNQDWL peptide from the human, melanocyte-specific gp100 antigen (hgp100₂₅₋₃₃), which is presented in the context of the mouse H-2Db major histocompatibility complex (MHC) class I molecule by C57BL/6 mice³⁰. The OT-I mouse strain is on a C57BL/6 background (H-2K^b) and expresses a transgenic T-cell receptor V α 2 specific for the SIINFEKL peptide of OVA in the context of MHC class I, H-2K^b (ref. 35). Spleen and lymph nodes from Pmel, OT-I or C57BL/6 mice were crushed through a 100- μ m filter to prepare a single-cell suspension. Red blood cells were removed by a 2-min incubation in ACK buffer. These pooled LN cell and splenocyte cultures were either used in the assays described or, where appropriate, CD8⁺ T cells were isolated using the MACS CD8⁺ (Ly-2) microbead magnetic cell sorting system (Miltenyi Biotec). Fluorescence-activated cell sorting (FACS) analysis demonstrated cultures typically of >98% CD8⁺ T cells, <2% CD4⁺ T cells, <0.1% contaminating, non-CD8⁺ NK1.1⁺ve cells. Viable cells were purified by density gradient centrifugation using Lympholyte-M (Cedarlane Laboratories).

In vivo studies

All procedures were approved by the Mayo Foundation Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from Jackson Laboratories at 6–8 weeks of age. TLR4 and TLR7 knockout (KO) mice (TLR4^{-/-}, TLR7^{-/-}) were purchased from The Jackson Laboratory at 6–8 weeks of age. MyD88 KO mice (MyD88^{-/-}) were a kind gift from L. Pease, Mayo Clinic. To establish subcutaneous tumors, 2×10^5 B16 cells in 100 μ l of PBS were injected into the flank of mice. Intravenous injections of virus were administered in 100 μ l volumes. For survival studies, tumor diameter in two dimensions was measured three times weekly using calipers, and mice were killed when tumor size was $\sim 1.0 \times 1.0$ cm in two perpendicular directions. Immune cell depletions were done by intraperitoneal injections (0.1 mg per mouse) of anti-CD8 (Lyt 2.43), anti-CD4 (GK1.5), both from the Monoclonal Antibody Core Facility, Mayo Clinic; anti-NK (anti-asialo-GM-1, Cedarlane) and IgG control (ChromPure Rat IgG, Jackson ImmunoResearch) at day 4 after tumor implantation and then weekly thereafter. FACS analysis of spleens and lymph nodes confirmed subset specific depletions.

Reverse transcriptase PCR

RNA was prepared from cell cultures with the Qiagen RNA extraction kit. 1 μ g total cellular RNA was reverse transcribed in a 20 μ l volume using oligo-(dT) as a primer. A cDNA

equivalent of 1 ng RNA was amplified by PCR with gene specific primers VSV 5'-ACTAACAGATATCACGCTCGAG-3' and 5'-CCAAACATGAAGAATCTGGCTA-3'.

ELISA analysis for IL-17, TGF- β or IFN- γ secretion

Typically, 10^6 splenocytes were incubated at 37 °C with freeze thaw lysates from tumor cells in triplicate, every 24 h for 3 d. Forty-eight hours later, cell-free supernatants were collected and tested by specific ELISA for IL-17 (R&D Systems), TGF- β or IFN- γ (BD OptEIA; BD Biosciences).

Dependence of suppression on TGF- β was assayed using recombinant human TGF- β sRII/Fc chimera (R&D Systems), a 159-amino acid extracellular domain of human TGF- β receptor type II fused to the Fc region of human IgG1 (refs. 34,41).

Statistics

Survival data from the animal studies were analyzed using the log rank test, and the two-sample unequal variance student's *t*-test analysis was applied for *in vitro* assays. Statistical significance was determined at the level of $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Validation of the altered self melanoma epitope VSV-cDNA library. **(a)** BHK cells were screened for expression of *gp100* and *TRP2*, *PSA*, or *GAPDH* by rtPCR after infection with no virus (lane 1), the ASMEL (MOI ~1) (lane 2) or VSV-GFP (lane 3). Lane 4, cDNA from the human LnCap prostate cancer cell line. **(b)** 10⁶ C57BL/6 splenocytes infected 24 h previously with either VSV-GFP, ASMEL or a VSV-cDNA library derived from normal human prostate tissue¹⁸ (MOI ~10) were co-cultured with either naive Pmel or OT-I T cells (effector/target ratio of 5:1) 4 and 28 h after virus infection. Supernatants were assayed 24–48 h later for IFN-γ, to detect transfer of expression of the hgp100_{25–33} KVPRNQDWL peptide³⁰ from the virus to infected splenocytes and subsequent presentation to the Pmel T cells. **(c)** Lane 1, splenocytes alone (no virus, no T cells); lane 2, splenocytes infected with VSV-GFP with added Pmel T cells; lane 3, splenocytes infected with the ASMEL with added OT-I T cells, which recognize the irrelevant SIINFEKL epitope of the OVA antigen³⁵; lane 4, splenocytes infected with ASMEL with added Pmel T cells, which recognize the hgp100_{25–33} KVPRNQDWL epitope³⁰; lane 5, splenocytes infected with VSV-cDNA from normal human prostate¹⁸ with added Pmel T cells; lane 6, naive Pmel alone (no splenocytes, no virus). Error bars, s.d.

Figure 2.

Intravenous ASMEL cures established B16 melanomas. **(a)** Mice bearing B16 tumors established for 5 d were either mock depleted (IgG), or were depleted of CD4⁺ or CD8⁺ T cells. Mice were then administered nine intravenous injections of the ASMEL or VSV-GFP (10^7 pfu/injection) on days 8–10, 15–17 and 22–24. Survival with time is shown. **(b,c)** Six months after treatment with nine intravenous injections of the ASMEL, the only overt symptoms of autoimmune vitiligo were a whitening of the whiskers **(b)** and tails **(b,c)** compared to age-matched mice treated with PBS. **(d)** Pooled lymphoid cells (10^6 /well) from mice treated with nine intravenous injections of the ASMEL were infected 24 h later with ASMEL or VSV-GFP (MOI ~10). The cultures were replenished 24 h later by an additional 10^6 lymphoid cells with a further round of virus infection 24 h after that. Cultures were stimulated 48 h after the final infection with virus, with either prostate tumor TC2 or melanoma B16 cell lysates and 48 h later, supernatants were assayed for IL-17 by ELISA. Lanes 1–4, lymphoid cells from ASMEL-treated mice treated with VSV-GFP and no tumor lysate (1); VSV-GFP and B16 tumor lysate (2); ASMEL and B16 tumor lysate (3) or ASMEL and TC2 tumor lysate (4) (ref. 40). Lanes 5–7, lymphoid cells from ASMEL-treated mice depleted of CD4 (5), CD8 (6) or NK cells (7) and treated *in vitro* with ASMEL and B16 tumor lysate. Error bar, s.d.

Figure 3.

VSV functions as an hsp70-mediated adjuvant. **(a)** Expression of hsp70 was assayed by rtPCR from cDNA prepared from pooled lymphoid cells (10^4 /well) of C57BL/6 mice 24 h after infection with either VSV-GFP or ASMEL at increasing MOI as shown. **(b)** Expression of hsp70 by rtPCR from pooled lymphoid cells (10^4 /well) of C57BL/6 mice at 24, 36 or 48 h after infection with either VSV-OVA, VSV-GFP or ASMEL at MOI of 10. **(c)** Pooled lymphoid cells (10^4 /well) from mice treated with nine intravenous injections of ASMEL were infected 24 h later with ASMEL or VSV-GFP at various MOI and either in the presence, or absence, of added recombinant hsp70 (10 μ g/ml). Representative wells were assayed by rtPCR 24 h later, for hsp70 expression. Similarly treated triplicate wells were replenished with an additional 10^4 lymphoid cells and treated with a further round of virus infection 24 h later. Forty-eight hours following this final infection with virus, cultures were stimulated with melanoma B16 cell lysates and 48 h later supernatants were assayed for IL-17 by ELISA. **(d)** Expression of hsp70 was assayed by rtPCR from cDNA prepared from pooled lymphoid cells (10^4 /well) of TLR7^{-/-} (lane 1), MyD88^{-/-} (2), TLR4^{-/-} (3) or C57BL/6 (4) mice 24 h after infection with the ASMEL at an MOI of 10. **(e)** Pooled lymphoid cells (10^4 /well) from C57BL/6, MyD88^{-/-}, TLR4^{-/-} or TLR7^{-/-} mice treated with nine intravenous injections of ASMEL were infected 24 h later with ASMEL at an MOI of 10 either in the absence (-rhsp70), or presence (+rhsp70), of added recombinant hsp70 (10 μ g/ml). Representative wells were assayed 24 h later, by rtPCR for hsp70 expression. Similarly treated triplicate wells were replenished with an additional 10^4 lymphoid cells and reinfected with a further round of virus infection 24 h later. Forty-eight hours after this final infection with virus, cultures were stimulated with melanoma B16 cell lysates and 48 h later supernatants were assayed for IL-17 by ELISA.

Figure 4.

VSV-induced TGF- β masks the tumor-specific IL-17 recall response. **(a,b)** Lymphoid cell cultures from ASMEL-vaccinated mice were screened for IL-17 **(a)** or TGF- β **(b)** secretion after infection with 12 single viruses purified by limiting dilution from the parental, unselected ASMEL stock (ASMEL nos. 1–12), with representative viruses selected from the screen of Supplementary Figure 1a. VSV-cDNA clone no. 3 (encoding NRAS sequences), VSV-cDNA clone no. 7 (encoding TYRP1 sequences), VSV-cDNA clone no. 11 (encoding CYC1 sequences) or with VSV-GFP (all infections at MOI 10). **(c)** The IL-17 screening assay of **a** above was repeated but in the presence of TGF- β RII Fc chimera to inhibit TGF- β in the cultures^{34,41}. Error bars, s.d.

Figure 5.

Tumor-specific immunity induced by VSV-cDNA libraries is mediated by combinations of antigens. **(a)** Lymphoid cell cultures from ASMEL-vaccinated mice were screened for IL-17 secretion after no infection (lane 13); or with infection with 10^7 pfu of individual single viruses from the parental, unselected ASMEL stock (ASMEL nos. 1–6) (lanes 1–3, 5–7); with the selected viruses VSV-cDNA clone no. 3 (NRAS), VSV-cDNA clone no. 7 (TYRPI), VSV-cDNA clone no. 11 (CYC1) (lanes 9–11); or with VSV-GFP (lane 13); in addition, infections were performed with three-way combinations (3×10^6 pfu of each virus) of ASMEL no. 1 + no. 2 + no. 3 (lane 4); ASMEL no. 4 + no. 5 + no. 6 (lane 8); or VSV-cDNA clones no. 3 + no. 7 + no. 11 (lane 12). Error bars **(a–c)**, s.d. **(b)** The experiment in **a** was repeated in the presence of TGF- β RII Fc chimera to inhibit TGF- β in the cultures^{34,41}. **(c)** Pooled lymphoid cell cultures (10^5 /well) from naive C57BL/6 mice were infected every 2 d with the ASMEL or VSV-GFP over a 2-week period, with regular replenishment of the lymphoid cells. Lymphoid cell cultures were either left uninfected (lane 1); or infected with single viruses (10^6 pfu) VSV-GFP (lane 2); ASMEL parental virus stock (3); VSV-cDNA clone no. 3 (NRAS) (4); VSV-cDNA clone no. 7 (TYRPI) (5), VSV-cDNA clone no. 11 (CYC1) (6); VSV-cDNA clone no. 12 (TRIM33) (7). In addition, lymphoid cell cultures were infected with two-way combinations of viruses: clone no. 3 (NRAS) + clone no. 7 (TYRPI) (8); clone no. 3 (NRAS) + clone no. 11 (CYC1) (9); clone no. 3 (NRAS) + clone no. 12 (TRIM33) (10); clone no. 7 + clone no. 11 (11); clone no. 7 + clone no. 12 (12); clone no. 11 + clone no. 12 (13). Lymphoid cell cultures were also infected with three-way combinations of viruses: clone no. 3 + clone no. 7 + clone no. 11 (14); clone no. 3 + clone

no. 7 + clone no. 12 (15); clone no. 3 + clone no. 11 + clone no. 12 (16); clone no. 7 + clone no. 11 + clone no. 12 (17). Finally cultures were also infected with a four-way combination of clone no. 3 + clone no. 7 + clone no. 11 + clone no. 12 (18). Forty-eight hours following the final infection with virus, cultures were stimulated with B16 melanoma cell lysates for three consecutive days. Forty-eight hours later supernatants were assayed for IL-17 by ELISA. **(d)** Mice bearing 5-d-old established B16 tumors were treated ($n = 6-8/\text{group}$) with intravenous injections of VSV-GFP or VSV-expressing the melanoma-associated antigen hgp100 as described³³. **(e)** Mice bearing 5-d-old established B16 tumors were treated ($n = 6-8/\text{group}$) with nine intravenous injections on days 6–8, 13–15 and 20–22 of two-way combinations (total viral dose 10^7 pfu/injection; 5×10^6 pfu of each individual virus) of either VSV-cDNA clone no. 3 (NRAS) + clone no. 7 (TYRP1); clone no. 3 (NRAS) + clone no. 11 (CYC1); or clone no. 7 + clone no. 11. A fourth group was treated with a three-way combination (total viral dose 10^7 pfu/injection; 3×10^6 pfu of each individual virus) of VSV-cDNA clone no. 3 (NRAS) + clone no. 7 (TYRP1) + clone no. 11 (CYC1). Survival with time is shown.