Broad Antigenic Coverage Induced by Viral cDNA Library-based Vaccination Cures Established Tumors

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Abstract

We show here that a cDNA library of normal tissue, expressed from a highly immunogenic viral platform, cures established tumors of the same histological type from which the cDNA library was derived. With suboptimal vaccination, immune escape was possible, but only when tumor cells were forced to acquire a radically new phenotype, readily treated by second line therapy. This approach has several major advantages. Use of the cDNA library leads to presentation of a broad repertoire of (undefined) tumor associated antigens, which reduces emergence of treatment resistant variants and also permits implementation of rational, combined modality approaches in the clinic. Finally, the viral vectors can be delivered systemically, without the need for tumor targeting, and are amenable to clinical grade production. Therefore, virus-expressed cDNA libraries represent a novel paradigm for cancer treatment by addressing many of the key issues which have undermined the efficacy of immuno/virotherapy to date.

Keywords

Vesicular Stomatitis Virus; cancer vaccines; tumor antigens; oncolytic virus; immune escape

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Several key issues have undermined effective cancer immuno/virotherapy, including lack of identification of tumor associated antigens (TAA), poor coverage of the antigenic repertoire expressed by tumors and difficulties of vector targeting to tumors *in vivo*[1–6]. To allow for direct *in vivo* immune selection of appropriate TAA, we showed that killing normal cells *in situ*, with the adjuvant hsp70, generated T cell responses to antigens which mediated rejection of tumors of the same histological type[7–11]. With this approach[12, 13], a broad repertoire of individually weak T cell responses is raised against multiple TAA imposing a cumulatively strong selective pressure against immune escape and no tumors have to be accessed by targeted vector delivery. However, injection of cytotoxic vectors into the normal tissues often resulted in toxicity[8, 9]. To alleviate these complications, we reasoned that the broad antigenic repertoire for *in vivo* immune selection of relevant TAA could be provided by expressing a cDNA library of a normal tissue from a systemically delivered, immunogenic vector to activate autoimmune/anti tumor T cell responses.

In this respect, viruses such as Vesicular Stomatitis Virus (VSV) can act as potent adjuvants for expression of antigens[14–17], including TAA[18–20], provided the TAA-expressing virus can access the draining lymph nodes (DLN)[20, 21–23, 24].

Combining these observations, we hypothesized that cDNA from a normal tissue could be expressed from the VSV platform[14–20, 23] to vaccinate against a wide range of TAA expressed on tumors of the same histological type. We also exploited the observation that altered self epitopes of TAA can be more immunogenic than the corresponding self epitopes[25–27]. We show here that a Viral Expressed Epitope Library (VEEL) from normal human prostate can induce rejection of established murine prostate tumors, without detectable autoimmunity. If tumors escaped immune selection, they adopted a radically new phenotype, which could be treated with second line therapy. Therefore, virus-expressed cDNA libraries represent a novel paradigm by which the ability of highly mutable tumor cells to escape selective pressures *in vivo* can be exploited to therapeutic advantage.

RESULTS

Viral Expressed Altered Self Epitope Library (VEASEL)

A cDNA library from normal human prostate, expressing Altered Self Antigens/Epitopes (in a murine context) from the Library (ASEL) was cloned into VSV (Fig.1a) in direct or reverse orientations. ASEL(Direct) and ASEL(Reverse) libraries had titers (1×10^7–6×10^7 pfu/ml) 1–2 log lower than VSV-GFP.

Sequences of human prostate antigens, but not the melanocyte/melanoma associated TRP-2 (Fig.1b), gp100 or tyrosinase antigens (not shown), were present in the ASEL libraries (Fig. 1b, 1&2). Expression of prostate-specific sequences (Fig.1b, 3&4), as well as, in some cases, full-length protein (PSA), was transferred by ASEL virus (Fig.1c). Using rtPCR (Fig. 1d), we estimated that the positive PSA signal in 10^5 infected BHK cells (Fig.1c) derived from infection by about 200 VSV-PSA particles, and yielded PSA at 10 fold lower levels than in 10^4 LN-Cap human prostate cells (Fig.1c). When VSV-expressed cDNA libraries from murine cells which either did, or did not, express chicken ovalbumin (OVA), were used to infect H2-K^b splenocytes, naive OT-I T cells were activated to secrete IFN-γ,

indicating transfer of expression of the SIINFEKL epitope[28] at levels similar to infection with VSV-ova at an MOI of 0.01 (Fig.1e). However, the PCR assay of Fig.1d estimated that the VSV-cDNA(ova) virus was present in the splenocyte infections of Fig.1e at a MOI of about one log lower.

Intravenous injection of ASEL does not induce autoimmunity

Within two days, prostates injected with VSV-GFP or ASEL were enlarged compared to PBS-injected controls (p=0.04 or 0.01) (Fig.2a). Within 10 days, prostate weights were significantly lower in mice injected with ASEL compared to controls (p<0.001) (Fig.2a), associated with loss of tissue architecture and immune infiltration (Figs.2b-d). Splenocytes from mice injected intra-prostatically with ASEL secreted both IFN-γ (Fig.2e) and IL-17 (Fig.2f) in response to normal prostate (not shown) or TC2 murine prostate tumor cells (Fig. 2e,f), but not to murine B16 melanoma cells (not shown). In contrast, after 60 days, prostates from mice injected i.v. with ASEL were not significantly different from controls in either weight (Fig.3a) or histology (not shown).

Intravenous injection of ASEL cures established tumors

Intravenous ASEL generated a prostate-specific Th17, but not an IFN-γ, response (Fig.3b). Moreover, 3 i.v. injections of ASEL gave the best survival compared to either i.t. ASEL (p=0.01) or i.t. VSV-GFP (p<0.0001) (Fig.3c). Neither i.v. VSV-GFP (Fig.3c), nor i.v. ASEL(Reverse) (not shown), generated therapy. In contrast, the ASEL had no effects against B16 melanomas (Fig.3d,e). Similarly, a VSV-cDNA library from human melanoma cells significantly slowed murine B16 melanomas, but had no effect against TC2 tumors (Fig.3d,e).

With more injections of ASEL, tumors were cured more effectively with i.v., compared to i.t., treatment (Fig.3f). Thus, 9 i.v. injections of ASEL cured over 80% of mice compared to 0% with VSV-GFP (p<0.0001) or ASEL(Reverse) (not shown) (Fig.3f) - with no detectable autoimmune prostatitis. Unlike i.v. (Fig.3b), no mice treated i.t. with ASEL, including three cures, developed Th17 (Fig.3g) or IFN–γ (not shown) responses to prostate. Consistent with an immune, as opposed to oncolytic, mechanism for i.v. ASEL, therapy was dependent upon CD4+ T cells but not CD8+ T cells or NK cells (Fig.3f).

In vivo selection of immune escape variants

3 i.v. injections of ASEL (Figs.3c-f) typically induced initial regression with subsequent aggressive recurrence. Recurrent TC2R tumors were significantly different from parental TC2 tumors histologically with extensive interstitial lymphocyte infiltrates (Figs.4a-d). Similarly, TC2R tumors had lost, or reduced, expression of murine homologues of the human prostate-specific antigens in the ASEL (Fig.4e), as well as increased expression of N-Cadherin, SNAIL and SLUG, associated with an epithelial-mesenchymal-like transition (Fig.4f)[29–32].

Sequential vaccination cures recurrences

Viral Expressed Immune Escape Epitope Libraries (IEEL), constructed from TC2R tumors from mice treated with ASEL (Fig.5a), contained sequences of TC2R-characteristic genes

(SNAIL and SLUG) (not shown) and transferred expression of TC2R-expressed N-Cadherin (Figs.4f,5b).

TC2 tumor-bearing mice treated with ASEL (days 7,9,11) followed by IEEL (days 25, 27 & 29) had no survival advantage over ASEL alone, probably due to neutralization of IEEL virus by NAb against VSV raised by injections of ASEL[23] (Fig.5c). Consistent with reports that VSV is protected from NAb by loading onto PBL at low MOI [21–23], treatment of VSV-immune, TC2-bearing mice with PBL pre-loaded with ASEL restored therapy (Fig.5c). Therefore, we repeated sequential ASEL/IEEL treatment with IEEL virus pre-loaded onto PBL [T(IEEL)]. In Figs.5c,d, 4/7 mice treated with ASEL/T(IEEL) developed recurrences, but slower than with ASEL alone (Fig.5d). The 3 remaining mice never developed recurrences were tumor-free for over 100 days (Fig.5d) (p=0.001 compared to ASEL).

For prolongation of survival in the presence of NAb from the start of therapy, delivery of ASEL required pre-loading onto PBL but still induced recurrence (Fig.5e). Sequential T(ASEL)/T(IEEL) generated long-term cures with, in Fig.5e, only a single recurrence. Starting IEEL at day 20 (Fig.5e) consistently led to more long term cures compared to starting at day 27 (Fig.5d). The sequence of vaccination was also critical as neither T(IEEL) nor IEEL alone delayed growth of TC2 tumors (Fig.5e).

Sequential vaccination induces IFN-γ responses to TC2R

Long-term survivors of ASEL/IEEL (Figs.5d,e) developed a prostate-specific, Th17 response against TC2 and normal prostate (Fig.5f, 2&5) but not against TC2R cells (Fig.5f, 3). Conversely, splenocytes from survivors did not secrete IFN-γ in response to TC2 or normal prostates (Fig.5g, 2&5), but consistently produced IFN-γ in response to TC2R tumors (Fig.5g, 3). In contrast, non-survivors of ASEL/IEEL did not secrete IFN-γ in response to TC2R tumors (Figs.5g,i). Over two separate experiments, 7/14 (50%) of mice treated with ASEL (days 7,9,11), followed by IEEL (days 25,27,29), as in Fig.5d, were cured long term, compared to no cures (0%) with ASEL alone. In contrast, 0/14 mice (0%) treated with ASEL followed by IEEL, but with CD8+ T cell depletion (days 34,35), were cured.

Altered Self Libraries Afford Better Protection than Self Libraries

Finally, to investigate the contribution of responses against xenogeneic proteins, we constructed the Self Epitope expressed Library, SEL, from normal mouse prostate. The ASEL conferred significantly better protection against TC2 than the SEL (p=0.04), although both ASEL and SEL were better than PBS (p=0.0064, SEL vs PBS) or VSV-GFP (p=0.0029 SEL vs VSV-GFP) (Fig.6a). Similarly, *in vitro* stimulation/infection of splenocytes by the SEL induced lower levels of Th17 against prostate targets compared to the ASEL (Figs.6b-e).

DISCUSSION

We show here that cDNA libraries transferred into VSV express tissue-specific sequences and proteins (Figs.1b-d) and are immunologically functional (Fig.1e). Using human prostate cDNA to express altered self epitopes[25, 26, 33], we generated VEASEL (Viral Expressed

<u>A</u>ltered <u>S</u>elf Antigen/<u>E</u>pitope <u>L</u>ibrary) virus. Intra-prostatic ASEL induced Th17 and IFN-γ responses against prostate antigens (Figs.2e,f) which were probably responsible for the associated prostatitis (Figs.2a-d)[9, 34]. In contrast, i.v. ASEL induced only Th17, and not IFN-γ, responses to prostate antigens (Fig.3b) and no overt signs of prostatitis[9, 34] (Fig.3a), probably because of a lack of inflammatory signals within the prostate induced by intraprostatic injection.

We observed both a Th17 response against prostate antigens (Fig.3b) and cures of established TC2 tumors (Fig.3f) mediated by CD4+ T cells (Fig.3h), upon repeated i.v. injections of ASEL. It is tempting to speculate that a CD4+/Th17 response was responsible for these cures, although further depletion studies are required to confirm this. We believe that the direct oncolytic activity of VSV may have contributed to, but was not responsible for, these cures, because i.v. VSV-GFP had no similar efficacy (Fig.3f) and intra-tumoral ASEL was less effective against TC2 tumors than intravenous injection (Fig.3c). This is probably because intravenous injection of virus allows for significantly better access to the lymph nodes for cross priming of APC.

Emergence of treatment-resistant tumor variants is of great clinical significance[1–3, 6]. When vaccination was insufficient to clear tumors (Fig.3c), rapid re-growth of aggressive recurrent TC2R tumors was observed, which had *induced* new differentiation programs similar to an epithelial to mesenchymal type transition[29–32] (Figs.4a-d,f). As a result, ASEL-treated mice could be vaccinated early during the immune-driven evolution of TC2 to TC2R tumors against emergence of aggressive, TC2R tumors by Virus Expressed IEEL, which transferred expression of TC2R, as opposed to TC2, characteristic genes (Figs.4f, 5a,b,e), using PBL loading to protect IEEL viruses from NAb[21–23, 35, 36] (Figs.5c,d). Efficacy depended upon the sequence in which the libraries were given (Fig.5E), early treatment with IEEL (Figs. 5d,e), and upon two distinct, but synergistic, immune re-activities differing in antigen specificity and effector phenotypes (Fig.5f-i). The first ASEL-primed response (Th17-associated, CD4-dependent, TC2/prostate specific, TC2R 'blind') forced TC2 tumors to evolve into a phenotype against which the second, IEEL-primed response (IFN-γ-associated, CD8-dependent, prostate 'blind', TC2R specific), could clear recurrent tumors as they emerged.

We observed poorer protection with the SEL (self antigens) compared to the ASEL (xenogeneic, altered self antigens) (Fig.6a) and weaker stimulation against prostate targets by the SEL *in vitro* (Fig.6b-e). Significantly, the ASEL protected mice against TC2 tumors, but not B16 melanomas, and failed to stimulate splenocyte reactivity against non-prostate targets (Fig.5). This suggests that immune reactivity generated by the ASEL is directed not simply against xenogeneic household proteins (on B16 cells and normal pancreas) but against lineage-specific antigens - possibly reflecting a difference in the levels of T cell precursors to these two classes of antigens which survive both thymic and peripheral selection. Conversely, since xenogeneic antigens associated with rejection of allogeneic transplants include minor histocompatibility antigens (not lineage specific), the antigenic targets of rejection of TC2 tumors may turn out to be more widely expressed than simply in the prostate.

In summary, we show here that it is possible to vaccinate mice against established tumors using a wide repertoire of self[7, 13], or near self[25, 26, 33], epitopes encoded by a cDNA library expressed from the platform of a highly immunogenic virus. This allows for *in vivo* immune selection of TAA relevant for tumor rejection, but does not require their identification. In addition, systemic delivery of VSV-based libraries is possible, prevents damage inherent in delivering vectors directly to normal tissues[8, 9] and did not induce autoimmunity.

METHODS

Cells and viruses

TRAMP-C2 (TC2) cells are derived from a prostate tumor that arose in a TRAMP mouse, grow in an androgen independent manner and are routinely grown as tumors in C57BL/6 male mice[9]. Murine B16ova melanoma cells (H2-K^b) were derived from B16 cells by transduction with a cDNA encoding the chicken ovalbumin gene [43].

cDNA from normal human prostate (Biochain, Hayward, CA) was amplified from the BioExpress shuttle vector by PCR and cloned into the VSV genomic plasmid pVSV-XN2 [44] between the G and L genes. VSV-cDNA libraries were generated from the human prostate cDNA by size fractionating PCR cDNA molecules to below 4kbp, since lower sized cDNA inserts were associated with both higher viral titers and lower proportions of Defective Interfering particles. The complexity of the ASEL cDNA library cloned into pVSV-XN2 between the *Xho*1-*Nhe*1- sites was 4.75×10^6 colony forming units (at dilutions of 10^{-6} and 10^{-5} there were 5 and 45 colonies respectively). Of 20 colonies picked at random, 3 had no insert, 5 had an insert of less than 0.5kbp and 12 had inserts between 0.5kbp and 4kbp. Virus was generated from BHK cells by co-transfection of pVSV-XN2-cDNA library DNA along with plasmids encoding viral genes as described in[44]. Virus was expanded by a single round of infection of BHK cells and purified by sucrose gradient centrifugation.

Additional cDNA libraries from tumor cells (TC2R, B16ova, B16) were cloned into the pCMV.SPORT6 vector (Invitrogen, CA), amplified by PCR, and cloned into pVSV-XN2 before being packaged and amplified.

VSV-GFP was generated by cloning the cDNA for GFP into pVSV-XN2, as described[44]. Monoclonal VSV-GFP was plaque purified on BHK-21 cells and concentrated by sucrose gradient centrifugation. Titers were measured by plaque assays on BHK-21 cells[44].

Histopathology

Prostates or tumors were fixed in 10% Formalin in PBS, paraffin embedded and sectioned. H&E-stained sections were prepared for analysis of tissue destruction and gross infiltrate.

Preparation of lymphocytes

The OT-I mouse strain, on a C57BL/6 background (H-2K^b), expresses a transgenic T-cell receptor Vα2 specific for the SIINFEKL peptide of ovalbumin in the context of MHC class I, H-2K^b [28]. Spleen and lymph nodes from OT-I or C57BL/6 mice were crushed through a 100μm filter to prepare single-cell suspensions. RBC were removed by a 2-min incubation in ACK buffer. CD8$^+$ T cells were isolated using the MACS CD8α (Ly-2) Microbead

magnetic cell sorting system (Miltenyi Biotec). FACS analysis demonstrated cultures typically of >98% CD8$^+$ T cells, <2% CD4$^+$ T cells, <0.1% NK1.1+ve cells. Viable cells were purified using Lympholyte-M (Cedarlane Laboratories).

Loading of lymphocytes with VSV

CD8$^+$ lymphocytes were pelleted and incubated with VSV in 100μl for 4h at 4°C as described in[23]. Cells were washed x3 in ice cold PBS and used directly for *in vivo* adoptive transfer[23].

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. To establish subcutaneous tumors, 2×10^6 TrampC2 cells in 100μl PBS were injected into the flank. Intra-tumoral injections were performed in a total of 50μl. Intravenous injections of virus were administered in 100μl volumes. For adoptive transfer experiments, mice were i.v. administered normal lymphocytes, or lymphocytes pre-loaded with VSV, typically at $2x10^6$ cells in 100μl. For survival studies, tumor diameter in two dimensions, was measured three times weekly using calipers, and mice were killed when tumor size was approximately 1.0x1.0 cm in two perpendicular directions.

Immune cell depletions were performed by i.p. injections (0.1mg per mouse) of anti-CD8 (Lyt 2.43), anti-CD4 (GK1.5), (Monoclonal Antibody Core Facility, Mayo Clinic); anti-NK (anti-asialo-GM-1, Cedarlane) and IgG control (ChromPure Rat IgG, Jackson ImmunoResearch). FACS analysis of spleens and lymph nodes confirmed subset specific depletions.

Reverse transcriptase PCR

RNA was prepared from cells with the QIAGEN RNA extraction kit. 1μg total cellular RNA was reverse transcribed in a 20μl volume using oligo-(dT) as a primer. A cDNA equivalent of 1ng RNA was amplified by PCR with gene specific primers (details of primers on request).

Protein expression

Expression of human PSA was detected with the Prostate Specific Antigen antibody ab53774 (abcam) (rabbit polyclonal) and murine N-Cadherin with the 32/N-Cadherin mouse anti-N-Cadherin antibody (BD Biosciences) using standard Western Blot procedures.

ELISA analysis

Typically, 10^6 splenocytes were incubated at 37°C with freeze thaw lysates from tumor cells, or normal tissues, in triplicate, every 24 hours for 3 days. 48hrs later, cell-free supernatants were tested by specific ELISA for IL-17 (R&D Systems) or IFN-γ (BD OptEIA IFN-γ; BD Biosciences).

Statistics

Survival data was 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 $p < 0.05$.

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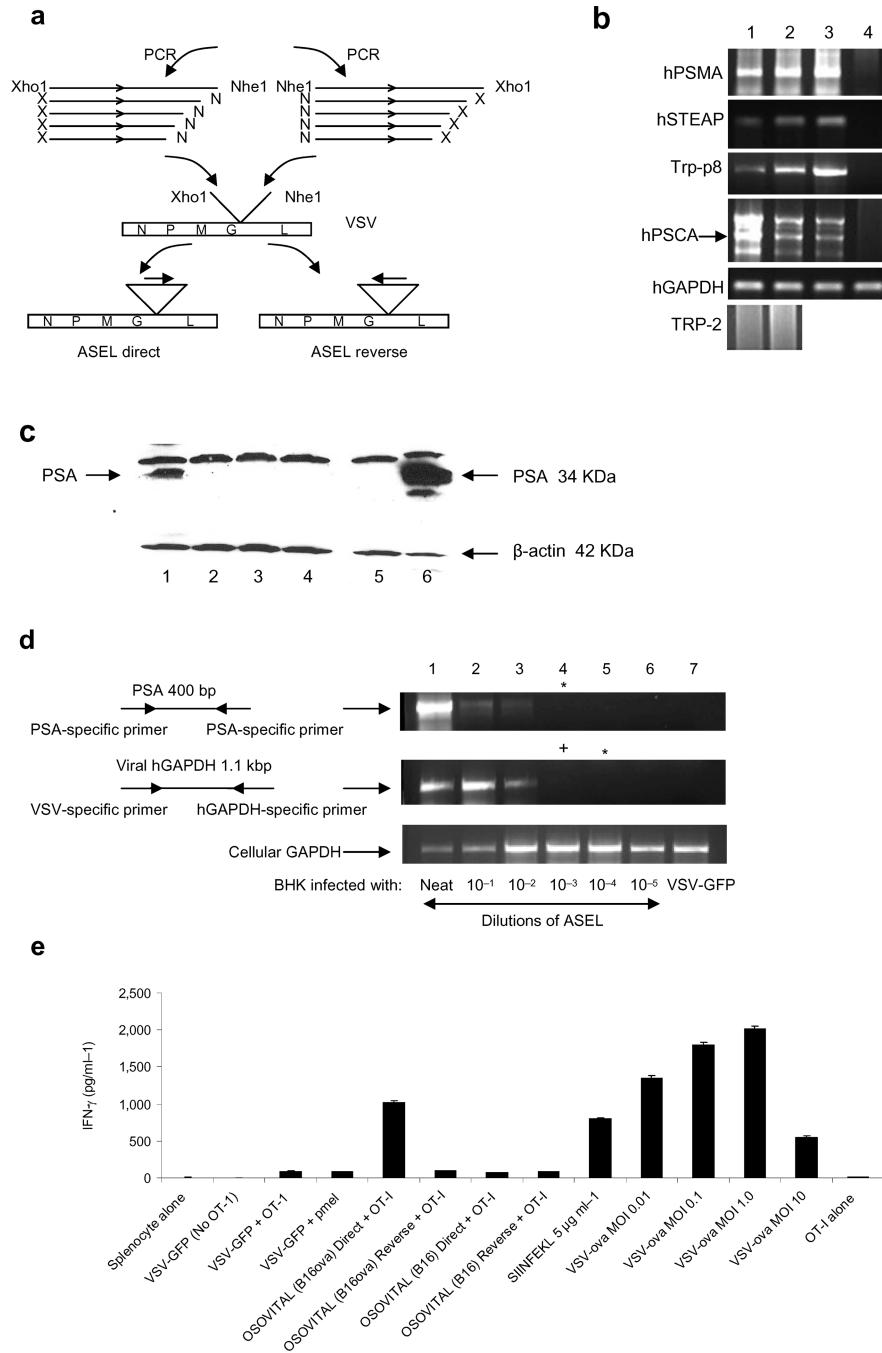
Figure 1. VSV-expressed cDNA libraries
a. The ASEL VSV-expressed cDNA library contains cDNA from normal human prostate cloned into VSV in direct, or reverse, orientation. **b.** Human prostate specific genes[37–40], but not the melanocyte-specific TRP-2[11], detected by PCR in the original human prostate plasmid library (lane 1) and in the VSV-cDNA plasmid library (2). rtPCR from cDNA of HT1080 cells infected with ASEL (MOI 0.1) (3) compared to uninfected cells (4) (predicted size for hPSCA shown with an arrow). **c.** BHK cells infected with ASEL Direct (1) or Reverse (2), or with control viruses (lanes 3&4) (MOI ~10) assayed for human PSA by

Western Blot. Lane 5, uninfected BHK cells; Lane 6, 10^4 human prostate LnCap cells. **d.** BHK cells infected with 10 fold dilutions of ASEL virus (1–6) assayed by rtPCR for PSA or human GAPDH. No PSA-specific signal was detected at dilutions lower than 1:100 of the original virus stock. (Expression of GFP from 100 pfu of VSV-GFP could be detected by this assay). +Positive/*negative for PCR upon nested PCR. **e**. Splenocytes infected with VSV-GFP or VSV-cDNA libraries from cells which did, or did not, express OVA (MOI 0.1), co-cultured with naive OT-I T cells and assayed for IFN-γ[28]. Lane 1, splenocytes alone; (2–4), splenocytes infected with VSV-GFP, without OT-I (2), with OT-I (3) or with irrelevant T cells[41]; (5&6), splenocytes infected with VSV-cDNA library from B16ova cells in Direct (5) or Reverse (6) orientation with OT-I; (7&8), splenocytes infected with VSV-cDNA library from B16 cells (no OVA) in Direct (7) or Reverse (8) orientations with OT-I. (9), OT-I activated by SIINFEKL peptide. (10–13), splenocytes infected with VSV-ova at MOI 0.01 (10), 0.1 (11), 1.0 (12) and 10 (13). (14), OT-I (no splenocytes, no VSV).
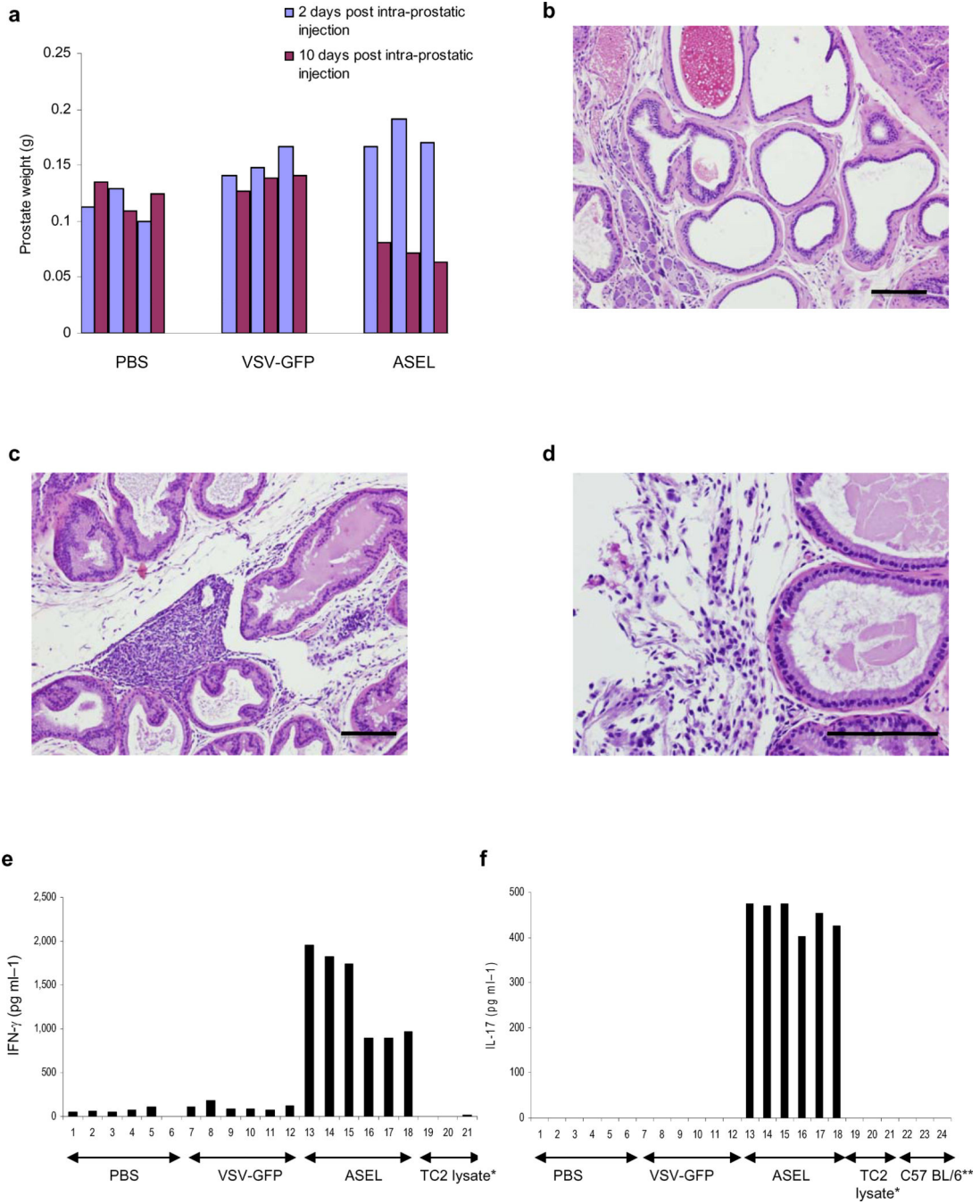
Figure 2. Intra-prostatic injection of ASEL induces autoimmunity
a. Prostate weights of mice injected intra-prostatically with PBS, VSV-GFP or ASEL 2 or 10 days after injection (n=3). **b-d.** Histology of prostates 10 days after intra-prostatic injection of PBS (**b**) or ASEL (**c,d**). Scale bars, 100μm. **e,f.** 10 days following intra-prostatic injection of PBS, or 10^7pfu VSV-GFP or ASEL (6 mice per group) splenocytes from each mouse co-cultured with lysates of TC2 cells were assayed for IFN-γ (**e**) or IL-17 (**f**). Mean values of cytokine from two ELISA wells per sample are shown for each mouse.

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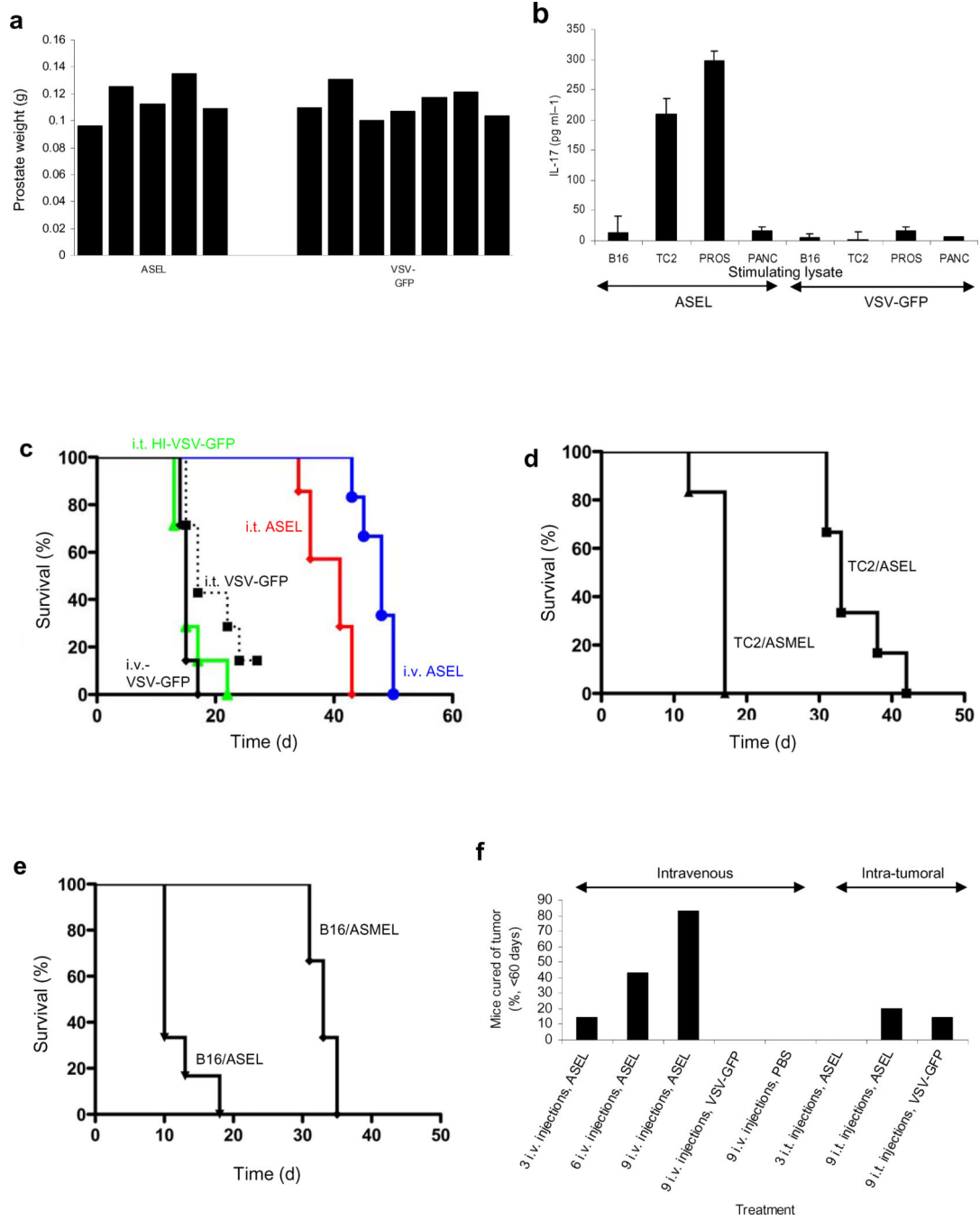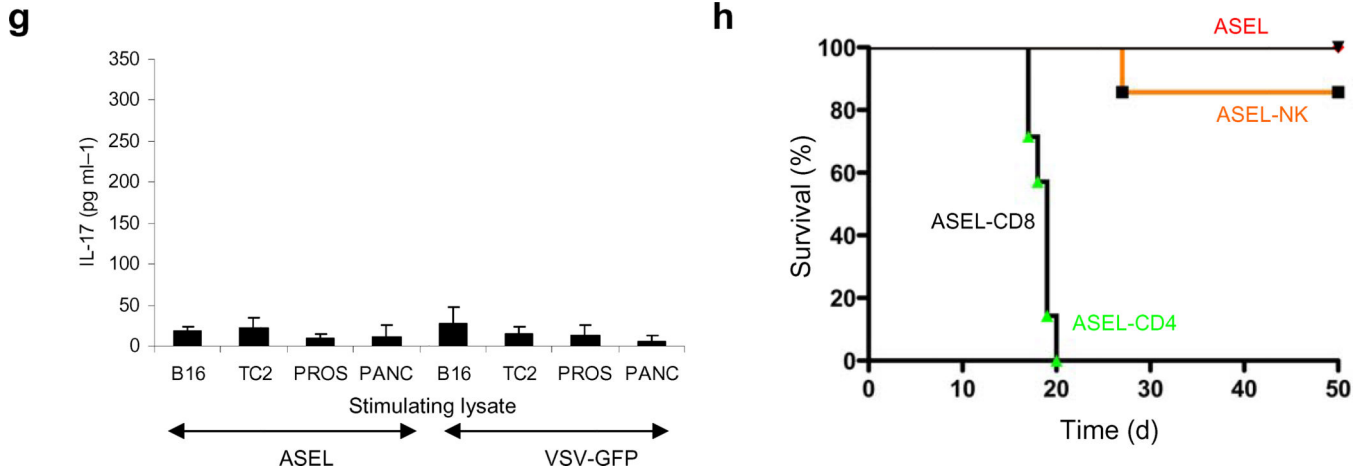
Figure 3. Intravenous injection of ASEL has anti-tumor efficacy

a. Prostate weights 60d following 10^7 pfu i.v. of ASEL (n=5) or VSV-GFP (n=7). **b.** Mean levels of IL-17 secreted by splenocytes from these mice co-cultured with lysates of B16 melanoma, TC2 prostate, normal mouse prostate or pancreas. **c.** Survival of mice bearing 7d TC2 tumors (n=7–8) injected intra-tumorally or intravenously with 10^7pfu of VSV-GFP, ASEL, or heat inactivated VSV-GFP (days 7,9,11). **d,e**. Survival of mice bearing 7d TC2 (**d**) or B16 (**e**) tumors injected intravenously with ASEL, or with a VSV-cDNA library from human melanoma cells (Altered Self Melanoma Epitope Library, ASMEL) (days 7,9,11). **f.** Cumulative percentages of mice cured of 7d TC2 tumors when administered 3, 6 or 9 injections of ASEL or VSV-GFP i.t. or i.v. every other day. **g.** IL-17 secreted by splenocytes from the three mice cured of TC2 tumors by 9 intra-tumoral injections of ASEL in **f.**, as well as from 3 mice treated similarly with VSV-GFP, co-cultured with lysates of B16, TC2, normal mouse prostate or pancreas. **h.** Survival of mice bearing 7d TC2 tumors (n=7/8), mock depleted, or depleted of CD4+, CD8+ or NK cells, injected intravenously with ASEL on days 7,9,11,14,16,18,21,23,25.
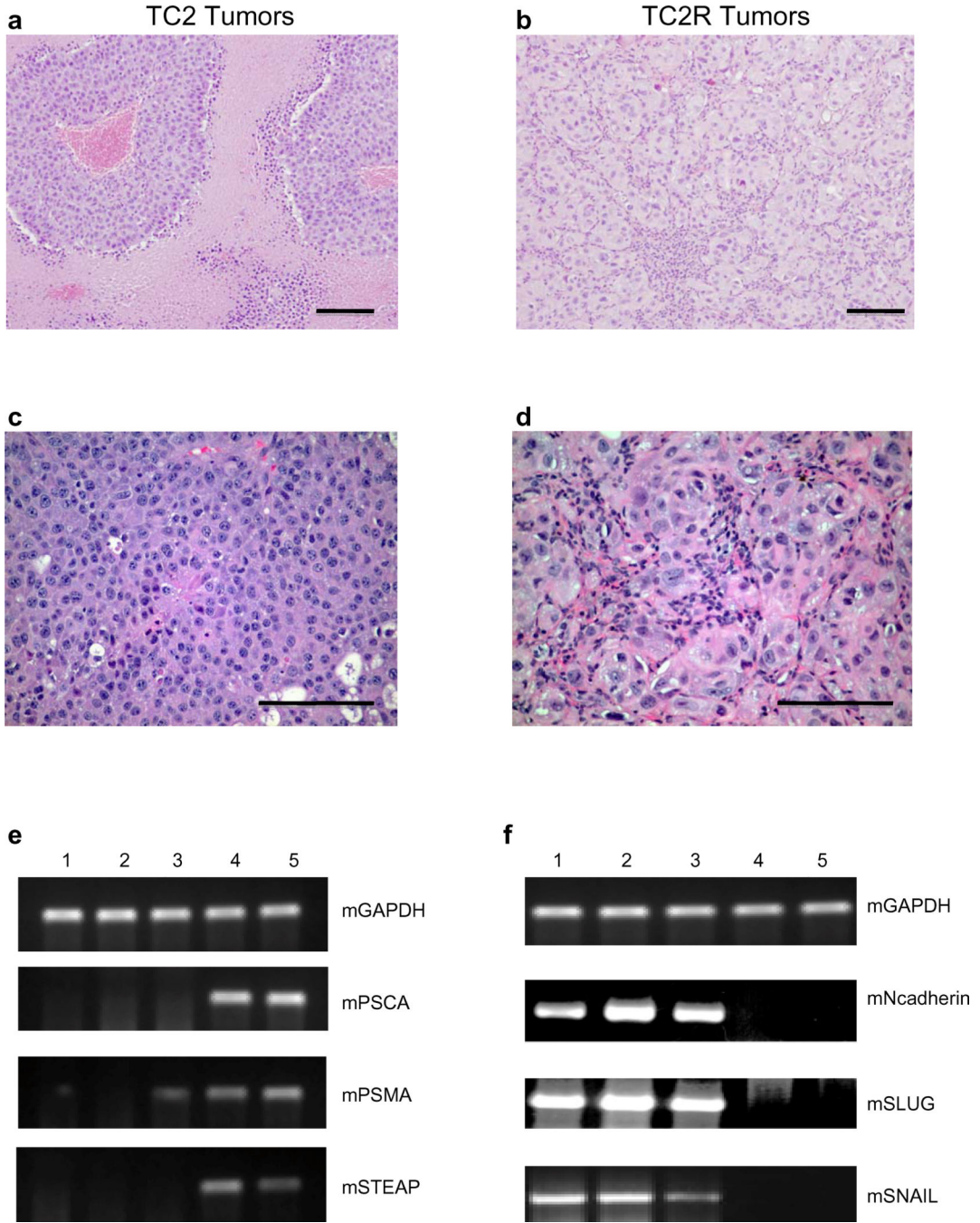
Figure 4. Sub optimal vaccination induces immune escape variants
a-d. H&E staining of tumors (1.0cm diameter) from mice bearing 7d TC2 tumors treated i.v.
with PBS (**a,c**) or ASEL (**b,d**) (days 7,9,11) (typically d20 for PBS (TC2) or d50 for ASEL
(TC2R)). Scale bars, 100μm. **e,f.** Three TC2R tumors from mice treated with ASEL (1–3),
one tumor from a mouse treated with PBS (4), as in **a-d.** above, and *in vitro* cultured TC2
cells (5) analyzed by rtPCR for murine prostate specific genes PSCA, PSMA and STEAP[42]
(**e**) or for N-Cadherin, SLUG or SNAIL[29, 31, 32, 30] (**f**). *Cultured TC2 cells positive for N-
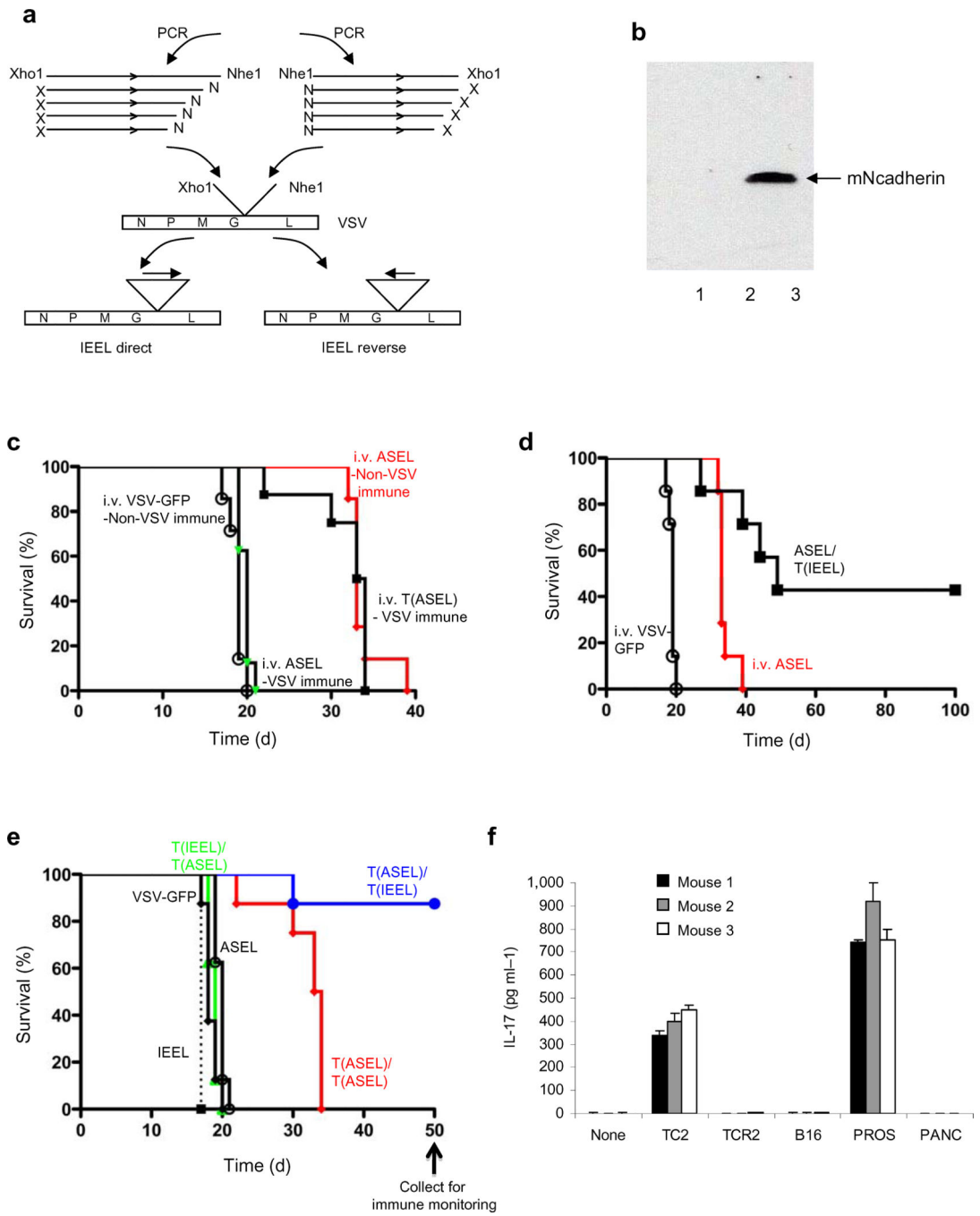
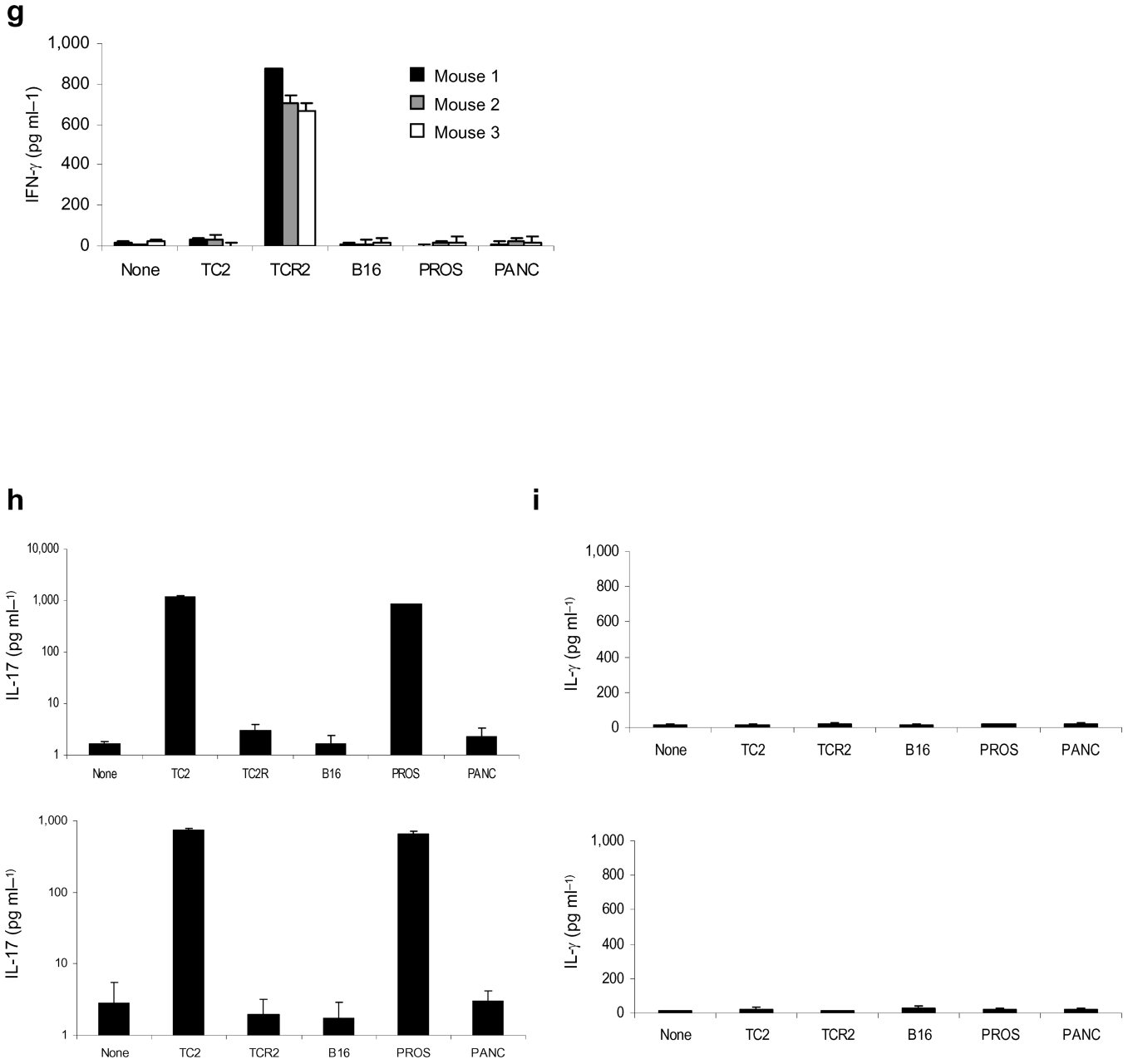Cadherin by nested PCR and weakly positive by Western Blot at lower levels than detected in TCR2 tumors.

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h **i**

Figure 5. TC2R tumors can be treated with a second vaccination

a. The IEEL contained cDNA from three TCR2 tumors cloned into VSV. **b.** Western blot for murine N-Cadherin from BHK cells infected with VSV (1), IEEL Reverse (2) or Direct (3) (MOI ~10). Equal loading confirmed by ß-actin probing. **c.** Survival of mock, or VSV-GFP,-vaccinated mice bearing 7d TC2 tumors treated with three i.v. injections of VSV-GFP or ASEL, either as viral supernatant (10^7pfu) or as virus pre-loaded onto CD8+ T cells [T(ASEL)]. **d.** Mice bearing 7d TC2 tumors were treated i.v. with VSV-GFP or ASEL (days 7,9,11). On days 25,27,29 mice initially treated with ASEL received i.v. IEEL virus pre-loaded onto CD8+ T cells [T(IEEL)]. **e.** VSV-vaccinated mice bearing 7d TC2 tumors were injected intravenously with VSV-GFP, ASEL or IEEL (10^7pfu) or with virus pre-loaded

onto CD8+ T cells [T(ASEL)/T(IEEL)] (d 7,9,11). On days 20,22,24, surviving mice were treated i.v. with T cells loaded with IEEL [T(ASEL)/T(IEEL)] or ASEL [T(ASEL)/ T(ASEL)]. **f-i.** Splenocytes from mice which either did (**f,g**) or did not (**h,i**) reject TC2/ TC2R tumors following i.v. ASEL + T(IEEL) (**d**) or T(ASEL) + T(IEEL) (**e**) were co-cultured with lysates of TC2, TC2R, B16, normal mouse prostate or pancreas and assayed for (**f,g**) IL-17 or (**h,i**) IFN-γ. Results are from three survivor mice (**f,g**) or two mice which succumbed to TC2R tumors (**h,i**).
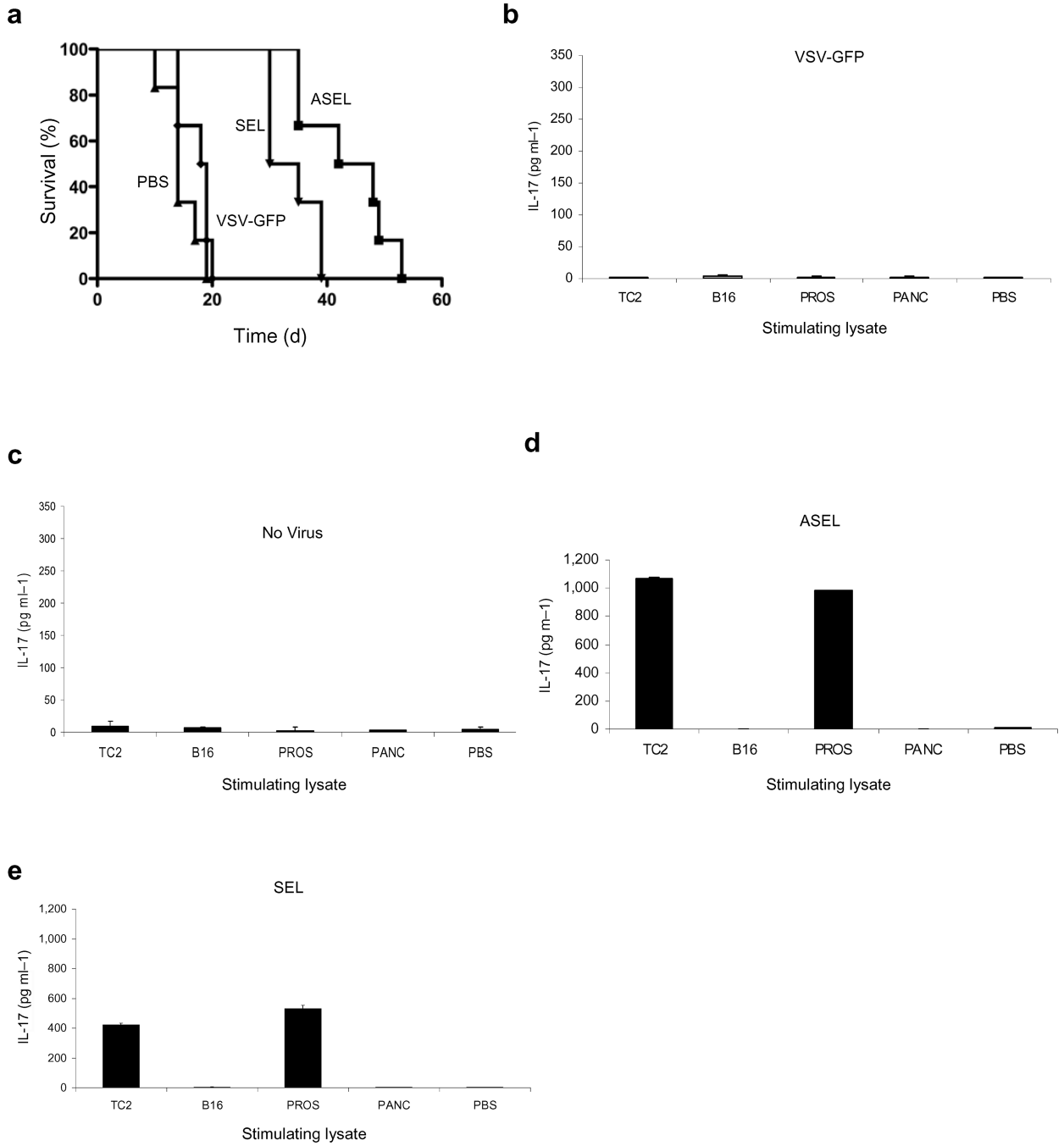
Figure 6. Immunogenicity of Altered Self/Self Libraries
a. Survival of mice bearing 7d TC2 tumors (n=7–8) injected intravenously with 10^7 pfu VSV-GFP, ASEL, or SEL (days 7,9,11). **b-e**. IL-17 secreted from lymph node cells/ splenocytes infected with (**b**) VSV-GFP, (**c**) no virus, (**d**) ASEL or (**e**) SEL (MOI 1) for 2 weeks and co-cultured with lysates of TC2, B16, normal mouse prostate, pancreas or PBS.