Both treated and untreated tumors are eliminated by short hairpin RNA-based induction of target-specific immune responses.

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Abstract

RNA interference for cancer treatment relies on the ability to directly kill cancer cells via down-regulation of target genes but issues of delivery and efficacy have limited clinical adoption. Furthermore, current studies using immune–deficient animal models disregard potential interactions with the adaptive immune system. It has previously been observed that certain viral antigens appear to be more rapidly presented to the immune system than normal proteins due to the production of defective ribosomal products by the virus. Given that RNA interference could potentially result in the generation of truncated mRNAs we wondered if a similar mechanism of immune presentation of a target gene was possible. Here we show that RNAi-cleaved mRNAs can be translated into incomplete protein, and if cleavage was downstream of cytotoxic T cell epitopes, resulted in increased presentation of target protein and the generation of a tumor-protective immune response. We show that mice inoculated with tumor cells treated with such shRNA were protected from subsequent challenge with untreated tumors. However, protection was only found if shRNAs were targeted downstream of the dominant CTL epitope. Our work suggests RNAi can alter immunity to targets and shows that not all tumor cells require direct RNAi exposure for treatment to be effective in vivo and points the way to a new class of RNAi-based therapy.


Introduction

RNA interference (RNAi) has proved to be a powerful tool to specifically silence gene expression and holds great promise for the treatment of genetic disorders, viral diseases, and cancer. RNAi is mediated by small interfering RNAs (siRNAs) that direct the sequence-specific degradation of target mRNA. To date, the use of RNAi for cancer treatment has mostly relied on the concept of targeting overexpressed or mutated oncogenes to directly kill the cells and while in vitro results have been promising, in vivo experiments have been less impressive. This is in part due to a major impediment to the clinical use of RNAi therapy, namely the need to deliver these macromolecules to the desired cells, tissues or organs in order to elicit gene silencing. Furthermore, to work effectively RNAi therapy needs to be delivered to each and every cancer cell in order to elicit direct and specific killing. Several studies have demonstrated some in vivo efficacy in the delivery of siRNAs using various strategies such as complexing siRNAs with cationic lipids, nanoparticles, polyethyleneimine, cyclodextrin, chitosan, and collagen (see (1) for a review).

One way to overcome these barriers is to harness the immune response to augment RNAi treatment. Some studies have concentrated on manipulating the immune system ex vivo by using RNAi to silence immune response regulatory factors (2, 3), while others have investigated the role of specific siRNA sequences that are able to induce innate immune responses and upregulate cytokines such as the interferons (4-9). However, to the best of our knowledge no one has investigated the use of RNAi to trigger specific adaptive immune responses against the RNAi target. This is partially due to the fact that most previous pre-clinical studies on anti-cancer agents, including RNAi, have used human cancer cells in immune-deficient small animal models such as Rag<sup>−/−</sup> or NOD-SCID mice. While this approach allows direct measurement of the RNAi effect, the role of the adaptive immune system cannot be assessed. The adaptive immune system constantly monitors the internal protein content of cells via the MHC/proteosome system allowing the
detection of non-self proteins. It has previously been observed that certain viral antigens appear to be more rapidly presented to the immune system than normal proteins (10-12) and it has been proposed that this is due to the virus production of defective ribosomal products, incomplete proteins that are misfolded or malformed such that they are shunted to the proteosome for destruction. This is known as the defective ribosome products, (DRiP), hypothesis (13) and the immunoribosome model (13, 14). Given that RNA interference could potentially result in the generation of defective mRNAs we wondered if a similar mechanism of immune presentation of a target gene was possible.

To address this issue we investigated RNAi therapy using two different antigen systems, the E7 oncoprotein from the human papillomavirus (HPV), the causative agent of cervical cancer, and the classic antigen, ovalbumin. In this study we show that shRNA treatment was able to generate a target-specific immune response capable of protecting mice from challenge with untreated tumour cells and investigate the mechanism by which this occurs.

Results

ShRNA treatment results in truncated mRNA. We hypothesised that RNAi is able to act in the same manner as that proposed for viruses and produce defective ribosome products or DRiPs (13, 14). We firstly established if truncated mRNA products were generated in cells treated with shRNAs. As a model system of cancer we targeted the E6/E7 oncoproteins from the type 16 human papillomavirus and used TC-1 cells, created by transforming murine lung fibroblasts with E6, E7 and ras (15). E6 and E7 are expressed from the same mRNA in a bicistronic fashion. Several shRNAs targeting the E6/E7 mRNA were cloned in the vector pLL, which expressed shRNAs from the U6 promoter as well as GFP from the CMV promoter (Figure 1A). TC-1 cells were transduced with a low dose of shRNA-bearing lentivirus and FACS-sorted based on GFP expression to achieve 100% transfection of the target cells. We have previously shown that high doses of E6E7 shRNA (12 copies/genome) results in cell death while lower doses (4-5 copies/genome) results in slower
cell growth and come cells entering senescence (16). After four days transfected cells were tested for the presence of E6/E7 mRNA using RT-PCR and a series of primers designed to detect full-length or various truncated versions of mRNA. Treatment with both E6-1 and E7-1 shRNA resulted in the loss of full-length mRNA (Figure 1B, R1 fragment) and E7 protein (Fig 1C). However, a PCR fragment corresponding to the 5’ end of the mRNA was still found even in the presence of shRNA (Figure 1B, R3). Furthermore, the presence of an intermediate size fragment (R2), encoding most of E6, while not observed in cells expressing the E6-1 shRNA, was still present in E7-1 shRNA-treated cells. Quantitative PCR revealed that E6-1 shRNA-treated cells had 23% the level of R2 compared to E7-1. Thus, in conditions where shRNA treatment resulted in silencing of full-length message, mRNA upstream of the shRNA target site was still present.

**Truncated mRNA can be translated.** We next assessed if these truncated mRNAs could be translated into partial length protein. To investigate this, and further generalise our findings, we utilised another target, ovalbumin (OVA). As before shRNAs were generated to target sites along the length of the OVA gene and OVA-expressing thymoma cells (EG7) were transduced with lentiviruses expressing shRNA-OVA1, -OVA2 or –OVA3 (Figure 2A). All three OVA shRNAs were able to reduce the level of full-length OVA protein by 40-60% (Figure 2B). To detect the presence of truncated OVA protein shRNA-treated cells were treated with the protease inhibitor MG132, to limit protein degradation, and western blots were performed using antibodies reactive to the extreme N-terminus of ovalbumin. We observed the appearance of a 15kDa protein in cells treated with the OVA2 shRNA, but not in untreated, OVA1- or OVA3-treated cells (Figure 2C). The predicted size of a protein generated by mRNA cleaved by OVA2 shRNA is 14.7kDa. The predicted truncated product generated by OVA3 shRNA treatment was not observed due to cross reacting proteins on the blot. Thus it appears that truncated proteins can be generated in vivo by the translation of mRNAs cleaved by shRNA.

**ShRNA treatment alters CTL epitope presentation of target proteins.** CTL epitopes originate from the intracellular digestion of proteins by the proteosome and are critical in the recognition of
foreign antigens by both professional antigen-presenting cells and T-cells bearing cognate T-cell receptor, and thus the activation of a CTL response. To test if the use of shRNA was able to alter the presentation of cytotoxic T cell (CTL) epitopes on the surface of cells we treated EG7 cells with OVA-1, -2 or -3 shRNA and examined the level of the dominant OVA CTL epitope, SIINFEKL, loaded into the cell surface MHC. SIINFEKL is located between the OVA 2 and OVA3 shRNA target sites (Figure 2A, *). The presence of the SIINFEKL-Kb MHC class 1 complex was measured by FACS using the antibody 25-D1.16, as previously described (12). Untreated EG7 cells had a mean florescent intensity (MFI) of 27 while treatment with OVA-1 shRNA, whose target is upstream of the CTL epitope, resulted in reduced levels of this complex (MFI=21), corresponding to its modest silencing ability (Figure 2D). OVA-2 shRNA gave a similar result to OVA1 (data not shown). However, the level of MHC-SIINFEKL complex on the surface of OVA3-treated cells was increased (MFI=34) despite the reduction in full-length protein as a result of shRNA expression (Figures 1C and 2D). These data are consistent with the our hypothesis that truncated OVA mRNA results in enhanced antigen processing of a partial OVA protein leading to greater numbers of EG7 cells expressing SIINFEKL on the cell surface.

**ShRNA treatment alters in vivo immune responses to give tumour protection.** While our data suggested downstream shRNAs resulted in increased numbers of cells expressing detectable CTL epitope/MHC complex, it was not clear if this would alter the immune response to the target antigen in any way. We therefore tested the hypothesis that immunization of mice with antigen-bearing tumour cells treated with an shRNA downstream of the CTL epitope would protect those mice from subsequent challenge with a second, untreated tumour. We inoculated C57BL/6 (H-2b) mice with TC-1 cells transduced by lentiviruses expressing the shRNAs E6-1, E7-1, E7-2 or empty vector. We took advantage of a unique H-2b-restricted tumour protective CTL epitope, RAHYNIVTF, in E7 protein (17, 18) located between the E6-1 and E7-1 shRNA sites (Figure 1A, *). After 10 days mice were challenged in the opposite flank with untreated TC-1 cells and tumours allowed to grow until day 20. It can be seen that in all treatments except E7-1, the weights of the primary and
challenge tumours were no different to untreated controls (Figure 3A). However, in mice treated with tumours bearing the E7-1 shRNA, both the primary and challenge tumours were absent in all mice expect one (Figure 3A). Such a result cannot be explained as a direct shRNA effect as challenge tumours were not treated with shRNA. Furthermore, both E6-1 and E7-1 are equally effective at knocking down E7 protein levels (Figure 1C) and have similar potency at directly reducing TC-1 cell growth and colony forming ability (Supplementary Figure 1).

In order to prove an adaptive immune response was required for this effect we repeated the same experiment above using RAG-/- mice, which lack B-, T-, and NKT-cells and thus are unable to mount an adaptive immune response. We now observed no significant differences in either primary or challenge tumours (Figure 3B), indicating an adaptive immune response was required. A reduction in E7 levels may result in senescence of tumour cells and senescence has been shown to result in clearance of tumours in a p53 inducible liver carcinoma system(19). This clearance required macrophages, neutrophils and NK cells, all of which are still present in Rag-/- mice suggesting the innate immune system is not involved and excluding senescence-induced immune clearance as a potential mechanism. Preliminary data indicated that depletion of CD8 T cells removed protection against tumour in 16E7-1 inoculated mice. Previous studies have shown that tumour cells undergoing apoptosis can prime CD8+ T cells and induce an immune response via “cross-presentation” to professional antigen-presenting dendritic cells (20), or via dendritic cell aggresome-like induced structures (21). To address this possibility we immunized with E7-expressing EL4 cells treated with shRNA. Unlike TC-1 cells, where complete loss of E7 can result in apoptosis, EL4 cells are already transformed and loss of E7 does not cause cell death. However, challenge tumours where still cleared in mice immunized with the E7-1 shRNA, but not the E6-1 shRNA and thus apoptosis of the primary tumour was not required for tumour clearance (Supplementary Figure 2). Furthermore, we did not observe non-specific toxicity in HPV18+ve HeLa cells treated with our HPV16-specific shRNAs (Supplementary Figure 3B). Finally, to prove the response was target antigen-specific we repeated the experiment once more, immunising
C57BL/6 mice with TC-1 cells treated with shRNA, but challenging with another E7-expressing H-2b cell line, C2. Once again, only in mice immunised with E7-1 was there a loss of tumour formation, thus indicating tumour clearance was specific to our shRNA target, E7 (Figure 3C).

It could be argued that we were observing an E7-specific effect in which silencing the E7 gene prevented some key immunomodulatory process. Therefore, to prove the generality of our observation we tested if RNAi-enhanced immunity would work using OVA as the target antigen. The experiments were repeated as above, but immunizing mice firstly with OVA-1, -2 or -3 shRNA-transduced EG7 cells and subsequently challenging with untreated EG7 cells. We observed that only mice inoculated with OVA3-transduced cells, that is the shRNA downstream of the major CTL epitope, SIINFEKL, had significantly reduced tumour formation following challenge (Figure 3D). These data further suggest clearance is dependent on the shRNA target and is not specific to E7 as a target antigen. Also, OVA3-shRNA treatment had no affect on the cell viability, thus eliminating non-specific toxicity as the reason for tumour clearance (Supplementary Figure 3A).

**ShRNA-treated tumours induce target specific CTLs in mice.** Finally in order to address the mechanism by which RNAi-mediated immune enhancement was occurring we directly measured the activity of E7-specific CTLs in mice immunised with our shRNAs. Twenty days after inoculation with shRNA-treated TC-1 tumour, *ex vivo* splenocytes were tested in an IFNγ ELISPOT assay in the presence of the E7-CTL epitope peptide. Significantly more IFNγ-secreting cells were recorded in the spleens of mice inoculated with E7-1 treated tumour than E6-1 treated tumour (Figure 4A), despite the fact that both reduced total protein levels by the same amount (Figure 1C). Moreover, these cells were capable of specifically killing syngeneic target cells pulsed with E7-CTL epitope peptide in a MHC (H-2b)-restricted fashion, after restimulation (Figure 4B). Additionally, restimulated splenocytes from mice inoculated with E7-1-treated tumour were significantly more effective than splenocytes from E6-1-inoculated mice in killing C2 target cells which endogenously express whole E7 oncoprotein and present the E7 CTL epitope (22) (Figure 4C).
Discussion.

We show here that shRNA targeting of E7 results in the presence of intermediate 5’mRNA products in the absence of full length mRNA. Furthermore, in the case of the OVA protein, shRNA targeting can result in the translation of truncated OVA protein. ShRNAs are processed into siRNAs for incorporation into the RNA-induced silencing complex (RISC) but following RISC-mediated cleavage the fate of the target mRNA is not completely understood. It has been shown that rapid and direct endonucleolytic cleavage occurs at the exposed and unprotected ends of the mRNA (23), leading to 5’ or 3’ exonuclease attack and rapid decay of target mRNA (24). However, RISC-mediated cleavage has previously been observed to produce 5’ decay intermediates both \textit{in vitro} and \textit{in vivo} (25-28). These 5’ intermediate products contain a cap structure but lack a poly(A) tail and stop codon and would be candidates for either non-stop decay or nonsense-mediated decay. The efficient turnover of these RISC-cleaved 5’ mRNA intermediates has been shown to require their translation (24). It is known that mRNAs that lack a poly(A) tail can be translated, albeit at 10-fold lower efficiency than poly(A)-containing mRNA (29). We propose that the production of such truncated proteins would cause preferential proteosomal processing by yet to be defined mechanism. It is clear that despite the reduction or loss of full length mRNA and protein, shRNAs targeted downstream of CTL epitopes resulted in increased presentation of these motifs on the cell surface in the context of MHC class 1. This may work in a similar manner to enhanced presentation of truncated proteins shown to result from microsatellite instability in certain tumours (30) which results in truncated proteins that are presented to the immune system or more often frameshifts that result in novel CTL epitopes (31).

Although each shRNAs silenced expression equally well they gave remarkably different outcomes \textit{in vivo}. The shRNA targeted downstream of the E7 CTL epitope shRNA had the ability to induce E7-specific immune responses against the tumor such that both primary, shRNA-treated, and secondary, untreated tumors were eliminated. However the upstream shRNA had no effect \textit{in
vivo on either primary or secondary tumors. There are a number of possible explanations for this effect that may be related to loss of E6 and E7 itself. For example, Xue et al recently showed that restoration of p53 in liver carcinoma cells resulted in cellular senescence followed by tumor clearance that was mediated by the innate immune system (19). This clearance required macrophages, neutrophils or NK cells. We have previously shown that the loss of E6, via shRNA treatment, causes increased p53 levels and induction of cellular senescence, depending on the dose of LV used (16). However, we did not observe immune clearance in Rag-/- mice, which lack B-, T-, and NKT cells but have macrophages, neutrophils and NK cells. Furthermore, depletion of CD8+ T cells reduces tumor clearance. Therefore we conclude that an adaptive immune response is required and senescent-induced innate immune clearance is not occurring here. Another possible explanation is that immune recognition was occurring via apoptosis of tumor cells, resulting in the priming of CD8+ T cells via professional antigen presenting dendritic cells (20) or through dendritic cell aggresome-like induced structures (21). However, using the thymoma cell line EL4, transduced to express full length E7, as the primary tumor inoculation and where apoptosis due to loss of E6/E7 does not occur, still resulted in elimination of untreated, E7-expressing secondary tumor cells. Importantly, using OVA as the antigen still resulted in clearance of OVA-expressing challenge tumors as long as immunization was with EG7 cells treated with shRNA targeted downstream of the CTL epitope. The loss of OVA in EG7 cells has no effect on cell death (Supplementary Figure 3).

Further analysis of the nature of the immune response shows that E7-specific CTLs are generated in mice immunised with E7-1 shRNA whereas using of E6-1 gave a reduced CTL response corresponding with the loss of protein production. From our data it is clear that E7-1-treated tumours elicit an immune response equally potent to untreated cells despite the loss of full length E7 protein. However, this raises the question of why mice inoculated with untreated TC-1 cells are unable to control either primary tumour or a subsequent untreated tumour challenge (Figure 3A and C) despite the presence of a quantitatively robust CTL response. We speculate this relates to sub-
optimal priming of T cells by full-length E7. The rapid generation of MHC class I peptides from
defective DRiPs (in this case truncated E7-1) in contrast to that of whole protein(12) likely allows
the control of nascent E7-expressing tumour immediately following primary inoculation.
Furthermore, the specialised antigen processing for DRiPs (12) may allow for a higher affinity CTL
population sufficient to control subsequent tumour challenge. This awaits further experimental
confirmation.

Overall, our data have revealed a novel means by which shRNA-driven RNAi is able to induce
an immune response that protects against tumours, including those that have never been treated with
RNAi. We show that RNAi leads to the production of truncated target gene mRNA transcripts that
are translated into incomplete proteins that are subsequently processed via the proteosome and
presented to the immune system in the context of MHC class 1. Tumour clearance occurs only
when RNAi is targeted downstream of the message sequence encoding the CTL epitope. Taken
together, our results point the way to a new form of antitumour therapy based on RNAi, a therapy
that need not be delivered to every cancer cell, thus overcoming a major obstacle to the clinical
translation of RNAi.

Materials and Methods.

shRNA design, cloning, and lentiviral packing plasmids. Four shRNAs were selected for HPV
E6/E7 mRNA; 2 targeting at E6 and other 2 targeting at E7 (Figure 1). Three shRNAs were selected
for ovalbumin (OVA). The shRNA expression cassettes were (from the 5’ end) HPV 16E6-1:
TGACCGGTCGATGTATGTCTTCAAGAGAGACATACATCGACGGTCTTTTTTC; HPV
16E6-2: TCGACGTGAGGTATAGACTTCAAGAGAGTCATATACCTCACGTCGTTTTTTC;
HPV16E7-1:
TGTGTGACTCTACGCTTCGGTTCAAGAGACCGAAGCGTAGAGTCACATTTTTTC;
HPV 16E7-2:
TCATGGAGA TACACCTACATTCAAGAGATGTAGGTGTATCTCCATGTGTTTTTTC;
OVA1,
TACCAAATGATTTATTCGGTTTACAGGAGAAATACATTCATTTGGTATTTTTTC;
OVA2,
TGGAACGTATAGAGGGCTTTTCAAGAGAAGCCTCCTCTATACAGTTCCATTTTTTC;
OVA3,
ATACAACCTCATCTGTCTTTTCAAGAGAGACAGATGTGAGGTTGTATTTTTTC).
The shRNA cassettes and their complementary strands were synthesised (PROLIGO, Lismore, Australia) and annealed and the resulting double-strand oligo DNAs were cloned into plasmid pLentiLox3.7 (pLL3.7) at the HapI and XhoI sites. The lentiviral packaging plasmids pRSVRev, pMDLgpRRE, and pMD.G (contains VSV.G gene) were third generation lentiviral packing plasmids.

**Cells, lentiviral-shRNA production, cell transduction, and flow cytometry sorting.** Lentiviral production and titration were as previously described (16). TC1 and 293T cells were maintained in DMEM supplemented with 10% heat-inactivated foetal calf serum, 100 units/ml penicillin G, 100µg/ml streptomycin sulphate and 0.29mg/ml of L-Glutamine (Gibco-Invitrogen). TC-1(H-2^b), C2 and EG7 cells have been previously described (15, 22, 32). EL4.A2 and P815 cells are susceptible to specific CTL lysis restricted through H-2^b and H-2^d respectively. C2, EG7, and EL4 cells were maintained in Click’s medium (equal volume of Eagle’s Ham Amino Acids (Sigma) and RPMI Medium 1640 (Gibco); 10% heat-inactivated foetal calf serum; 100 U/ml penicillin, 100µg/ml streptomycin, 0.292 mg/ml L-glutamine; and 0.0035% 2-mercaptoethanol (Sigma)). For transduction, the cells were plated in 6-well plates (1x10^5 cells /well) or in T75 flasks (3 x 10^5 /flask) and were cultured overnight. LV-shRNAs diluted (in cultural medium containing polybrene 8µg/ml) or not diluted were added to the cells containing 0.5ml (6-well plate) or 5ml (flask) polybrene-medium for incubation for 1 hour at 37°C. After this, 1ml or 5ml of fresh polybrene-medium was added to the cells and incubation continued for 48 hours. Polybrene-medium was then replaced with fresh culture medium and the cells were further cultured for assays. TC1 cells were
harvested by trypsin and washed 2 times with PBS before being resuspended in 0.5ml 1% paraformaldehyde/PBS for flow cytometry analysis using FACS Calibur. Transduced C2 or EG7 cells were harvested by centrifugation and washed in PBS followed by cell sorting using flow cytometry MoFlo.

**RT-PCR for mRNA and short-form mRNA detection.** Transduced and sorted TC1 cells were cultured for 2 to 4 days and harvested for total RNA extraction using TRizol® reagent (Invitrogen) following the manufacturers’ instructions. However, for truncated mRNA detection, the isopropanol precipitation procedure was prolonged for two hours in -20°C freezer. Reverse transcription reactions were carried out using the kit protocol with oligo-dT primer (Qiagen) or random hexamers. Primers for RT-PCR were as follows: HPV 16E/E7: Forward (F) CTGCAATGTTCAGGACC; Reverse 1 (R1) CAATTCCTAGT GTGCCCAT; Reverse 2 (R2) GAGATCAGTTGTCTCTGGTTG; Reverse3 (R3) GGACACAGTTGGCTTTTGAC. The PCR was performed in 20µl volume with 2.5µl of reverse transcription product; other components were same as standard PCR. PCR program was pre-heating 95°C for 5 min; the cycle of 94°C for 45sec; 56°C for 1min; and 72 °C for 2 min. Twenty-seven cycles were used for detecting shRNA degraded mRNA and 40 cycles for truncated mRNA detection.

**FACS analysis for MHC- SIINFEKL expression.** Transduced and non-transduced EG7 cells were treated with IFN-γ (Pharningen, San Diego, California, USA) at 100U/ml for 48 hrs. The cells (1X10^5 cells per tube) were then transferred into centrifuge tubes and were incubated with first antibodies: mouse anti-SIINF/H2K^b, 50μl/tube, provided by Dr. Tony Purcell (University of Melbourne, Melbourne, Australia), and mouse anti-H-2K^b antibody (1:50 in 5%FCS in PBS, 50μl/tube, Pharningen) for 1hr at 4 °C. The isotype control was incubated with mouse anti-rat IgM antibody (1:50 in 5% FCS in PBS, 50μl/tube, Pharningen), for 1 hour at 4 °C. The cells were then washed twice at 1300 rpm X 5min and incubated with the secondary antibody (APC-goat anti-mouse Ig antibody, (1:25 in 5% FCS, 50μl/sample, Sigma) for 1 hour at 4 °C. Cells were washed twice again at 1300 rpm X 5min before fixation with 5% formaldehyde in PBS and analysis on a
flow cytometer FACS Calibur. Flow cytometry data were analysed with WinMDI 2.8 version (Joseph Trotter, Scripps Research Institute, USA).

**Western blotting** Western blotting was conducted as previously described(16). Briefly, the cell lysates were prepared by trypsinizing the cells and resuspending in RIPA buffer plus protein inhibitory cocktails (0.1%). For electrophoresis, 30-40μg of total protein in 2x loading buffer was loaded to each well of a 10% (w/v) SDS-PAGE gel. After electro-transferring, the blot was blocked and probed with primary antibody at 4°C followed by incubation with HRP-conjugated secondary antibody (1:2000 dilution) and then for ECL. For truncated protein analysis, cells were treated with 10μM MG132 proteosome inhibitor (Sigma) for 3-4 hours prior to resuspending in RIPA buffer plus protein inhibitory cocktails. Anti-HPV-16E7 was from Santa Cruz Biotechnology, anti-PARP antibody from Cell Signalling Technology and anti-OVA polyclonal antibody from Chemicon. Anti-mouse β-tubulin antibody and mouse monoclonal antibody to OVA were from Sigma. HPV 16E7 antibody was used at 1/200 dilution, OVA polyclonal antibody at 1/5000 dilution, OVA monoclonal at 1/4000, and other primary antibodies were used at 1/1000 dilutions.

**Animals and animal experiments** Specific pathogen-free female C57BL/6, Rag<sup>−/−</sup> and RAH TcR mice of 5-6 weeks old were used in this study. RAH TcR mice(33) expressing a transgenic TCR β-chain derived from an H-2<sup>b</sup>–restricted E7 peptide–specific CTL clone and recognize the HPV16 E7 CTL epitope RAHYNIVTF in the context of H-2D<sup>b</sup>. Transduced TC1 cells were cultured and harvested by trypsinization and were counted and resuspended in PBS at 1x 10<sup>7</sup>/ml. For the transplant model, the mice were subcutaneously injected (5 mice/group) with 0.1 ml cell suspension to the neck scruff and the tumor growth was monitored until day 21. The mice were then sacrificed and tumors were collected and weighed. In the challenge model transduced TC1 or C2 cells or EG7 and controls were prepared as above and subcutaneously injected to one side of the flank, ten days later the non-transduced TC1 or C2 or EG7 cells were prepared in the same way and similarly injected to the other side of the flank. Ten days after second injection, mice were sacrificed and tumors were harvested and weighed. For peptide immunisation mice were immunised s.c. at the tail
base with 50ug peptide + 0.25ug tetanus toxoid (TT) as a source of T-helper epitopes + 10ug Quil A adjuvant(34). The University of Queensland animal ethics committee approved all animal experiments.

**Murine IFN-γ ELISPOT assay.** Epitope-specific gamma interferon (IFN-γ) secreting spleen cells were enumerated *ex vivo* by an enzyme-linked immunospot (ELISPOT) assay with or without E7 CD8\(^+\) T-cell epitope peptide, essentially as described (35). IFN-γ spots were counted using an AID ELISPOT reader system. Results were calculated as IFN-γ positive cells/10\(^6\) spleen cells.

**\(^{51}\)Cr-release CTL assay.** CTL assays were conducted as previously described (35). In summary, target cells (10\(^4\) per well) sensitised at 37\(^0\)C for 1 hour with 1μg/ml cognate or irrelevant peptide, or medium alone, and labelled with 100μCi \(^{51}\)Chromium (Cr), were incubated with effector cells at various effector: target cell ratios in triplicate in 96-well microtiter plates. Negative controls included wells containing target cells but no effector cells (= ‘background’). Supernatants were harvested from CTL assays at 4 hours, and \(^{51}\)Cr release quantified by gamma counting. Results are expressed as percent cytotoxicity +/- standard deviation (\(^{51}\)Cr release in experimental wells minus background/detergent-mediated total release minus background) x 100%. For restimulation, *ex vivo* splenocytes were restimulated *in vitro* for 6 days as described (35).

**Data analysis** Data were collected and were appropriate statistical comparison was made using the student’s unpaired t-test.

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References


24. Orban, T. I. & Izaurralde, E. (2005) Decay of mRNAs targeted by RISC requires XRN1, the
     Ski complex, and the exosome Rna 11, 459-469.
25. Elbashir, S. M., Lendeckel, W., & Tuschi, T. (2001) RNA interference is mediated by 21-
27. Martinez, J. & Tuschi, T. (2004) RISC is a 5' phosphomonoester-producing RNA
     endonuclease Genes Dev 18, 975-980.
     a Mg2+-dependent endonuclease Curr Biol 14, 787-791.
     accentuate expression of the yeast transcriptome Rna 13, 982-997.
30. Ripberger, E., Linnebacher, M., Schwitalle, Y., Gebert, J., & von Knebel Doeberitz, M.
     (2003) Identification of an HLA-A0201-restricted CTL epitope generated by a tumor-
     specific frameshift mutation in a coding microsatellite of the OGT gene J Clin Immunol
     23, 415-423.
31. Schwitalle, Y., Linnebacher, M., Ripberger, E., Gebert, J., & von Knebel Doeberitz, M.
     (2004) Immunogenic peptides generated by frameshift mutations in DNA mismatch repair-
     deficient cancer cells Cancer Immun 4, 14.
     the class I pathway of antigen processing and presentation Cell 54, 777-785.
     effectiveness of combined active/passive immunotherapy for epithelial tumors J Natl
     Cancer Inst 96, 1611-1619.

Figure Legends

Figure 1. Treatment of cells with shRNA results in the generation of truncated mRNA and protein from target gene. **A.** A diagrammatic representation of the target mRNA encoding full-length HPV16 E6 and E7. The target sites of shRNA 16E6-1 (417-434bp), 16E6-2 (136-153), 16E7-1 (660-677), and 16E7-2 (483-500bp) are indicated, as is the site of the cytotoxic T cell epitope (RAHNIVTF, 626-652bp - *). The primers used to detect the truncated mRNAs are shown (F, 17-35bp; R1, 729-747bp; R2, 525-545bp; and R3, 338-356bp). **B.** Total RNA was extracted from lentivirus-transduced TC-1 cells 4 days after infection and RT-PCR carried out using the primers in (a). **C.** Western blotting results for HPV 16 E7 protein expression in TC-1 cells following lentiviral transduction. TC1 are untreated cells while PLL are cells transduced with empty lentiviral vector. 18E6-1 is a non-specific control shRNA targeting HPV type 18 E7 gene expression. Mouse ®-tubulin was assayed as the loading control (Tub).

Figure 2. Cells treated with shRNA targeted downstream of the OVA CTL epitope, SIINFEKL, have reduced full-length OVA expression, translate truncated protein, and show increased levels of the SIINFEKL-Kb MHC class 1 complex. **A.** Three distinct shRNAs against different regions of the OVA gene were designed and cloned into lentiviral expression vectors as described in Materials and Methods section. The site of the cytotoxic T cell epitope, SIINFEKL, is indicated (*) **B.** Western blot showing that shRNAs result in a reduction in full length OVA. **C.** Western blot using an N-terminal anti-OVA antibody showing that the OVA2 shRNA resulted in the appearance of a 15kDa band in EG7 cells. Cells were treated with the proteosome inhibitor MG132. **D.** Cells were treated with lentiviruses expressing the OVA-specific shRNAs indicated in 2A followed by 100U/ml IFN-γ for 48 hrs before complexes were detected using control anti-mouse H-2Kb (EG7 (-)) or the anti-SIINFEKL/H2Kb (all others) antibody, 25-D1.16, as previously described (12).
Figure 3. ShRNAs targeted downstream of CTL epitopes result in clearance of both shRNA-treated and untreated tumours that bear the target antigen. A. TC-1 cells (1 x 10^6), untreated or transduced with lentivirus (PLL, 16E6-1, 16E7-1 or 16E7-2) were subcutaneously (s.c.) injected into the left flank of C57BL/6 mice. Ten days later, untreated TC-1 cells (1 x 10^6) were injected s.c. in the opposite flank. Tumors were harvested and weighed ten days after the second injection. Primary and challenge tumor weights are shown. B. The experiment in A was repeated but using immune deficient Rag^-/- mice. C. The experiment in A was repeated but the challenge tumor was C2 cells (EL4 thymoma cells expressing HPV 16 E7). D. EG7 cells (1 x 10^6), untreated or transduced with lentivirus (PLL, OVA1, OVA2 or OVA3) were subcutaneously injected into the left flank of C57BL/6 mice. Ten days later, untreated EG7 cells (1 x 10^6) were injected s.c. in the opposite flank. Tumors were harvested and weighed ten days after the second injection. Primary and challenge tumor weights are shown.

Figure 4. Mice exposed to tumour expressing a truncated E6/E7 transcript which encodes the E7 CTL epitope, display enhanced E7-directed CTL responses compared with mice exposed to tumour expressing a truncated E6/E7 transcript which does not encode the E7 CTL epitope. A. Ex vivo splenocytes from RAH TcR transgenic mice inoculated with 1x 10^6 TC1 tumour cells treated with E7-1 or, E6-1 shRNA, or with vector alone, were tested 20 days later in γIFN ELISPOT assay, in the presence or absence of cognate E7 peptide. γIFN-secreting cells were evaluated 18h later. Splenocytes from control mice immunised with E7 peptide contained 263 +/- 42 γIFN-secreting cells per 10^6 splenocytes (not shown). B. Splenocytes from RAH TcR transgenic mice inoculated with E7-1 treated TC-I tumour cells as above were restimulated with 1μg/ml E7 peptide for 6 days, then incubated with ^51^Cr-labelled H-2^b^ or H-2^d^ target cells pulsed with E7 peptide or not, at the indicated splenocyte:target cells ratio. Specific lysis of target cells was determined at 4h. C. splenocytes from C57/BL6 mice inoculated with 1x10^6 TC-1 tumour cells
treated with E7-1 or, E6.1 shRNA, or with vector alone, were restimulated with E7 peptide as above then incubated with $^{51}$Cr-labelled C2 tumour cells at an effector:target cell ratio of 50:1. Specific lysis of C2 cells was determined at 4 h. Control groups of mice were immunised with E7 peptide or with PBS.
Figure 2

A

B

C

D

Cell Counts

SIINFE/Kb complexes
Figure 3

A. Primary Tumour  Challenge Tumour

B.

C.

D.
Figure 4

A. Acute Pneumonia Elipsoid

B. G440H(0.0)

C. Cytokine assay in EL4/ET cells