HBsAg-vectored DNA vaccines elicit concomitant protective responses to multiple CTL epitopes relevant in human disease.

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Summary.

Vaccines capable of controlling neoplastic and infectious diseases which depend on the cellular immune response for their resolution, have proven difficult to develop. We, and others, have previously demonstrated that the potent immunogenicity of hepatitis B surface antigen (HBsAg), the already- licensed human vaccine for hepatitis B infection, may be exploited to deliver foreign antigens for cytotoxic T-lymphocyte (CTL) induction. In this study we demonstrate that recombinant (r) HBsAg DNA delivering a CTL polyepitope appended at the C' terminus elicits concomitant responses to multiple epitopes restricted through a diversity of MHC class I haplotypes, which are relevant in a number of human diseases. We show that the rHBsAg DNA vaccine elicits concomitant protection against neoplastic and infectious disease. These studies vindicate the use of HBsAg as a powerful vector to deliver CTL responses to foreign antigens, and have implications for a multi-disease vaccine applicable to the HLA-polymorphic human population.

Keywords; DNA vaccine, cytotoxic T-lymphocyte, hepatitis B surface antigen, cancer, infectious disease, epitope

Introduction

Vaccine strategies other than attenuated or killed whole organism vaccines, capable of safely and effectively inducing cellular and humoral responses in humans have not proven easy to develop [1]. The small envelope protein of hepatitis B surface antigen (HBsAg-S) self-assembles into highly organised virus-like particles (VLPs) in yeast, insect cells and mammalian cells [2]. HBsAg VLPs are exploited as the current globally licensed vaccine for Hepatitis B virus infection in humans (including children). The vaccine has a long history in millions of recipients, inviting the usage of HBsAg as a vector for delivery of immunogens from other infectious diseases and tumors. Regulatory issues surrounding HBsAg vaccines containing foreign disease-associated immunogens may be less stringent than for other approaches.

The HBsAg VLP vaccine generates strong B cell immunity, comparing favourably with traditional strong immune response inducers [3]. The ability of this vaccine to also induce cellular responses may be linked to CTL epitope density that occurs in VLPs and/or the ability of VLPs to be easily endocytosed [4]. HBsAg may also be delivered as a DNA vaccine [5,6]. Using DNA as immunogen, CTL induction by intracellular translated HBsAg protein occurs through the 'classical' endogenous pathway and may occur through the 'alternative' exogenous pathway via secreted HBsAg particles or protein [7,8]. However powerful immune responses to HBsAg DNA vaccine are obtained even in the absence of VLP formation. Whether the HBsAg protein has a specific characteristic that complements the way that DNA vaccines are presented to the immune system, whether it is from some intrinsic adjuvant-like characteristic or provision of T-'help', remains unclear.

DNA-based vaccines hold particular promise as an option to prevent and treat infections and some tumors [9]. While clinical trials have hitherto shown that the magnitude of immune responses primed by standard DNA vaccines is generally weaker in humans than in small mammals, a number of strategies (eg. targeting antigen to the endoplasmic reticulum or dendritic cells, using adjuvants, prime-boost regimens and/or use of cytokines) have been explored to overcome this. One approach which particularly shows promise for HBsAg is electoporation or ballistic delivery of DNA directly into the skin [10]. A number of these 'new generation' approaches to DNA vaccination in humans is now underway [11], and the immunological results of trials appear similar to the results we and others have obtained in mice [12].

We have previously reported a novel strategy for generating cellular immunity using HBsAg vector by *deleting* HBsAg-specific CTL epitopes and *replacing* them with foreign CTL epitopes of similar physical properties (ie. size and hydrophobicity) [13,14]. In addition, other groups have extended the C' and/or N' termini of HBsAg with whole antigens and shown that such vaccines can induce protective immunity[15-18]. While we have previously highlighted the enormous

potential of HBsAg as a potential generic vaccine framework for inducing potent protective immunity[19], it is clear that this potential is only just being realised .

The demonstration that multiple CTL epitopes may by linked linearly to form a polyepitope vaccine [20] suggests the possibility of simultaneous protection against multiple diseases, by encoding epitopes from a number of different pathogens. In the present study we explored HBsAg DNA as a vector to deliver DNA encoding a polyepitope comprising eight murine and human disease-protective CTL epitopes. We demonstrate that immunization of mice induced CTL responses to all eight foreign epitopes. We also demonstrate that CTL responses were associated with protection against neoplastic and infectious diseases.

These data underscore the efficacy of HBsAg DNA as a powerful vector to elicit CTL responses to multiple foreign epitopes encoded within a DNA polyepitope. They also demonstrate the capacity of rHBsAg DNA to deliver simultaneous protective immunity against multiple diseases.

Results

pHBsAg-Polyepitope#3

The essential features of the HBsAg-polyepitope construct (pHBsAg-Polyepitope#3) are depicted in Fig 1**.** Mammalian codon optimisation of HBsAg and the CTL polyepitope C'terminal extension was used to predispose to enhanced polyprotein production. To minimise internal initiation of truncated proteins, we preferentially selected epitopes without methionine residues, and those two epitopes (LLM and YLL) which did contain a methionine residue were included at the extreme N' terminus. The epitopes included in the polyepitope were previously described to elicit disease-protective CTL responses in murine models of human disease (Table 1). Individual epitopes were separated by hydrophilic 'spacer' sequences to reduce the overall hydrophobicity of the polyepitope. Additionally, an arginine residue was included at the C' terminus of each epitope, in order to maximise antigen processing and immunogenicity [21].

Immunization with pHBsAg-Polyepitope#3 DNA elicits CTL responses to each encoded foreign epitope.

To investigate effector CTL responses, we quantified epitope-specific IFN-γ secretion by splenocytes harvested *ex vivo* from mice receiving a single immunization with pHBsAg-Polyepitope#3 . A significantly higher number of splenocytes from mice immunized with pHBsAg-Polyepitope#3 secreted IFN-γ when cultured *in vitro* with peptides RAH, ESY,GIL, VGA, and SII (Table 1, 3-letter code) than without peptide ($p<0.001$) (Fig. 2). *Ex vivo* splenocytes from mice immunized with HBsAg wild-type DNA (pHBsAg W/T) cultured *in vitro*

with these peptides did not secrete IFN-γ above the level observed when cultured without peptide (data not shown).

We also investigated whether immunization with pHBsAg-Polyepitope#3 would induce memory CTL capable of being restimulated *in vitro* and of killing target cells displaying the encoded foreign epitopes. Splenocytes from pHBsAg-Polyepitope#3- or pHBsAg W/T-immunized mice were restimulated for 6 days *in vitro* with each of the encoded foreign epitopes individually, and then reacted in ELISPOT assay. Significantly higher numbers of restimulated splenocytes secreted IFN-γ when cultured *in vitro* with peptides RAH, ESY,GIL, SII (each $p<0.001$), VGA, KLI (each $p<0.007$) and YLL ($p=0.05$) (Fig. 3A), than without peptide . Numbers of IFN-γ secreting cells were significantly higher in restimulated splenocytes than in *ex vivo* splenocytes (14-114 fold, $p<0.001$; Figs. 2, 3A). Restimulated splenocytes specifically killed epitope-pulsed , but not unpulsed, target cells ($p<0.001$, except LLM, Fig. 3B). Restimulated splenocytes from pHBsAg W/T- immunized mice did not secrete levels of IFN-γ above background , or kill peptide pulsed target cells (data not shown).

We were concerned that although the above immunization regimen elicited responses to seven of the eight foreign epitopes encoded by pHBsAg-Polyepitope#3, no response was elicited to epitope LLM. To address this we adopted a prime-boost approach. Mice immunized once the pHBsAg-Polyepitope#3 were boosted with a low dose of LLM peptide, and CTL response subsequently evaluated. Restimulated splenocytes from mice immunized with pHBsAg-Polyepitope#3 and boosted with LLM peptide, but not mice immunized with pHBsAg-Polyepitope#3 without peptide boost, or mice immunized with peptide without prior pHBsAg-Polyepitope#3 prime, specifically killed target cells expressing the LLM epitope (Fig. 4).

Together, the above data indicates that a single immunization with recombinant HBsAg plasmid DNA encoding eight human disease-relevant epitopes as a polyepitope appended at the C'terminus, primes for IFN-γ associated effector and memory T cell responses which are cytotoxic for target cells expressing each of the epitopes.

Immunization with pHBsAg-Polyepitope#3 confers protection against growth of tumors expressing tumor-associated antigens.

We investigated whether mice immunized with pHBsAg-Polyepitope#3 were simultaneously protected against tumors expressing either the HPV 16 E7 or ovalbumin tumor-associated antigens. We first confirmed in two representative mice per group of seven that immunization with pHBsAg-Polyepitope#3 evoked a IFN-γ secreting effector immune response directed to RAH epitope (of HPV 16 E7) and to the SII epitope (of ovalbumin) as per Fig. 2 (data not shown). The remaining five mice per group were challenged with E7-expressing TC-1 tumor or with ovalbumin-expressing B16-OVA tumor. In the TC-1-challenged mice, significantly fewer

mice acquired tumors, and at later time points after challenge, in the group immunised with pHBsAg-Polyepitope#3 compared with the group immunised with pHBsAg W/T (Fig 5A). Additionally, individual tumor growth was significantly slower in the pHBsAg-Polyepitope#3-immunized mice which developed tumors (Fig 5B $p<0.008$). Similarly, in the B16.OVA-challenged mice, significantly fewer mice acquired tumors, and at later time points after challenge, in the group immunised with pHBsAg-Polyepitope#3 compared with the group immunised with pHBsAg W/T (Fig 5C). Additionally, individual tumor growth was slower in the pHBsAg-Polyepitope#3-immunized group (Fig 5D $p=0.05$).

These data indicate that immunization with a recombinant HBsAg DNA vaccine encoding a polyepitope containing multiple tumor-associated epitopes confers concomitant protection, associated with epitope-specific IFN-γ secreting CTL responses, against the growth of multiple tumors.

Immunization with pHBsAg-Polyepitope#3 confers protection against pulmonary hRSV infection.

We asked whether immunization of groups of mice with pHBsAg-Polyepitope#3 (which expresses a hRSV CTL epitope; ESY, Table 1) would confer protection against pulmonary infection in mice challenged with hRSV. Groups of H-2^d mice (seven per group) were immunized twice with 100 ug pHBsAg-Polyepitope#3 or pHBsAg W/T id. We first confirmed in two representatives mice per group that immunization with pHBsAg-Polyepitope#3 evoked a IFN-γ secreting effector immune response directed to ESY as in Fig. 2 (data not shown). The remaining five mice per group were inoculated intranasally with hRSV and four days later, lungs were removed for virus quantitation. The mean hRSV titer was significantly reduced in the group immunized with pHBsAg-Polyepitope#3, compared with the group immunized with pHBsAg W/T (Fig. 6).

Taken together, the data in Figs 5 and 6 indicate that immunization with pHBsAg-Polyepitope#3 affords protection against two tumors and one respiratory virus infection in murine disease models.

Immunization with pHBsAg-Multiepitope#2, encoding foreign CTL epitopes inserted into the HBsAg backbone, elicits multiple CTL responses.

We have previously shown that rHBsAg DNA vaccines in which sequences encoding endogenous HBsAg CTL epitopes are *deleted* from the HBsAg backbone and *replaced* with DNA encoding a foreign epitope, elicit CTL responses to the inserted foreign epitope [22]. We wished to examine whether a rHBsAg vaccine encoding multiple epitopes inserted into the HBsAg backbone might also be effective in eliciting CTL responses relevant to multiple diseases. We constructed, using

this epitope replacement strategy , a rHBsAg DNA vaccine (pHBsAg-Multiepitope#2) containing six of the eight CTL epitopes listed in Table 1, inserted into the HBsAg backbone (Fig 7). We examined the efficacy of induction of CTL responses, capable of killing target cells displaying the encoded foreign epitopes. Splenocytes from mice immunized with HBsAg-Multiepitope#2 or pHBsAg W/T were restimulated for 6 days *in vitro* with each of the six encoded foreign epitopes, and reacted in ELISPOT assay. A higher number of splenocytes from mice immunized with pHBsAg-Multiepitope#3 secreted IFN-γ when cultured *in vitro* with peptides GIL, ESY, RAH, and KLI, (but not but not VGA or LLM (not shown)), than without peptide (Fig. 8A).In addition, restimulated splenocytes specifically killed cognate epitope-pulsed , but not unpulsed, target cells (Fig 8B). Restimulated splenocytes from pHBsAg W/T immunized mice did not secrete levels of IFN-γ above background , or kill peptide pulsed target cells (not shown).

These data indicate that CTL responses to foreign epitopes may be elicited by a rHBsAg DNA vaccine encoding multiple disease-relevant foreign CTL epitopes inserted into the HBsAg backbone.

Discussion

In this study we have constructed a human codon optimised recombinant HBsAg DNA vaccine encoding a polyepitope comprising eight mouse and human disease-protective CTL epitopes restricted through four MHC class 1 haplotypes. We demonstrate effector and memory CTL responses to the foreign epitopes following a single immunization (in the case of seven of the eight epitopes), and where tested, an association of CTL induction with protection against neoplastic and infectious disease. While animal ethics considerations precluded challenge of mice with multiple diseases simultaneously, the data are consistent with the notion of simultaneous protection of immunized recipients against multiple diseases, mediated by CTL responses restricted through multiple MHC class 1 (including HLA) haplotypes. Taken together, these observations are supportive of the concept of a currently licensed human vaccine (HBsAg) genetically modified to encode human disease- protective epitopes. Such a vaccine would be applicable to the human MHC class 1 polymorphic population to simultaneously protect against multiple human infectious diseases and some cancers which depend on the cellular immune response for their resolution.

The observation that CTL-mediated disease resolution is usually focussed on one or a few epitopes [23] and that a majority of human MHC class I polymorphism is contained with relatively few epitope cross-presenting class I 'supertypes' [24], suggests that wide population coverage against a substantial number of diseases may be feasible with C'terminal polyepitope extensions containing relatively few CTL epitopes.

In this study, we also extend our previous observations [25] that insertion of multiple foreign CTL epitopes *within* the HBsAg backbone allows the generation of multiple simultaneous CTL responses to the foreign epitopes. While a direct side-by-side comparison of the efficacy of C'terminal extension with CTL polytope (ie. pHBsAg-Polyepitope#3*)* versus insertion of individual epitopes into the backbone (ie.pHBsAg-Multiepitope#2), was not made, it is clear that both strategies are highly efficacious for induction of CTL responses to inserted epitopes. C'terminal extension is logistically preferable as vaccines using this rHBsAg strategy are more easily engineered. In other studies, we have shown that experimental DNA vaccines encoding foreign antigen simultaneously appended at *both* the C' terminus and the N' terminus of the HBsAg protein elicit protective CTL responses to antigen at both termini (O.Haigh, in preparation). Thus, the capacity for delivery of foreign CTL epitopes by HBsAg DNA vaccines may be enhanced by simultaneous intra-molecule insertion, and N'-terminal extension, in addition to C'terminal extension. Constraints on size of foreign insert may not be so limiting for vaccine derivation when DNA rather than VLP is used as the delivery modality.

For the induction of CTL-mediated immunity, comparison of delivery of wild-type HBsAg as a DNA vaccine compared with delivery as a VLP vaccine, suggests that the former is the preferred modality in terms of immunogenic efficacy, as well as economically and practically [26]. Continuous exposure to small doses of antigen [27] produced by on-going transcription from persisting HBsAg DNA (eg. in muscle cells [28] and follicular dendritic cells [29] is likely responsible for the persistence of effector and memory CTL responses for many months following a single intramuscular injection of HBsAg plasmid DNA [30,31] (R.Tindle, unpublished). We have demonstrated the efficacy of rHBsAg DNA vaccines given as a single 1000-fold lower dose, than that for conventional DNA vaccines in mice (R.Tindle, unpublished). Wild-type HBsAg-based DNA vaccination required a dose up to 2,500-fold lower in humans than used in previous clinical trials with conventional DNA [32]. Following injection, transfected antigen presenting cells (APCs) will activate the CTL response through the intracellular (endogenous) processing pathway [33]. This pathway has likely evolved to deal with nascent proteins from the ribosome machinery, and it has been demonstrated that particle formation within the APC is not required for endogenous processing of the HBsAg polyprotein. [34]. Whether secretion of HBsAg polyprotein and/or particles, eg. from DNA transfected muscle cells, provides a second mechanism for HBsAg CTL generation via the exogenous pathway [35] is controversial [36,37]. Data generated from a matched series of plasma DNA vectors expressing wild-type or several mutant forms of HBsAg that were secretion-defective, or severely truncated, indicated that neither VLP formation nor it's secretion or liberation plays a significant part in the development of the CTL response [38].

In summary, DNA-based HBsAg immunization is extremely potent, and may be explained by prolonged or higher expression, high epitope density, superior antigen processing,

the presence of multiple T-helper epitopes [39,40], biasing the response via cytokines to a Th-1profile [41] and, possibly in some circumstances, secretion for uptake of polyprotein/particle by APCs. The observation of long-lasting CTL responses (and antibody) in neonatally immunized mice by (wild-type) HBsAg DNA, but not HBsAg particles[42], reinforces the rationale for a DNA approach for recombinant HBsAg vaccines. Furthermore, that the capacity of rHBsAg DNA vaccines to elicit powerful CTL responses is undiminished in the presence of high-titer HBsAg antibody (R.Tindle, unpublished) further argues for a DNA approach where rHBsAg DNA vaccine may be given to recipients who sustain a HBsAg B-memory response from prior vaccination for hepatitis B virus. Finally, DNA vaccination effectively induces CTL responses in recipients bearing MHC class I haplotypes that do not respond to immunization with HBsAg VLP vaccine [43]

Indeed, recombinant HBsAg VLP vaccines are likely to prove impractical outside the laboratory setting; extensive studies in our laboratory (S.Thomson , M.Mather unpublished data) replacing endogenous HBsAg CTL with foreign CTL epitopes matched for size, charge, and hydrophobicity, and from other laboratories[44,45] have indicated that VLP formation is severely and unpredictably compromised by modification of the HBsAg protein, presumably relating to structural and/or stability constraints .

pHBsAg-Polyepitope#3 did not produce VLPs when used to transfect Huh cells (data not shown) even though we designed HBsAg-Polyepitope#3 protein to retain tertiary structure compatible with VLP formation. Thus, inclusion of hydrophilic spacers between epitopes reduced the overall hydrophobicity of the polyepitope extension to prevent it's insertion into the bilid membrane (as predicted by TMHMM topography algorithm (http:\\ca.expasy.org\tools\). This measure predisposes the polyepitope to an external (to the particle) location. We also elected to exclude epitopes containing cysteine residues, thereby minimising perturbation in secondary structure due to disulphide bonding. The lack of VLP formation is in contrast to rHBsAg C'-terminally extended with a HIV polyepitope where some VLP production was recorded.[46]. It underscores findings of our laboratory and others [47,48] on the relative lack of predictability of VLP-forming propensity by HBsAg engineered to contain foreign sequences.

The indifferent immunogenicity of sub-dominant epitopes in the presence of one or more immunodominant epitopes can compromise the efficacy of vaccines encoding multiple CTL epitopes . The results reported here indicate CTL responses to all eight CTL epitopes appended as a C'terminal polyepitope, even though the polyepitope contained at least two 'strong' CTL epitopes (ESY, GIL) which might have been expected to be immunodominant. The lower CTL responses elicited by 'weaker' epitopes eg VGA, KLI in this context (Fig. 3) were similar to responses elicited by these epitopes administered individually as high molar excesses of peptide in Quil A adjuvant (not shown), suggesting that level of response from pHBsAg-Polyepitope#3

was determined by intrinsic properties of these epitopes, and not by co-expression of other, 'immunodominant', epitopes

We were unable to elicit an antibody response to the encoded hRSV B-cell mimotope following two immunizations with pHBsAg-Polytope #3 DNA (data not shown).This may have reflected the id. route of immunization from which most injected DNA localises to professional APC in draining lymph nodes, predisposing to rapid antigen processing of nascent HBsAg polypeptide However, we have previously elicited antibody to the hRSV epitope via the id. route when expression was from hRSV DNA inserted into to the 'a'-loop region of HBsAg DNA (T. Doan, unpublished). In the latter case, the hRSV-HBsAg recombinant formed VLPs. This is consistent with the notion that secretion or release of rHBsAg polyprotein/VLP is a necessary prerequisite for induction of antibody by rHBsAg DNA vaccines.

In this study we demonstrate that recombinant HBsAg delivering a CTL polyepitope appended at the C' terminus elicits concomitant responses to multiple epitopes restricted through a diversity of MHC class I haplotypes, which are relevant in a number of human diseases. We show that the rHBsAg vaccine elicits concomitant protection against neoplastic and infectious disease. These studies vindicate the use of HBsAg as a powerful vector to deliver CTL responses to foreign antigens. They also have have implications for a multi-disease vaccine applicable to the HLA-polymorphic human population.

Materials and Methods

pHBsAg-Polyepitope#3

The hepatitis B surface antigen codon sequence of Valenzuela et.al.[49] was used as a basis for the codon optimization of the gene using codons described by Cid-Arregui et al [50]. The sequence data has been submitted to the Genbank database under accession number xxx.

Essentially, oligonucleotides were synthesized as 80mers (Geneworks, Australia) which covered the full length sequence of the HBsAg in the 5' to 3' direction. A series of CTL epitopes were interspersed with spacer regions comprising the sequences HWSISKPQ, RAKT, RADT, RDTA or RTKA. (Fig. 1). Spacer sequence HWSISKPQ is mimotope of a hRSV F-protein B-cell epitope [51]. Other spacer sequences are as described [52]. A set of complementary oligonucleotides were also synthesized which overlapped the termini of the forward oligonucleotides by 40 bases. Each oligonucleotide was then added to a PCR reaction at a concentration of 5ng/ul in the presence of PrimeSTAR HS DNA polymerase (Takara Bio Inc, Shiga, Japan). PCR reaction conditions were as described by the manufacturer. The PCR product was purified from a 1% agarose gel after electrophoresis using UltraClean GelSpin cartridges (Mo Bio Labs Inc, CA) according to the manufacturer's instructions and then incubated in the presence of dNTPs (10mM) and Taq DNA polymerase (Promega, USA) for the addition of 3'-teminal

adenosine residues. The DNA was ligated into pGEM-T easy vector (Promega, USA) using T4DNA ligase (Promega, USA) according to the manufacturer's instructions. The DNA insert into the pGEM-T easy plasmid was sequenced in both directions and any errors in the sequence corrected using the QuickChange II site-directed mutagenesis kit (Stratagene TX, USA).

pHBsAg-Multiepitope#2.

pHBsAg-Multiepitope #2 was derived essentially as described [53]. In summary, the plasmid pcD3-HBsAgS (ayw subtype) [54] was engineered to delete the HBsAg-specific CTL epitopes (^{13}VLQAGFFL^{21}, ^{28}IPQSLDSWWTSL^{39}, ^{41}FLGGTPVCL^{49}, ^{97}LLDYQGMLP^{105}, ^{184}GLSPTVWLS^{193} and ^{206}SILSPFIPLL^{215} [55,56], and to introduce the restriction enzyme sites BsiW1, NheI , BspE1, Afl1, BlpI and SacII respectively, by PCR-driven site directed mutagenesis. Synthetic oligonuleotides encoding the selected foreign epitopes were inserted into HBsAg through systematic sub-cloning into these restriction sites to create the construct depicted in Fig.7. Due to reading frame shifts caused by addition of restriction sites, codons for alanine and leucine (AA or A and AL) were included in the insert sequences at the 5' and 3' ends of the inserted epitope oligomers. Sequences of constructs were verified via Terminator Sequencing (ABI) Big Dye 3.1.

Immunization and restimulation of splenocytes

Mice were immunized intradermally (id.) in the ear as with 100 ug of purified plasmid DNA prepared by EndoFree Plasmid Giga Kit (Qiagen, Australia). Two weeks after immunization, spleens were removed and splenocytes were restimulated *in vitro* for 6 days as described [57]with 1ug/ml cognate peptide. For peptide immunizations, mice were immunized subcutaneously (sc.) at the tail base with 50ug peptide + 0.25ug tetanus toxoid (TT) as a source of T-helper epitopes + 10ug Quil A adjuvant [58]. Ten days later spleens were harvested and splenocytes were restimulated as above.

Cells.

EL4.A2 cells [59] are susceptible to specific CTL lysis through both H-2^b and HLA A*0201 restriction pathways. P815 is susceptible to specific CTL lysis through the H-2^d restriction pathway. Cells were maintained as described [60].

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 minimal CD8$^+$ T-cell epitope peptides,

essentially as described [61]. 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 [62]. In summary, target cells (10^4 per well) sensitised at 37^{O}C for 1 hour with 1ug/ml cognate or irrelevant peptide, or medium alone, and labelled with 100uCi ^{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 d) x 100%.

Tumor protection assays.

Groups of H-2^b mice (7 per group) were immunized twice with 100 ug pHBsAg-Polyepitope#3 or pHBsAg W/T id., or 100ug of E7 or OVA peptide + tetanus toxoid + Quil A sc. CTL responses to tumor epitopes RAH and SII were confirmed in two representative mice per group by IFN-γ ELISPOT on *ex vivo* splenocytes. The following day, the remaining 5 mice per group were injected sc.on the flank with $2x10^5$ TC-1 cells, which express the E7 tumor-associated antigen of human papillomavirus type 16 [63] or with 10^5 B16-OVA melanoma cells expressing ovalbumin [64]. (The tumor doses were pre-determined by titration experiments to discern a minimal dose giving rise to tumor in 80-100% of unimmunized mice). Mice were monitored for incidence of tumor with time to 43d. Tumor volume was derived at intervals from calliper measurements in two perpendicular dimensions by the formula $S^2 L$, where S is the shorter dimension and L is the longer dimension.

Data are also presented as Kaplan Meier tumor incidence curves of % tumor free mice at given time points after tumor injection.

Propagation of RSV and evaluation of pulmonary viral infection.

hRSV A2 (VR-1302) strain (American Type Culture Collection , Rockville, MD, USA) was propagated in HEp-2 cells essentially as described [65] and recovered from the supernatant following freeze/thawing of monolayers displaying a cytopathic effect.

hRSV was quantified by immunofocus assay, using goat anti-hRSV primary antibody (Chemicon, Australia), anti-goat Ig horse-radish peroxidase conjugated second antibody, and tetraaminobiphenyl hydrochloride substrate (Sigma, Australia), according to manufacturer's

instructions. Lungs were collected 4 days after mice were inoculated intranasally (in.) with $8x10^5$ plaque forming units (pfu) and RSV quantified in lung homogenates by immunofocus assay as described [66].

Statistics.

Experimental values were compared for significant difference using Student's t-test. Kaplan Meier tumor incidence curves were compared using the Log Rank statistic.

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Conflict of interest

The authors have no conflict of interest to declare.

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Epitope	Virus protein	Restriction	Reference
RAHYNIVTF	Human papillomavirus (HPV)16 E7	H-2D^{b}	[67]
ESYIGSINNITKQSA	Respiratory syncytial virus (hRSV) F	H-2K^{d}	[68]
GILGFVFTKL	Influenza A (Flu) matrix	HLA A*02	[69]
VGALIFTKL	Human metapneumovirus (hMPV)	H-2K^{b}	[70]
SIINFEKL	(Ovalbumin)	H-2K^{b}	[64]
KLILALLTFL	Human metapneumovirus (hMPV) SH	HLA A*02	[71]
LLMGTLGIV	Human papillomavirus (HPV) 16 E7	HLA A*02	[72]
YLLEMIWRL	Epstein Barr Virus (EBV) LMP1	HLA A*02	[73]
AMQMLKETI	HIVgagp24	H-2K^{d}	[74]

Table 1. Foreign CTL epitopes included in pHBsAg-Polyepitope#3

Titles and legends to figures

Fig.1 rHBsAg-Polyepitope#3 multi-disease vaccine. DNA encoding eight disease-protective CTL epitopes (AA sequence depicted as 3-letter code) was appended to the 3'-prime end of a codon optimised HBsAg minigene in a mammalian expression vector. The epitopes were restricted through murine (H-2^b, H-2^d) and human (HLA-A2) MHC class I alleles. Epitopes were separated by 'spacer' sequences.

Fig. 2 Immunization with pHBsAg-Polyepitope#3 elicits-epitope specific IFN-γ secreting splenocytes . *Ex vivo* splenocytes from $A2K^b$ or Balb/c mice (as appropriate to MHC restriction; 3 per group) immunized once id. with pHBsAg-Polyepitope#3 exhibit cognate epitope-directed CTL responses. Effector IFN-γ- secreting cells were quantified by IFN-γ ELISPOT assay using splenocytes harvested at 14 days (DNA immunization) or ten days (peptide immunizations) after immunization, and incubated for 15-18h. with cognate epitope peptide (black histogram bar) or without peptide (stippled histogram bar) , as indicated. Splenocytes from $A2K^b$ or Balb/c mice immunized with HBsAg wild type DNA (pHBsAg W/T) and incubated with the appropriate peptide showed no ELISPOT response above background (not shown). Bars represent means +/- standard deviation of 3 replicates.

Fig.3 Splenocytes from mice immunized with pHBsAg-Polyepitope#3 and restimulated *in vitro* with cognate peptide exhibit enhanced IFN-γ secretion and specific cytotoxicity (A) $A2K^b$ or Balb/c mice (as appropriate to MHC restriction; 3 per group) were immunized once id with pHBsAg-Polyepitope#3. Splenocytes harvested 14 days after last immunization were restimulated *in vitro* with cognate peptide for 6 days, then (A) reacted in IFN-γ ELISPOT with (black bar) or without (stippled bar) peptide (bars represent means +/- standard deviation of 3 replicates), or (B) reacted in ^{51} Chromium release cytotoxicity assay with peptide-pulsed (squares) or unpulsed (triangles) EL4.A2 (H-2^b and HLA A*02) or P815 (H-2^d) target cells as appropriate. Data points represent means of three replicates +/- standard deviations. (Where standard deviations are <3%,they are masked by symbols).

Fig 4. Immunization with pHBsAg-Polyepitope#3 primes for a CTL response to epitope LLM which can be boosted with peptide immunization. Two groups of $A2K^b$ mice (A and B ; three mice per group) were immunized once with 100ug id. of pHBsAg-Polyepitope#3. Fourteen days later, the groups were boosted with (A) 5ug LLM peptide plus adjuvant, or (B) adjuvant

alone. A third group (C; three mice) received 5ug LLM peptide plus adjuvant, without prior priming with pHBsAg-Polyepitope#3. Splenocytes harvested 8 days later were restimulated with LLM peptide *in vitro* for six days, then reacted with EL4.A2 target cells pulsed (square symbols) or not pulsed (triangle symbols) with LLM peptide in a ^{51}Chromium cytotoxicity assay. Data points represent means of three replicates +/- standard deviations (standard deviations were <5%).

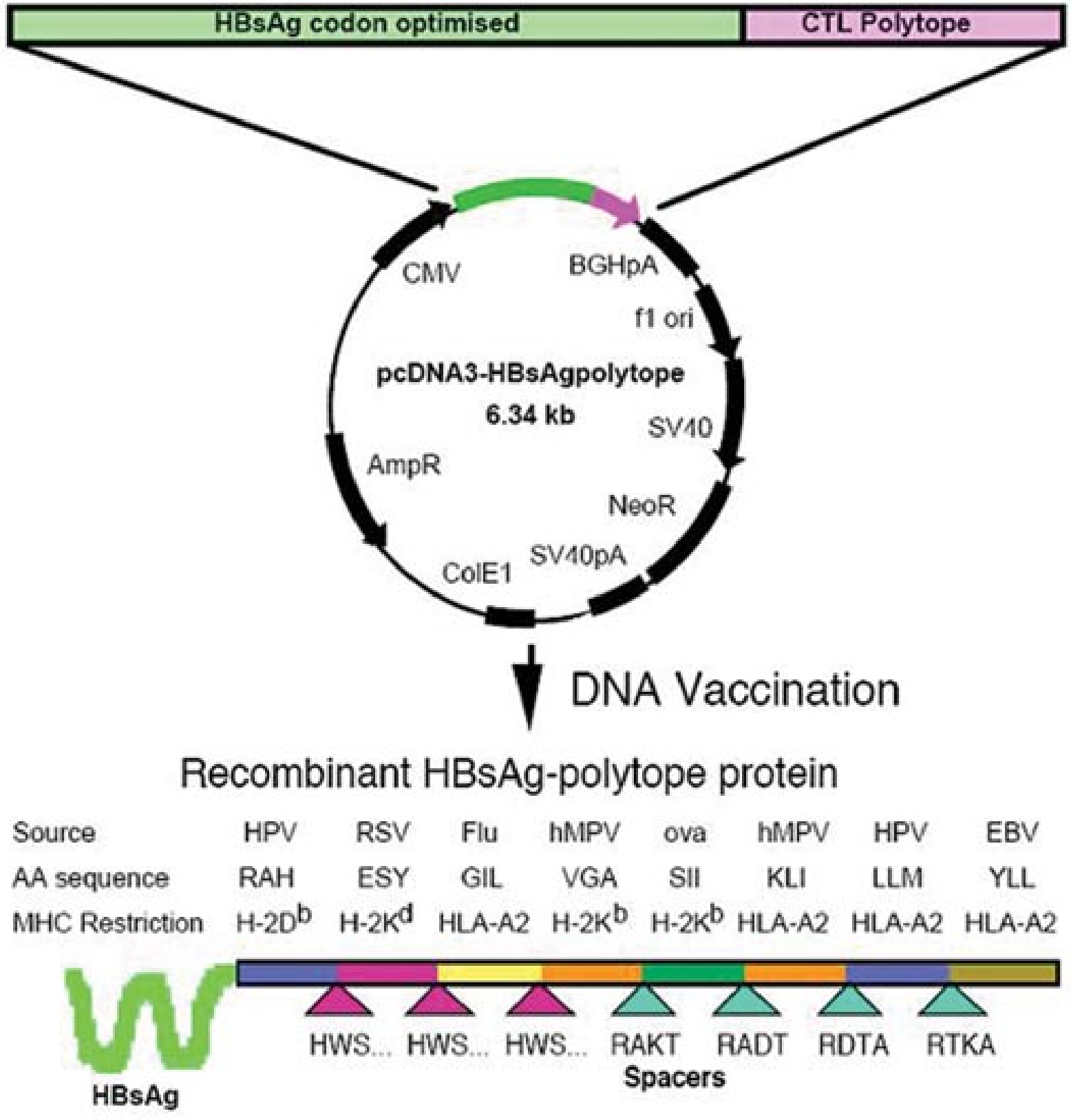
Fig 5.Immunization with pHBsAg-Polyepitope#3 simultaneously protects mice against challenge with tumors each expressing a different tumor-associated antigen (HPV 16 E7 or ovalbumin). H-2^{b} mice (seven per group) were immunized twice id.with pHBsAg-Polyepitope#3 , or with pHBsAg W/T at a 14d interval. Induction of RAH (HPV E7 epitope)- and SII (OVA epitope)- specific IFN-γ secreting splenocytes was confirmed by ELISPOT 14days later from two representative mice per group (not shown). The remaining five mice per group were challenged with (A,B) 2x10^{5} TC-1 tumor cells , or (C, D) 10^{5} B16-OVA melanoma cells sc. on the flank. Tumor growth was monitored to 42 days as the percentage of mice with palpable tumor, and by calliper measurement of tumor size. Mice with tumors in excess of 1000mm^{3} were euthanized in accordance with animal ethics requirements. Results are expressed as tumor-free mice (%) at the indicated time points (A,C), and as tumor volume in mice with time (B, D. Closed symbols, individual mice immunized with pHBsAg-W/T; open symbols, individual mice immunized with pHBsAg-Polyepitope#3).

Fig.6 . Immunization with pHBsAg-Polyepitope#3 reduces hRSV viral load in lungs. H-2^{d} mice (seven per group) were immunized twice id. with pHBsAg-Polyepitope#3 or with pHBsAg W/T at an interval of 10 days. Induction of hRSV epitope (ESY)-specific IFN-γ secreting splenocytes was confirmed by ELISPOT 14days later from two representative mice per group (not shown). The remaining five mice per group were challenged in. with 8x10^{5} pfu hRSV. hRSV in the lungs was quantified 4 days later by plaque assay. Results are expressed as mean viral titer per lung per group +/- standard deviation.

Fig. 7 rHBsAg-multiepitope#2 vaccine. Six DNA minigenes encoding different disease-protective CTL epitopes (AA sequence depicted as 3-letter code) were inserted into DNA encoding HBsAg in pSwitch2 expression plasmid, to replace endogenous HBsAg CTL epitopes, as described ([75]and 'Experimental procedures').

Fig. 8 Splenocytes from mice immunized with rHBsAg-multiepitope#2 and restimulated *in vitro* with cognate peptide exhibit enhanced IFN-γ secretion and specific cytotoxicity. A2K^{b}

or Balb/c mice (as appropriate to MHC class I restriction ; 3 per group) were immunized once id. with rHBsAg-multiepitope#2 . Splenocytes harvested 14 days after last immunization were restimulated *in vitro* with cognate peptide for 6 days, then (A). reacted in IFN-γ ELISPOT with (black bar) or without (stippled bar) peptide or (B) reacted in ^{51}Cr release cytotoxicity assay with peptide-pulsed (square symbols) or unpulsed (triangle symbols) EL4.A2 (H-2^{b} and HLA A*02) or P815 (H-2^{d}) target cells as appropriate. Data points represent means of three replicates +/- standard deviations. (Standard deviations were <3%)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CMV
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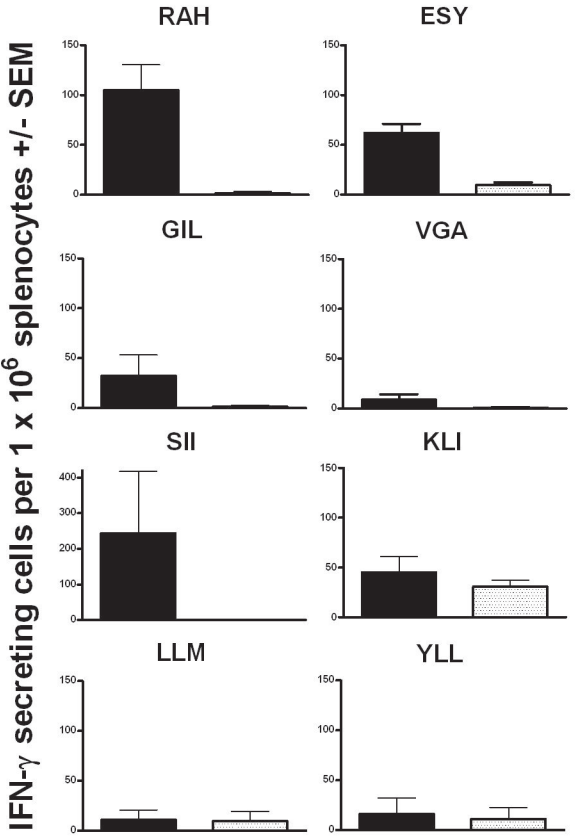
f1 ori
pcDNA3-HBsAgpolytope
6.34 kb
SV40
AmpR
NeoR
SV40pA
ColE1
DNA Vaccination
Recombinant HBsAg-polytope protein
Source HPV RSV Flu hMPV ova hMPV HPV EBV
AA sequence RAH ESY GIL VGA SII KLI LLM YLL
MHC Restriction H-2D^b H-2K^d HLA-A2 H-2K^b H-2K^b HLA-A2 HLA-A2 HLA-A2
HWS... HWS... HWS... RAKT RADT RDTA RTKA
Spacers
HBsAg

Fig. 1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VGA
SII
KLI
LLM
YLL
IFN-γ secreting cells per 1 x 10^6 splenocytes +/- SEM

Fig. 2

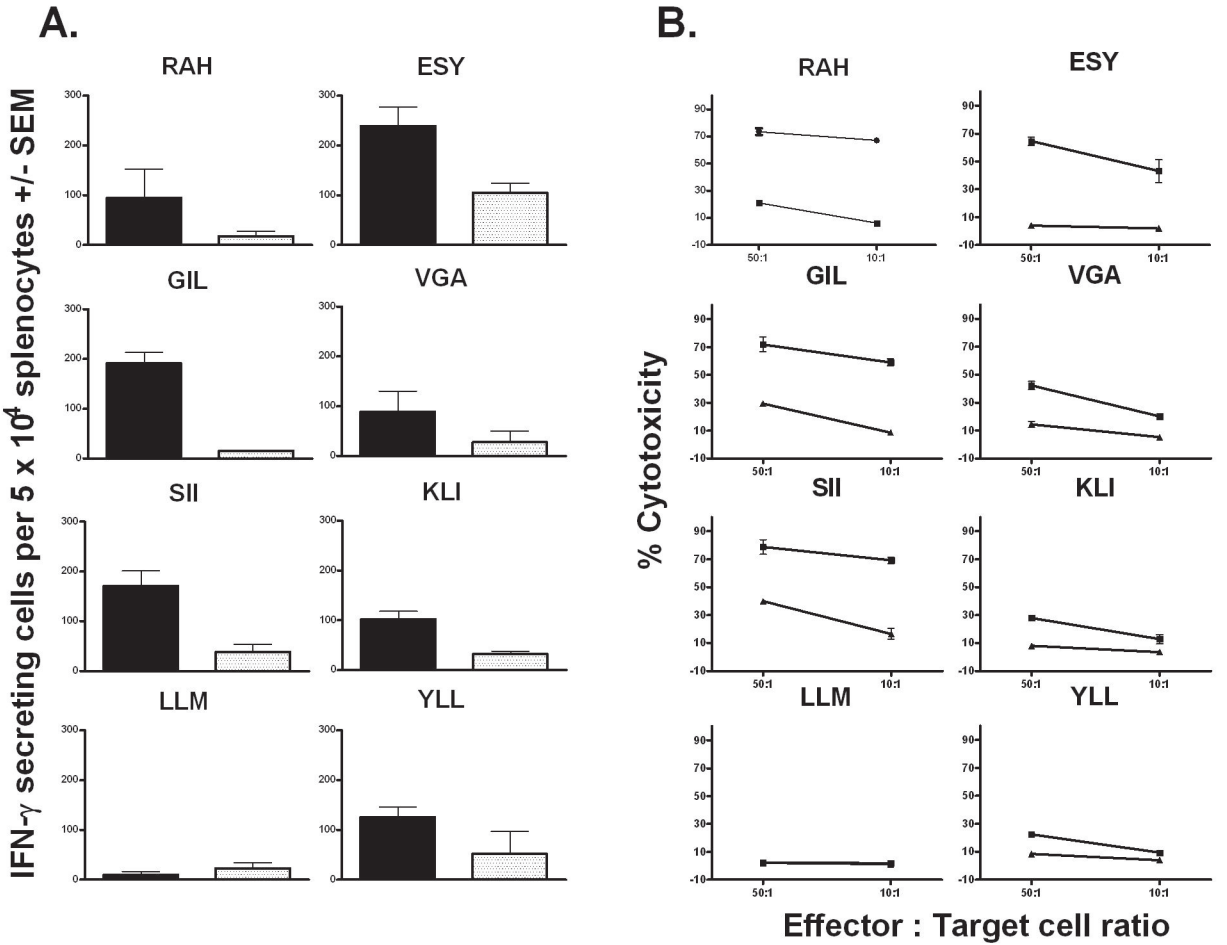

A.
RAH
ESY
GIL
VGA
SII
KLI
LLM
YLL
IFN-γ secreting cells per 5 x 10⁴ splenocytes +/- SEM
B.
% Cytotoxicity
50:1
10:1
Effector : Target cell ratio

Fig. 3

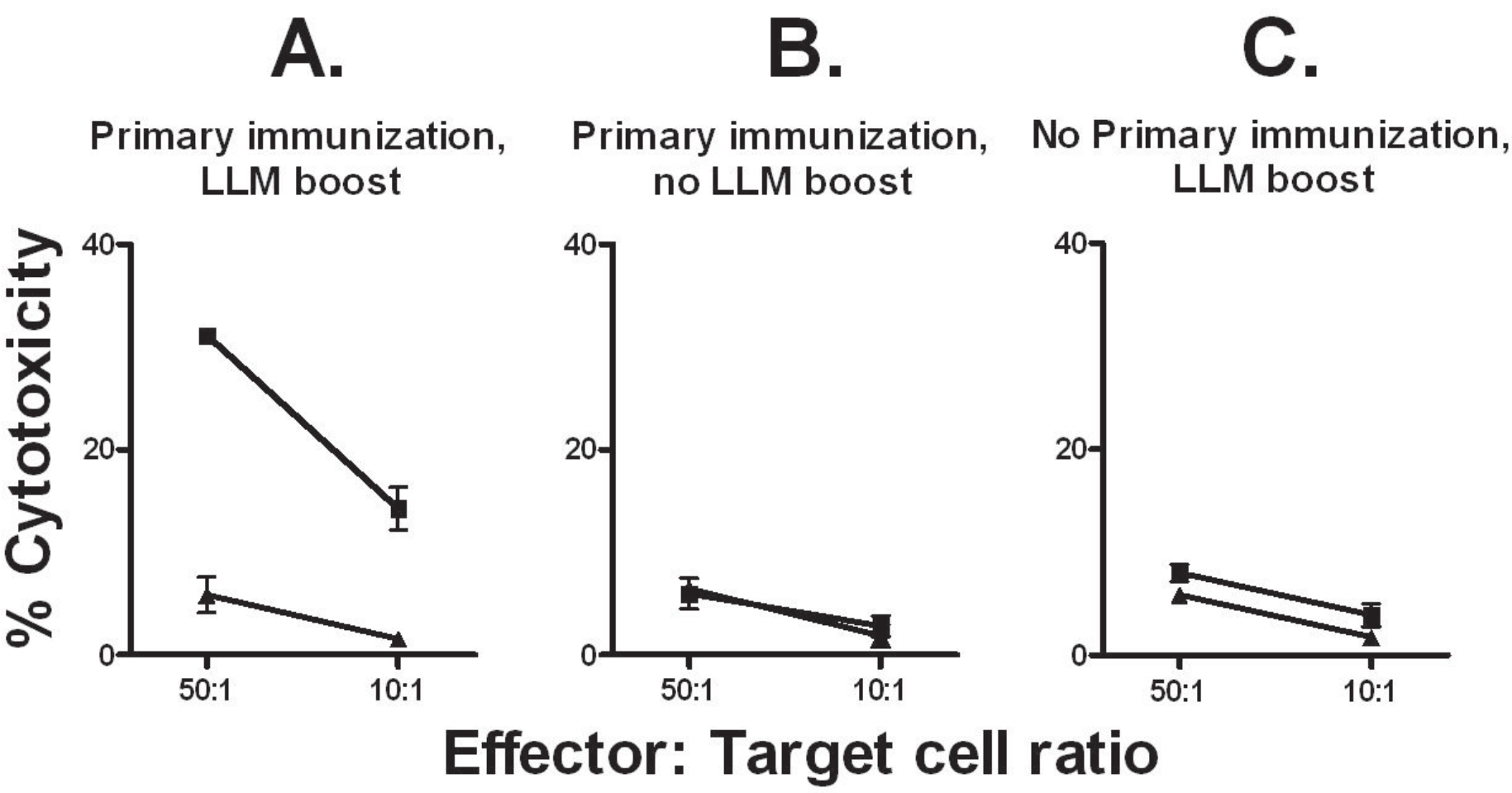

A.
B.
C.
Primary immunization, LLM boost
Primary immunization, no LLM boost
No Primary immunization, LLM boost
% Cytotoxicity
40
20
0
50:1
10:1
Effector: Target cell ratio

Fig. 4

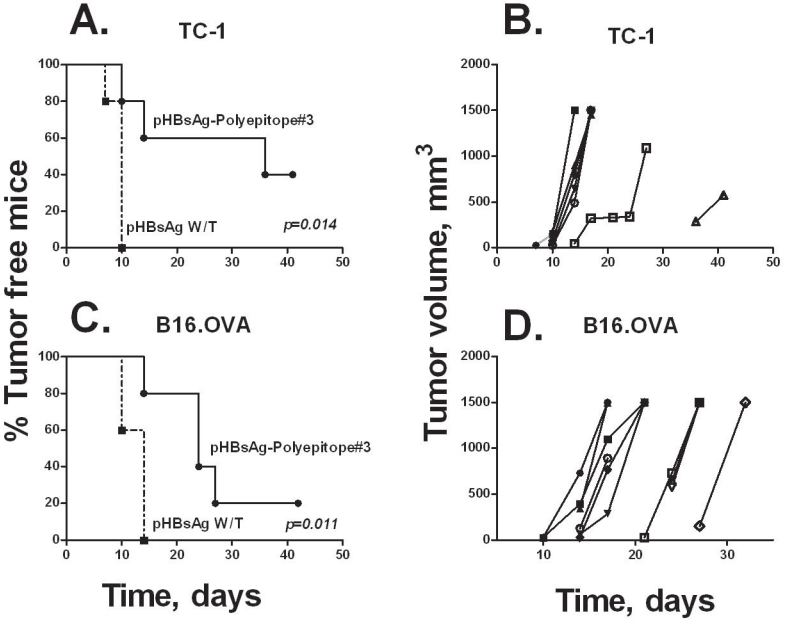

A.
TC-1
pHBsAg-Polyepitope#3
pHBsAg W/T
p=0.014
% Tumor free mice
B.
TC-1
Tumor volume, mm3
C.
B16.OVA
pHBsAg-Polyepitope#3
pHBsAg W/T
p=0.011
Time, days
D.
B16.OVA
Time, days

Fig. 5

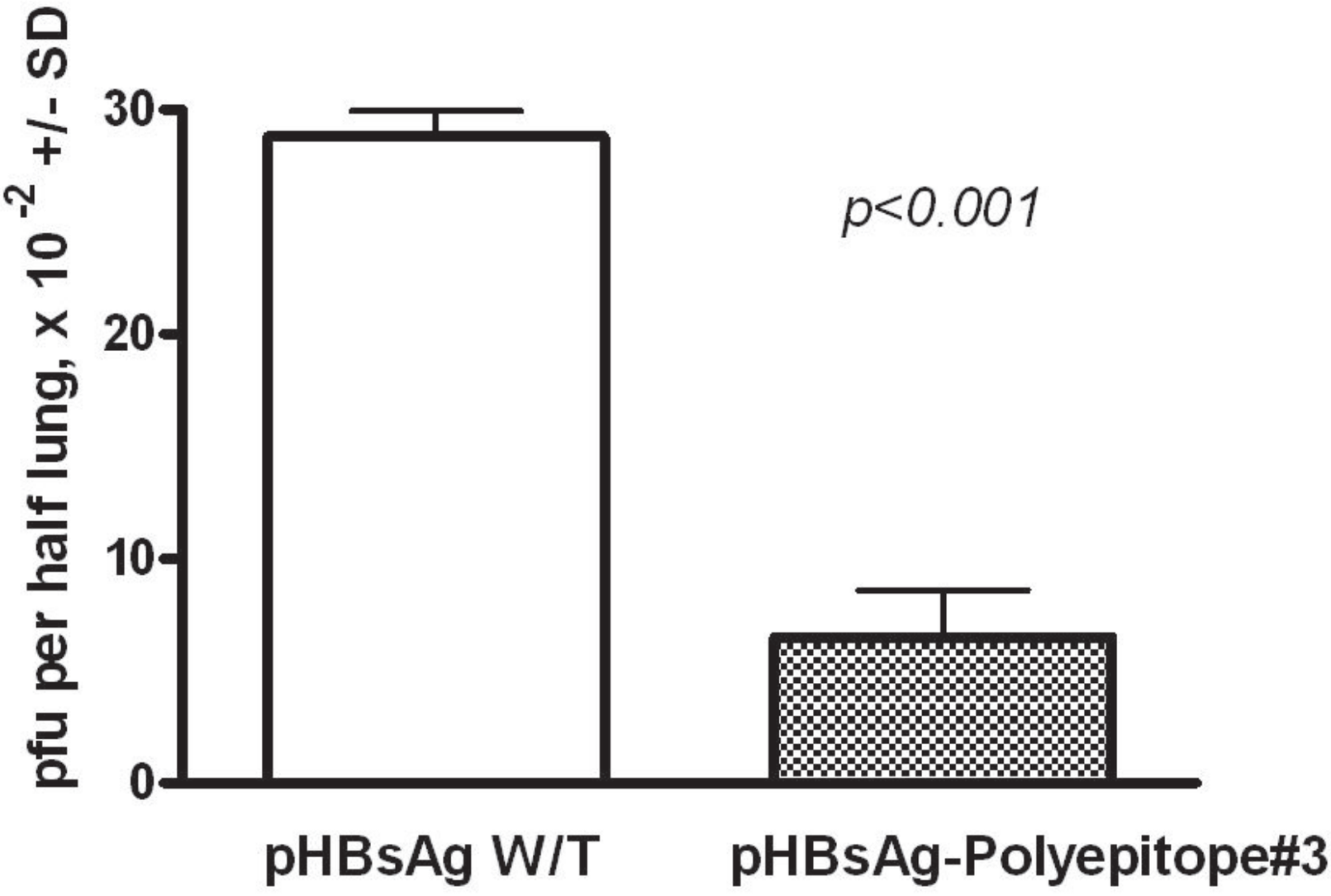
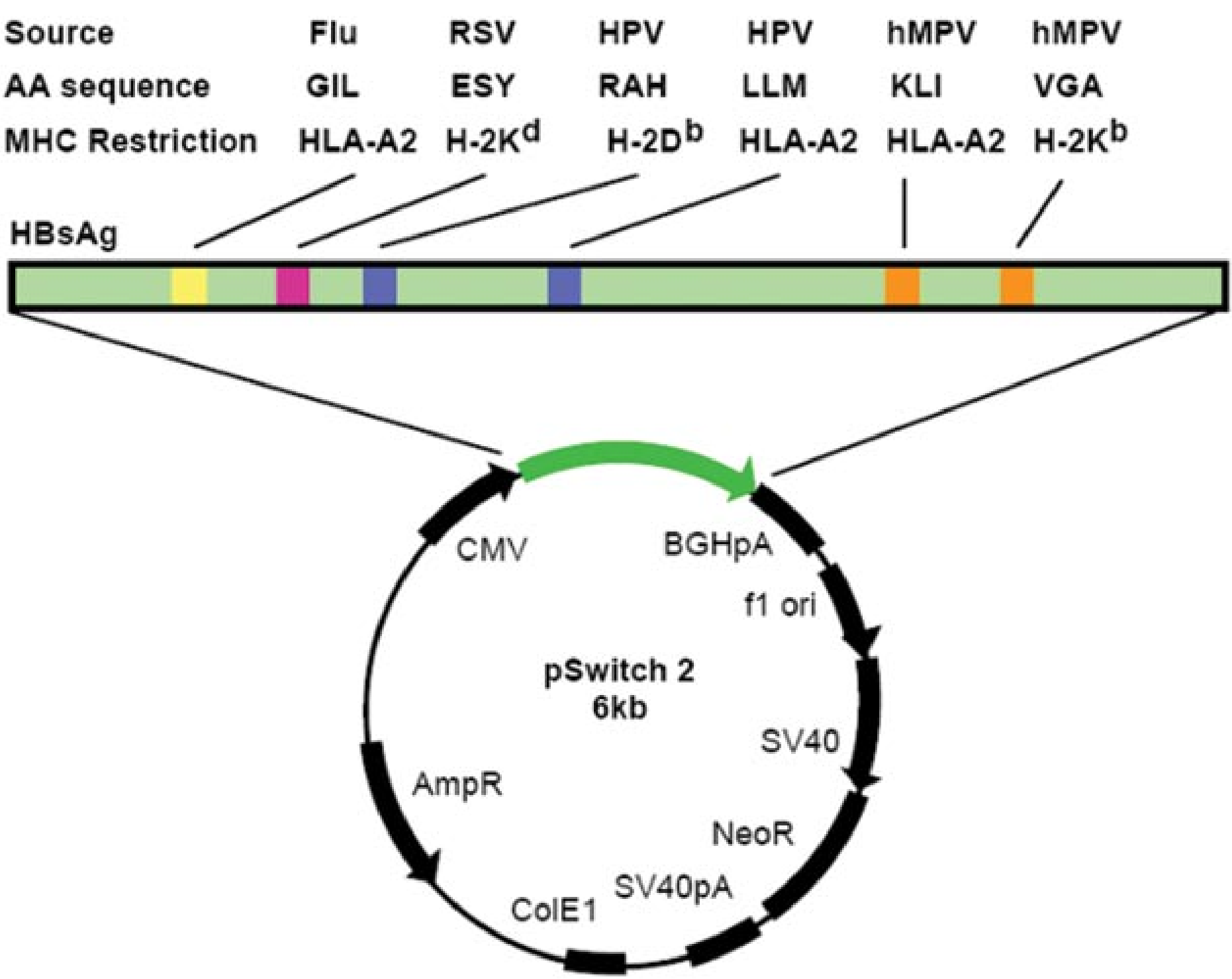

pfu per half lung, x 10 -2 +/- SD
30
20
10
0
p<0.001
pHBsAg W/T
pHBsAg-Polyepitope#3

Fig. 6


Source
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KLI
VGA
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H-2K^d
H-2D^b
HLA-A2
HLA-A2
H-2K^b
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pSwitch 2
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AmpR
NeoR
SV40pA
ColE1

Figure 7

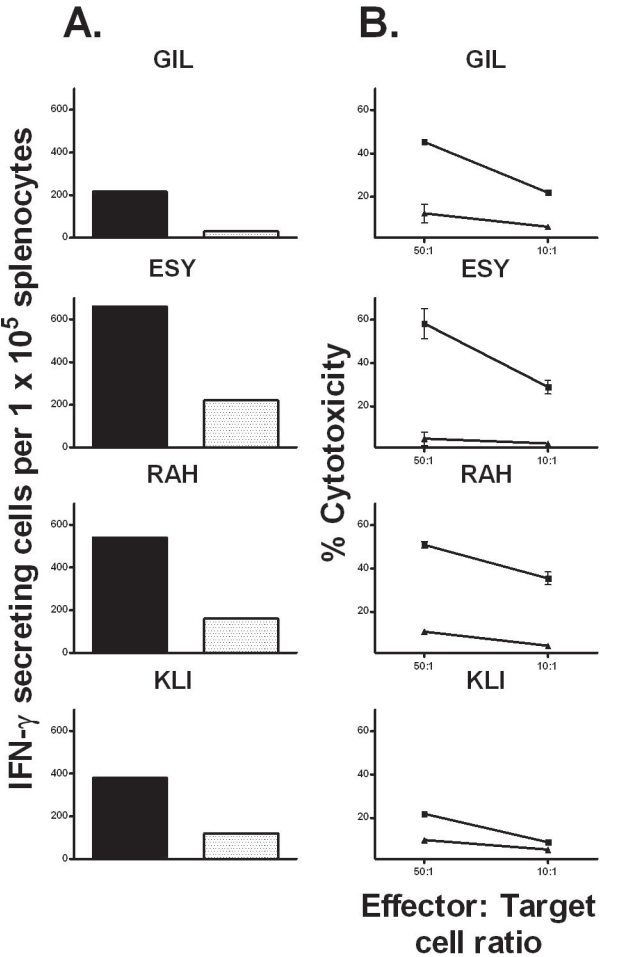
A.
B.
GIL
ESY
RAH
KLI
IFN-γ secreting cells per 1 x 10^5 splenocytes
% Cytotoxicity
Effector: Target cell ratio
50:1
10:1

Fig. 8