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DNA vaccination with T-cell epitopes encoded within Ab molecules induces high-avidity anti-tumor CD8$^+$ T cells

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Stimulation of high-avidity CTL responses is essential for effective anti-tumor and anti-viral vaccines. In this study we have demonstrated that a DNA vaccine incorporating CTL epitopes within an Ab molecule results in high-avidity T-cell responses to both foreign and self epitopes. The avidity and frequency was superior to peptide, peptide-pulsed DC vaccines or a DNA vaccine incorporating the epitope within the native Ag. The DNA Ab vaccine was superior to an identical protein vaccine that can only cross-present, indicating a role for direct presentation by the DNA vaccine. However, the avidity of CTL responses was significantly reduced in Fc receptor γ knockout mice or if the Fc region was removed suggesting that cross presentation of Ag *via* Fc receptor was also important in the induction of high-avidity CTL. These results suggest that generation of high-avidity CTL responses by the DNA vaccine is related to its ability to both directly present and cross-present the epitope. High-avidity responses were capable of efficient anti-tumor activity *in vitro* and *in vivo*. This study demonstrates a vaccine strategy to generate high-avidity CTL responses that can be used in anti-tumor and anti-viral vaccine settings.

Key words: CTL · Epitopes · Tumor immunity · Vaccination

Introduction

CTL play a pivotal role in anti-viral and anti-tumor immunity. Vaccination to date has been unsuccessful for treatment of cancer patients with established disease. It is accepted that the generation of high-frequency T-cell responses is not necessarily an indication of the induction of a competent immune response. The presence of Ag-specific T cells rarely correlates with positive clinical responses in patients, whereas T-cell avidity may be a better indicator of clinical response [1–4]. In both viral infection and tumor models, only high-avidity and not low-avidity CTL mediate viral clearance and tumor eradication [1, 3, 5]. Avidity is defined by the amount of peptide required for activation of effector function [3, 6, 7] and is therefore a measure of the overall strength of the interaction between a CTL and a target cell [3, 8, 9]. Although avidity has been shown to be important, the mechanisms by which high CTL are generated *in vivo* remains unclear. Several factors have however been implicated in the regulation of functional avidity, *e.g.* the cytokines IL-12 and IL-15 [10, 11], CD8$\alpha\beta$ expression [7, 12], TCR affinity, the level of co-stimulatory molecules expressed by APC [10, 13] and the maturation state of DC. The challenge is therefore to find a vaccine approach that mimics these conditions.

Several groups have used Ab to stimulate immune responses [14]. They showed that it was possible to genetically replace CDR-H3 with helper and B-cell epitopes and stimulate immune responses [15, 16]. Zaghouani *et al.* also attempted to replace CDRH3 with class I restricted CTL epitopes. Although they

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showed that transfectomas expressing recombinant Ig were capable of inducing CTL responses, the purified Ig was unable to do so [17, 18]. Recent studies with this mouse IgG2b expressing a nucleoprotein CTL epitope (NP-Ig) have shown that it is possible to stimulate CTL responses if co-administered with the TLR agonist dsRNA, which upregulates Fer receptor IV (FerγIV) receptor IV (FcγRIV) and downregulates FcγRIIb [19]. This group did not assess T-cell avidity.

We have shown that a human monoclonal IgG1 anti-idiotypic Ab, which expressed a T-cell mimotope of CD55 Ag within its CDR, can stimulate helper and cytotoxic T-cell responses in over 300 cancer patients with no associated toxicity [20–22]. Two of the osteosarcoma patients were cured of their disease and survived for at least 10 years post treatment. When the Fc region of this Ab was removed it displayed 1000-fold less efficiency at stimulating T cells [23]. Immature circulating DC in the blood express only low levels of FcγRI to avoid binding serum Ig, but this is transiently upregulated by IFN-γ on extravasation into inflamed tissue [24]. It can then bind, internalize and process any IgG whether free or forming small immune complexes within the inflamed tissue. Large immune complexes can be cross-presented by FcγRIIa (FcγRIV in mice) but only if the inhibitory FcγRIIb is blocked or downregulated [25]. We have shown that immunizing with a DNA vaccine incorporating CTL and helper epitopes within a human IgG1 or mouse IgG2a framework without any additional adjuvants stimulates high-frequency responses to a wide range of epitopes but the functional avidity of these responses was not assessed [26]. In this study, we address the question: can Ab targeting the high affinity FCR engineered to express CTL epitopes stimulate high-avidity CTL responses that are capable of efficient anti-tumor activity?

Results

We have previously shown that Ab–DNA vaccines engineered to express CTL epitopes can stimulate high-frequency responses to self and foreign epitopes but it was unclear if these were of high avidity [26]. Initially a DNA vaccine incorporating the H-2Kb OVA epitope, SIINFEKL, within a human IgG1 molecule was screened for stimulation of high-avidity CTL responses.

CTL epitopes engineered into an antibody molecule elicit high-frequency responses compared to peptide

The SIINFEKL epitope OVA was grafted into CDRH2 region alongside an I-Ab restricted CD4 helper epitope from Hepatitis B (HepB) surface Ag. C57BL/6 mice immunized with this DNA construct demonstrated high-frequency epitope-specific responses compared to a control irrelevant peptide ($p<0.0001$) (Fig. 1B). It was next assessed if encoding an epitope within an Ab–DNA vaccine could break tolerance to a self Ag. An epitope from the melanoma Ag tyrosinase related protein 2 (TRP2) was engineered into a human IgG1 Ab alongside the HepB CD4 epitope. Immunized C57BL/6 mice also demonstrated high-frequency TRP2-specific responses, although these were lower than OVA-specific responses ($p<0.0001$) (Fig. 1C). The ELISPOT assays in this study use total splenocyte populations and it is possible that other IFN-γ producing cells reside within this population. To address this, $CD8^+$ cells were depleted prior to use in the ELISPOT assay. Depletion of the $CD8^+$ cells eliminates the TRP2-specific response but has no effect upon the HepB helper peptide-specific response (Fig. 1D).

To determine if there was any advantage in immunizing with Ab–DNA vaccine as compared to simple peptide immunization, T-cell responses to OVA/HepB or TRP2/HepB human IgG1 DNA vaccines were compared to vaccination with HepB/OVA or TRP2/HepB linked peptides. Mice immunized with peptide show significantly lower frequency responses compared to human IgG1 DNA immunized mice ($p<0.0001$ and $p=0.003$, respectively) (Fig. 1e).

High-avidity responses are induced from human IgG1 DNA vaccine

Functional avidity of CD8 responses has been shown to be important in the induction of anti-tumor immunity. Analysis of the functional avidity revealed that responses induced in human IgG1 DNA immunized mice were over 100-fold higher compared to peptide immunized mice for both OVA and TRP2 epitopes ($p<0.0001$ and $p=0.0009$, respectively) (Fig. 2A and B). OVA human IgG1 DNA shows avidity of 1×10^{-11} M compared to OVA peptide at 1.3×10^{-9} M. TRP2 human IgG1 DNA demonstrates an average avidity of 6×10^{-12} M compared to TRP2 peptide at 1.7×10^{-9} M. Furthermore, lysis of B16F10 tumor cells was considerably enhanced in human TRP2 IgG1 DNA vaccinated mice as compared to TRP2 peptide immunized mice ($p<0.0001$) (Fig. 2C).

The establishment of functional T-cell memory is vital for the success of an immunization protocol. To assess if functional CTL responses could be generated by a single immunization or if a prime boost regime were required, C57BL/6 mice were given single or multiple immunizations with TRP2/HepB human IgG1 DNA. No epitope-specific responses were detectable 20 days after a single immunization with TRP2/HepB human IgG1 DNA, but high-frequency responses were detectable after two immunizations ($p=0.026$) which increased further with another immunization ($p<0.0001$) (Fig. 2D). The avidity of responses after two or three immunizations was analyzed. The responses induced in mice receiving two or three DNA immunizations were of high avidity (1.4×10^{-12} M and 1.8×10^{-12} M, respectively). There is no significant difference in avidity between these two groups ($p=0.89$) (Fig. 2E).

High-avidity does not correlate with high-frequency and is not influenced by xenogeneic human Fc

As both the frequency and avidity of the CTL response appear enhanced, the question "was avidity related to frequency?" arose.

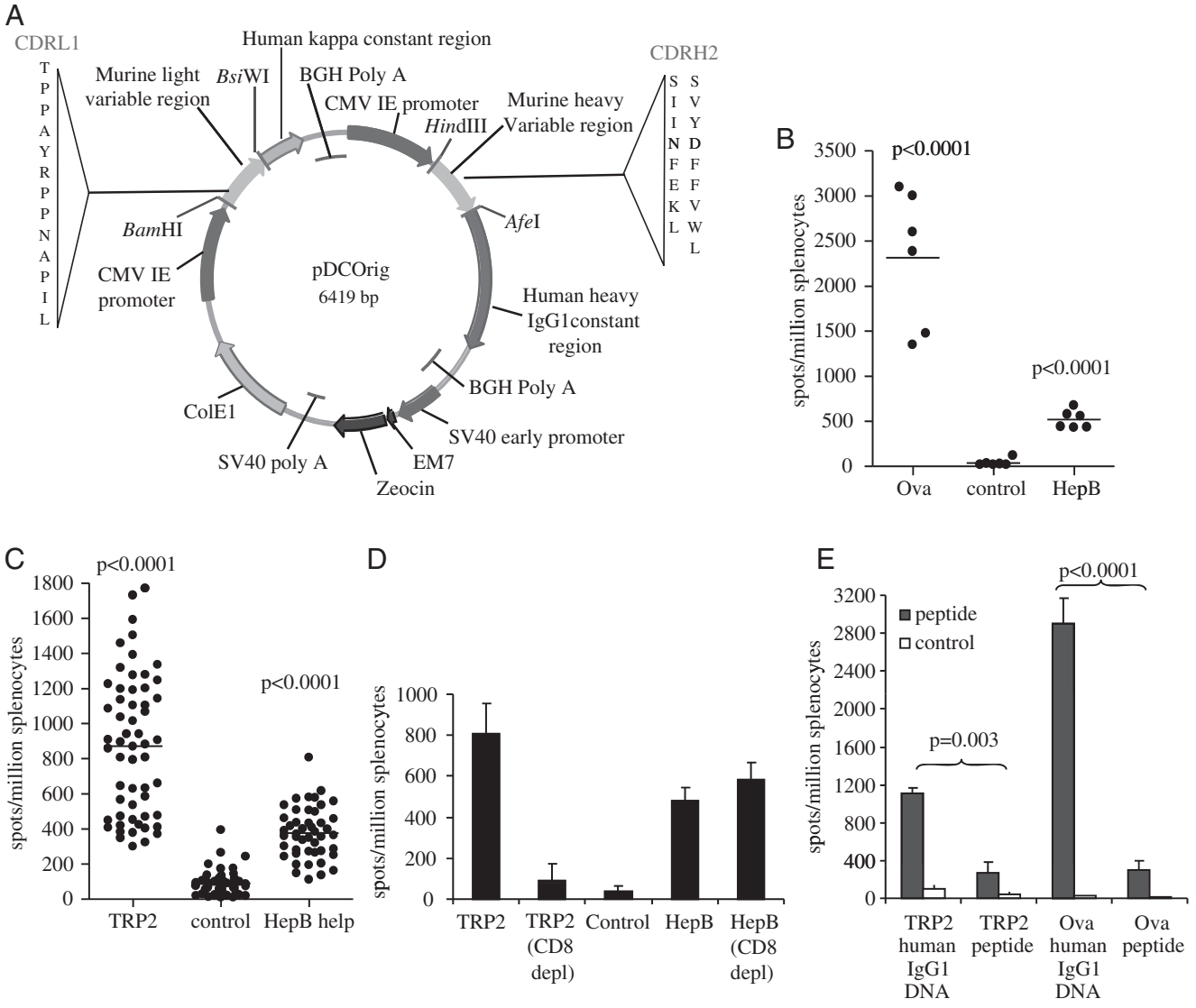

Figure 1. Foreign and self CTL and helper epitopes incorporated into human IgG1 framework are processed and presented to elicit immune responses *in vivo*. (A) Schematic diagram depicting features of the double expression vector pDCOrig. C57BL/6 mice were immunized with human IgG1 constructs. On day 19, splenocytes were analyzed by IFN-γ ELISPOT assay against relevant CTL and helper peptides and an irrelevant peptide control at 0.5 μM concentrations. (B) A construct containing the SIINFEKL epitope in CDR H2 and HepB CD4 epitope in CDR L1 ($n = 6$). (C) A construct containing the TRP2 epitope in CDR H2 and HepB CD4 epitope in CDR L1 ($n = 60$). (D) Splenocytes from TRP2/HepB HuIgG1 DNA immunized mice were depleted of $CD8^+$ T cells prior to analysis ($n = 4$). (E) OVA/HepB HuIgG1 and TRP2/HepB HuIgG1 DNA constructs were compared to OVA/HepB or TRP2/HepB peptide immunizations ($n = 6$). Data show mean spots/million splenocytes+SD and are representative of two independent experiments.

Over 80 mice were immunized with TRP2/HepB human IgG1 DNA and the frequency and avidity of responses measured. The avidity of the TRP2-specific responses ranged from 5×10^{-8} M to 5×10^{-13} M peptide. No significant correlation between avidity and frequency of TRP2 peptide-specific responses was identified, suggesting they are independent events (Fig. 3A).

It is possible that xenogeneic human Fc influences the frequency and avidity of responses induced. Comparison of responses from immunization with human IgG1 or an equivalent murine IgG2a construct reveals similar frequency and avidity (Fig. 3B), suggesting that the xenogeneic human Fc was not influencing the response.

Human IgG1 DNA immunization stimulates similar frequency but higher avidity than peptide-pulsed DC

Synthetic peptides have short half lives *in vivo* and are poor immunogens as they have no ability to specifically target professional Ag presenting cells such as DC. Current therapies are showing DC pulsed with peptide induce an efficient immune response.

TRP2/HepB human IgG1 DNA immunization was compared to DC pulsed with HepB/TRP-2 linked peptide. TRP2/HepB human IgG1 DNA demonstrated similar frequency responses compared to those elicited by peptide-pulsed DC, both of which

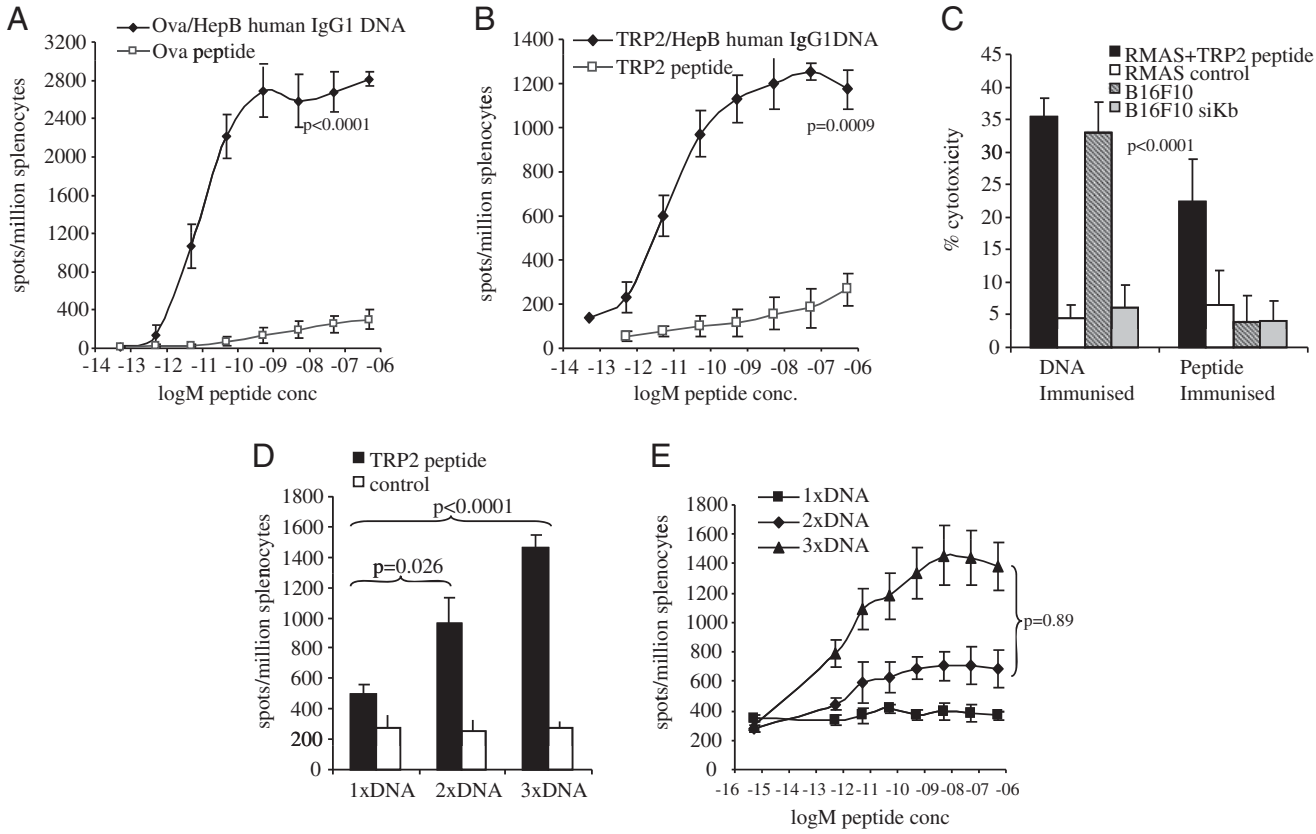

Figure 2. Prime boost immunization regime is essential for the induction of high-avidity epitope-specific responses. (A) Splenocytes from mice immunized with OVA/HepB human IgG1 DNA construct or synthetic peptide were assayed for avidity to the SIINFEKL epitope by measuring responses to increasing peptide concentration in IFN-γ ELISPOT assay. (B) Splenocytes from mice immunized with TRP2/hepB human IgG1 DNA construct or synthetic peptide were assayed for avidity to the TRP2 epitope. (C) Splenocytes from TRP2/HepB human IgG1 DNA or synthetic peptide immunized mice were assessed for the ability to kill peptide labeled RMA-S targets or B16F10 melanoma cells in a cytotoxicity assay at 100:1 effector to target ratio after 6 days *in vitro* TRP2 peptide stimulation with 10 μM peptide. B16F10 cells transfected with a siRNA (siKb) to knock out H-2Kb were used as a negative control. (D) C57BL/6 mice were immunized with TRP-2/HepB human IgG1 DNA at days 0, 0 and 7, or 0, 7 and 14. Splenocytes were analyzed on day 20 by IFN-γ ELISPOT assay in triplicate using 0.5 μM TRP2 peptide. (E) Splenocytes from immunized mice were assayed for avidity to the TRP2 epitope. Data show mean+SD ($n = 6$) and are representative of at least two independent experiments.

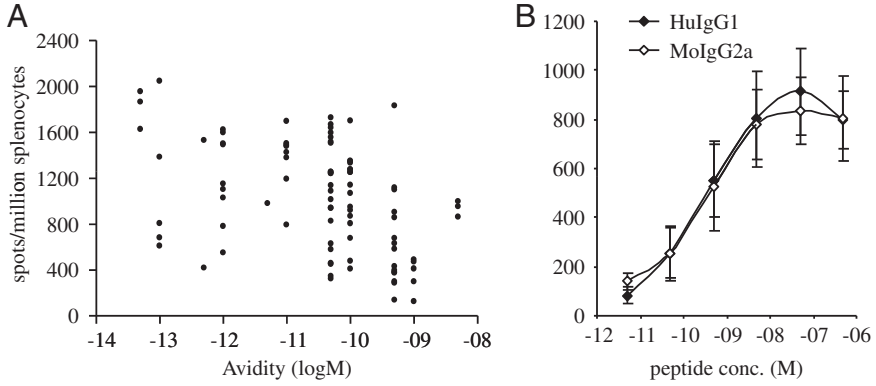

Figure 3. High-avidity responses do not correlate with high frequency and are not a result of influence from xenogeneic human Fc. (A) Correlation of frequency and avidity of responses generated in C57BL/6 mice with the TRP2/HepB human IgG1 construct CDRL1 ($n = 87$). (B) Splenocytes from mice immunized with identical TRP2/HepB human IgG1 or murine IgG2a constructs were assayed for avidity to the TRP2 epitope by measuring responses to increasing peptide concentration in IFN-γ ELISPOT assay. Data show spots/million splenocytes, and avidity is assigned as the concentration that gives 50% maximal effector function. An average avidity is shown. Data are representative of at least two independent experiments.

were superior to peptide immunization ($p = 0.0051$ and $p = 0.0053$) (Fig. 4A). Analysis of the avidity of responses reveals that the avidity in TRP2/HepB human IgG1 DNA immunized mice is 10-fold higher than with peptide-pulsed DC ($p = 0.01$) (Fig. 4B). The TRP2 specific responses were analyzed for ability to kill the B16F10 melanoma cell line *in vitro*. Figure 4C shows that although responses from peptide and peptide-pulsed DC immunized mice demonstrate a good peptide-specific lysis, mice immunized with TRP2/HepB human IgG1 DNA showed better killing of the B16 melanoma cells ($p = 0.003$).

Human IgG1 DNA elicits higher frequency and avidity responses compared to a native antigen DNA vaccine

The enhancement of avidity could be related to direct presentation of the epitopes by the Ab–DNA vaccine and similar responses may be elicited by a DNA vaccine incorporating the native TRP2 Ag. TRP2/HepB human IgG1 DNA immunization was compared to the whole murine TRP2 Ag. Higher frequency and avidity responses were observed to human IgG1 DNA when compared to Ag DNA ($p = 0.0047$) (Fig. 4D).

Human IgG1 DNA immunization protects against tumor challenge and delays tumor growth

High-avidity CTL responses should result in effective anti-tumor responses. The TRP2/HepB human IgG1 DNA vaccine was screened for prevention of lung metastases and inhibition of growth of established subcutaneous lesions.

The B16F10 cells expressing IFN-α (B16F10 IFN-α) have a moderate growth rate of 4 wk, which is more representative of human cancer and were thus chosen for preliminary *in vivo* studies. Forty days post final immunization and forty nine days

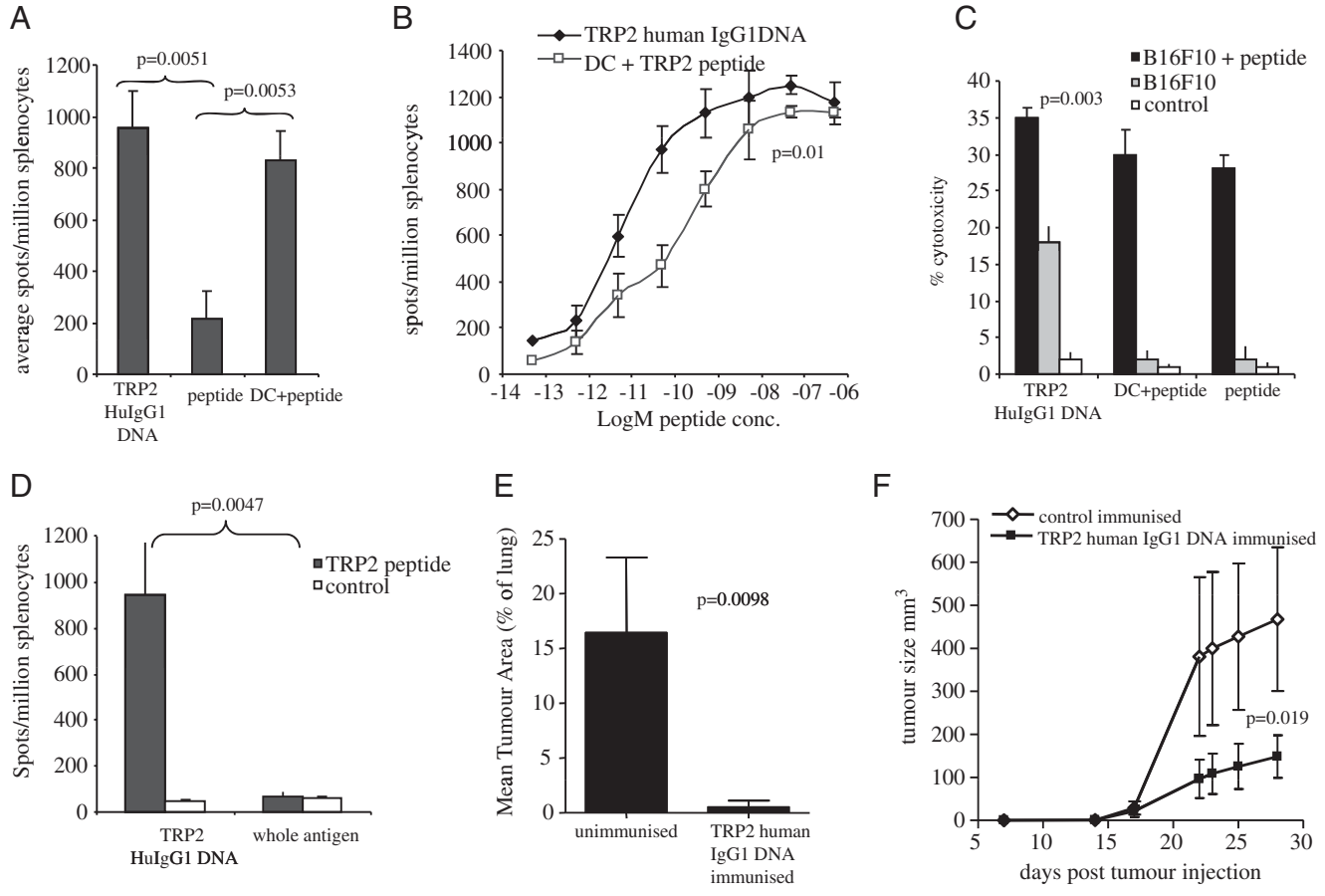

Figure 4. TRP2/HepB human IgG1 DNA immunisation is comparable to immunisation with peptide-pulsed DC. (A) C57BL/6 mice were vaccinated with human IgG1 DNA, peptide or peptide-pulsed DC. Splenocytes were assayed for frequency of TRP2-specific responses in IFN-γ ELISPOT assay against 0.5 μM peptide. (B) TRP2 epitope-specific responses were assessed for avidity. (C) Cytotoxicity of splenocytes assessed by ^{51}Cr-release assay. Data is shown at effector to target ratio of 25:1. (D) TRP2/HepB human IgG1 DNA was compared to immunization with DNA encoding whole antigen TRP2. Data show mean+SD ($n = 6$). (E) C57BL/6 mice were challenged with B16F10 IFN-α cells i.v. post immunization. Tumor burden in the lungs was assessed at 49 days post tumor challenge and expressed as a mean tumor area as a percentage of total lung area ($n = 10$). (F) C57BL/6 mice were injected with B16F10 cells s.c. prior to immunization. Tumor burden was assessed at 3-4 day intervals ($n = 10$). All data are representative of at least two independent experiments.

after tumor cell injection TRP2/HepB human IgG1 DNA immunized mice exhibited peptide and tumor-specific immune responses (data not shown). The tumor area was quantified and expressed as percentage of total lung area. TRP2/HepB human IgG1 DNA immunized mice demonstrated a significant reduction in tumor burden compared to untreated control mice ($p = 0.0098$) (Fig. 4E). When the hair was permitted to grow back after last immunization, mice immunized with TRP2/HepB human IgG1 DNA were observed to have growth of white hair at the site of immunization, which was not apparent in control mice.

TRP2/HepB human IgG1 DNA was evaluated for its ability to prevent the growth of the aggressive parental B16F10 tumor line in a therapeutic model. Figure 4f shows that immunization with TRP2/HepB human IgG1 DNA significantly ($p = 0.019$) delays growth of the aggressive B16F10 melanoma compared to a control human IgG1 DNA vaccine. This suggests that delivering epitope-based DNA vaccines in the context of an inert carrier (*i.e.* Ab) has advantages.

High-avidity responses result from a combination of direct and Fc-mediated epitope cross-presentation

We have previously shown that Ab protein vaccines can target Ag presenting cells through the high affinity FcγR1 receptors. Ab–DNA vaccination was therefore compared to protein vaccination and also to vaccination in Fcγ knockout mice. DNA vaccination gene gun can stimulate naïve T-cell responses by direct transfection of DC allowing direct presentation CTL epitope. Alternatively, transfection of non-professional APC and secretion of protein leading to cross presentation can occur. In contrast, generation of an immune response from protein immunization can only occur by cross presentation. TRP2 human IgG1 DNA vaccine was compared to an identical protein vaccine. TRP2 human IgG1 DNA immunized mice generate superior frequency and avidity epitope-specific responses ($p = 0.0028$) (Fig. 5A). The results indicate that DNA vaccine is superior to protein possibly by allowing both direct and cross-presentation of CTL epitopes. A suggested mechanism for the cross presentation

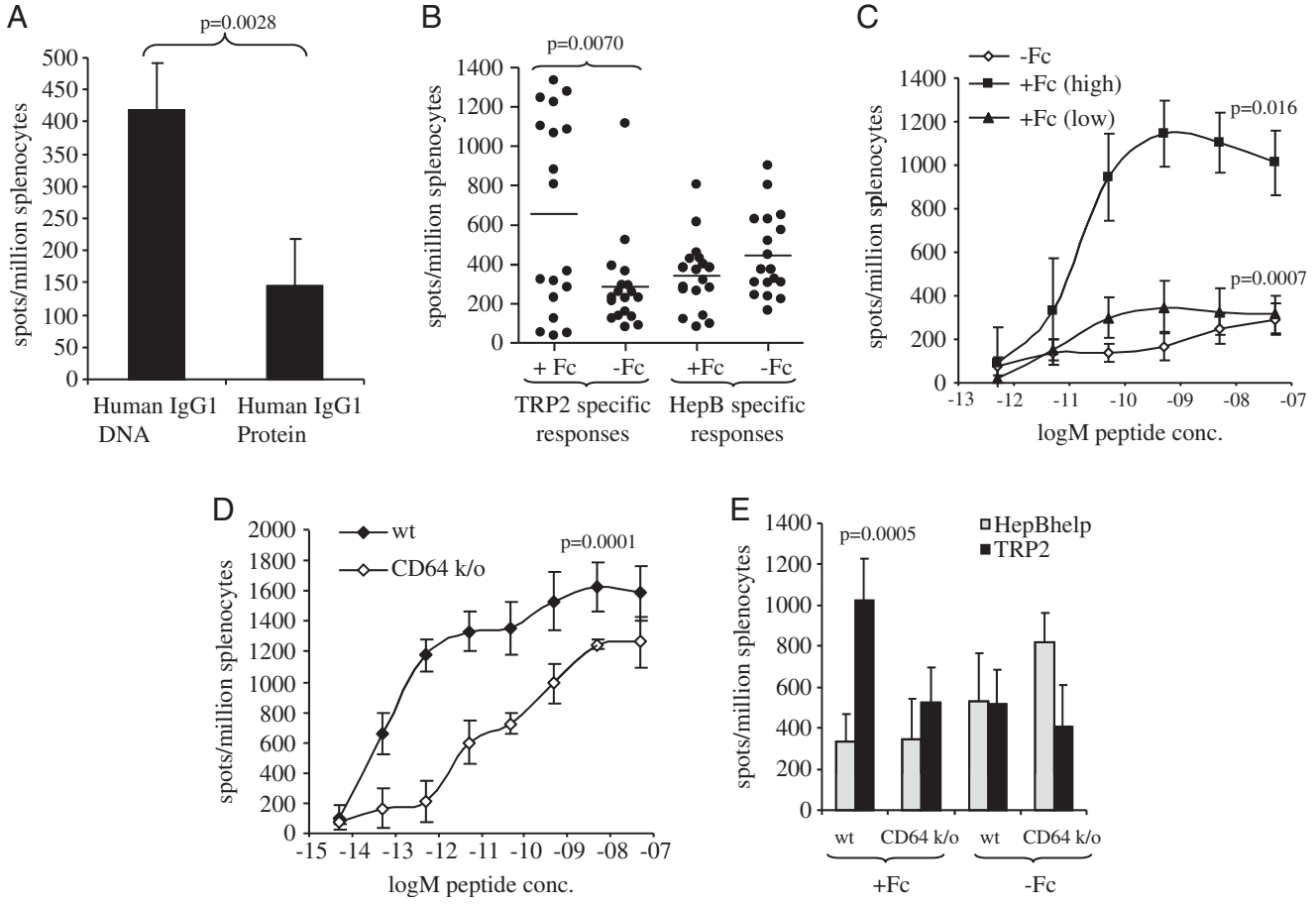

Figure 5. Fc region is important for the induction of CD8 responses. (A) Responses from C57BL/6 mice immunized with a human IgG1 DNA were compared to an identical protein construct in IFN-γ ELISPOT assay. (B) TRP2/HepB human IgG1 DNA was compared to a similar construct lacking the Fc region. (C) Avidity of responses from C57BL/6 mice immunized with constructs containing or lacking the Fc region IFN-γ ELISPOT assay. (D) Avidity of responses from C57BL/6 and γ chain-deficient mice immunized with constructs containing or lacking the Fc region were analyzed. (E) TRP2 and HepB helper peptide-specific responses are shown at low peptide concentration from C57BL/6 or γ chain-deficient mice immunized with human IgG1 constructs containing or lacking Fc region. Data show mean ± SD ($n = 6$ (A, C–E); $n = 18$ (B)) and are representative of at least two independent experiments.

of epitopes from human IgG1 DNA is the binding and uptake of protein by the FcγR1.

To examine if the Fc region was important mice were immunized with TRP2/HepB human IgG1 DNA constructs lacking the Fc region. Mice immunized with the vaccine lacking the Fc region demonstrate a significantly reduced response specific ($p = 0.007$) for the TRP2 epitope but the helper peptide-specific response remains unchanged (Fig. 5B). Immunization with the full length human IgG1 DNA construct appears to show high- and low-frequency responder populations. The high-frequency population have an average avidity of 1.4×10^{-10} M and the low frequency population has an average avidity of 8.1×10^{-11} M (Fig. 5C). Despite the disparity in frequency, the avidity of these two populations is not significantly different ($p = 0.14$). The avidity of the responses from mice immunized with the construct lacking the Fc region demonstrate an average avidity of 3.7×10^{-9} M (Fig. 5C). The avidity of TRP2-specific responses in mice immunized with the full length construct is significantly enhanced for both the high and low frequency responders when compared to the Fab fragment immunized mice ($p = 0.016$ and $p = 0.0007$, respectively). These results suggest that the targeting of the high affinity FcR, FcγR1, plays a role in the generation of efficient immune responses.

This was further confirmed by the immunization of Fcγ$^{-/-}$ mice. WT and Fcγ$^{-/-}$ mice show high frequency. However, analysis of the avidity of these responses reveals that Fcγ$^{-/-}$ mice generate lower avidity (2.1×10^{-11} M) responses than WT mice (1.9×10^{-13} M) ($p = 0.0001$) (Fig. 5D). This is emphasized by comparison of the TRP2-specific responses at low peptide concentration in WT and Fcγ$^{-/-}$ mice which shows a significantly lower response in Fcγ$^{-/-}$ mice ($p = 0.0005$) (Fig. 5E). This response is comparable to that induced by a construct lacking Fc region in WT mice. In contrast, analysis of the helper peptide-specific response shows no significant difference between WT and Fcγ$^{-/-}$ mice when Fc region is present or absent. The role of FcγR1 was further suggested as there was no change in responses in FcγRIIb$^{-/-}$ mice suggesting that this inhibitory receptor plays no role in the cross-presentation of this vaccine (data not shown).

Discussion

Vaccination to date has been relatively unsuccessful for treatment of cancer patients with established disease. It is widely accepted that the generation of high-frequency T-cell responses is not necessarily an indication of a competent immune response. In contrast T-cell functional avidity correlates well with an efficient anti-tumor immune response [1–4]. Is the failure of most vaccinations in cancer patients therefore due to an attenuated T-cell repertoire or an inability of the vaccination to generate high-avidity responses? Several studies have shown that CTL can modulate their functional avidity. Recent studies in TCR transgenic mice have shown that an individual cell can give rise to progeny with different avidities suggesting that avidity modulation at the level of an individual cell may play an important role in the CD8$^+$ T-cell response *in vivo* [27]. We have previously demonstrated that an Ab–DNA vaccine encoding defined T-cell epitopes is an efficient means to generate CD8$^+$ and CD4$^+$ T-cell responses but did not assess avidity [26]. Here we demonstrate that this vaccine induces high-avidity CD8 responses compared to peptide for both the foreign OVA epitope the self TRP2 epitope. Interestingly, the avidity of response to the OVA was similar (1.7×10^{-9} M) to the response to TRP2 (1.3×10^{-9} M) suggesting that there is no deletion of the repertoire to this self Ag. However responses to both epitopes could be increased over 100-fold, by using an Ab–DNA vaccine compared to peptide immunization. These results suggest that at the each peptide MHC complex interacts with a defined number of TCR within the repertoire playing an important role in determining the original avidity [28] but this can then be further modulated at the clonal level.

The range of avidities observed in the mice analyzed spans five logs, yet within individual experiments this variation is less. This probably reflects the plasticity of the avidity to any given TCR:MHC/peptide combination with optimal immunization leading to a high avidity. The avidity with DNA vaccination depends upon the degree of direct v cross presentation, which may vary between experiments. However this does not explain the reduced variability within one experiment. Our explanation is that despite careful operating procedures, this is related to the efficacy of immunization/monitoring of the response. We are aware that timing for harvesting the splenocytes to plating into an assay is a key parameter and endeavor to keep this constant. Finally experiments were performed over a 2-year period and factors such as subtle changes in mice, environment and batches of DNA have to be considered. Within the small groups these factors would be more consistent. The avidity of the responses to the TRP2/HepB human IgG1 DNA vaccine varied from 5×10^{-13} M to 5×10^{-8} M in different mice but was on average 5×10^{-10} M. Is this avidity sufficient to result in effective immune response? An elegant study by Dutoit *et al.* demonstrated that T cells cloned from cancer patients exhibited an exponential increase in killing with T-cell avidity greater than 10^{-9} M [2]. A similar study with T-cell clones showed that only high-avidity clones adoptively transferred caused tumor rejection in mice [1]. The avidity resulting in tumor killing will depend upon the expression level of the Ag/MHC. Our study is in agreement with these demonstrating that selective vaccination can increase avidity to a level sufficient for therapy.

The frequency and avidity of the responses from human IgG1 DNA immunization was significantly higher than that observed from peptide immunization. Initially unlinked peptides were used but due to lack of T-cell help, these gave very weak responses (results not shown). To give a more reasonable comparison, the CTL epitopes were linked to a well known helper epitope which still gave poor responses. This was perhaps not surprising as even linked helper-CTL peptides have a very short half life and are poor immunogens *in vivo* [29]. Peptide stability can be enhanced by pulsing onto DC to provide the APC which has shown promise

in the clinic. TRP2/HepB human IgG1 DNA stimulated similar frequency but higher avidity responses to peptide-pulsed DC. Other studies have failed to show protection from established tumors in TRP2 peptide immunized mice but peptide-pulsed DC induced tumor rejection [30]. If the technology described here can be transferred into a clinical setting, it would allow a vaccine to be manufactured that is superior to DC vaccination. It would also overcome the variability, expense and patient specificity problems associated with conventional DC-based therapies.

Previous studies have shown xenogeneic DNA immunization breaks tolerance to self epitopes but using syngeneic DNA is only successful if Ag is linked to a foreign immunogenic protein [31], if it is encoded within a viral vector [32] or if various adjuvants are used [33, 34]. The generation of therapeutic anti-tumor immunity has also been demonstrated in the absence of regulatory T cells [35]. Enhanced responses of TRP2/HepB human IgG1 DNA immunization compared to syngeneic Ag DNA suggests that epitope removal out of the whole Ag context overcomes the inhibition by any regulatory elements within that whole Ag sequence.

How does immunization with TRP2/HepB human IgG1 DNA enhance avidity? *In vitro* stimulation of splenocytes, from B16 GM-CSF-immunized mice with low doses of TRP-2 180–188 peptide generates high-avidity responses. These results indicate that a repertoire of T cells specific for the TRP2 180–188 epitope exists and that they can be modulated to high functional avidity [27]. It is therefore possible that TRP2/HepB human IgG1 DNA may be working by providing a low dose of Ag to stimulate high-avidity responses. The difference in responses generated from TRP2 human IgG1 DNA compared to the protein equivalent suggests that the direct transfection of skin APC plays a role in the generation of these immune responses. The gene gun was initially believed to stimulate CTL by direct transfection of skin APC but has more recently been shown to also induce CTL *via* cross presentation [36, 37]. We have also shown that the FcγR is important in generating high-avidity but not high-frequency responses from the DNA vaccination. It is of interest that there is often low and high-frequency groups within the immunized mice (see Fig. 3A). This probably reflects the degree of direct *versus* cross presentation. If immunization fails to transfect a significant number of APC they will have a lower response than mice with efficient APC transfection. This is a parameter which is hard to control with either gene gun or electroporation and is not enhanced with the use of cytokines such as GM-CSF or adjuvants such as imiquimod (result not shown). Reports in the literature have previously demonstrated that vaccine induced T-cell responses can be enhanced by Ab [38–40]. A recent elegant study by Saenger *et al.* demonstrates that anti-tumor immunity is dramatically enhanced by combination of DNA vaccination and treatment with an anti-TRP-1 Ab [40]. They propose that the immune enhancement observed is explained by the cross-presentation of tumor Ag by the Ab and subsequent activation of FcR. Our data would suggest that the human IgG1 DNA vaccine exploits both pathways of direct presentation and cross-presentation through FcγR1 to induce high-frequency and high-avidity $CD8^+$ T-cell responses, a phenomenon that is not possible with a similar protein vaccine. The CD4 T-cell responses appears to be unaffected by the absence of the Fc region. Recently the literature describes a variety of intracellular autophagic routes by which Ag can gain access to MHC class II [41]. It is possible that the CD4 epitope is processed *via* one of these routes upon direct transfection of APC. We also observe no difference in the CD4 responses generated when secretion is of HuIgG1 construct is prevented (data not shown). Further studies into the precise mechanism of Ag presentation will be necessary to clarify this.

In conclusion, a DNA vaccine incorporating CTL epitopes within an Ab molecule results in high-frequency and high-avidity T-cell responses that result in effective tumor immunity. The vaccine appears to work by presenting low doses of CTL epitopes within an inert carrier for both direct and Fc-mediated cross-presentation. Further studies will determine if the avidity to other viral and self Ag can also be enhanced by this method of immunization.

Materials and methods

Cell lines and media

B16F10 and RMAS mouse cell lines were obtained from the ATCC and were maintained in RPMI (Cambrex, Wokingham, UK) with 10% FBS (Sigma, Poole, UK). To knockdown expression of H-2Kb in the cell line B16F10, RNA interference was utilized. The complimentary oligonucleotides siKB forward and reverse targeting H-2Kb (Table 1) were annealed cloned into the vector psiRNA-h7SKGFPzeo (Invivogen, Calne, UK). The stable cell line B16F10 siKb was generated by transfection using genejuice (Novagen, Nottingham, UK) and selection in the presence of 200 μg/mL of zeocin. B16F10 cells were transfected with the plasmid pORF-IFN-α (Invivogen, Calne, UK) and selected by growth in the presence of 500 μg/mL of G418. To confirm the expression of IFN-α and psiKb-h7SKGFPzeo, the levels of MHC class I on the cell surface was analyzed by flow cytometry.

Media used for splenocyte culture was RPMI-1640 with 10% FBS (Sigma), 2 mM glutamine, 20 mM HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin and 10^{-5} M 2-mercaptoethanol.

Plasmids

CDRs within ImmunoBody™ single heavy and light chain vectors had been replaced with unique restriction sites enabling rapid insertion of epitope sequences [26].

In brief, to generate the human IgG1 TRP2 and OVA constructs, oligos encoding the TRP2 epitope SVYDFFVWL [42] and OVA epitope SIINFEKL [43] were incorporated into CDRH2 or in direct replacement of CDRH3 (Table 1). Into the same plasmids the I-Ab restricted helper CD4 epitope from the HepB nucleoprotein TPPAYRPPNAPIL [44] was inserted in replacement of CDRL1 of the kappa chain.

Table 1. Primers used in this study

Primers	Sequence
TRP2/H2 FORWARD	5′-TAGTGTTTATGATTTTTTTGTGTGGCTCCGATTCA-3′
TRP2/H2 REVERSE	5′-TGAATCGGAGCCACACAAAAAAATCATAAACACTA-3′
TRP2/H3 FORWARD	5′-GAAGTGTTTATGATTTTTTTGTGTGGCTCTG-3′
TRP2/H3 REVERSE	5′-CAGAGCCACACAAAAAAATCATAAACACTTC-3′
OVALBUMIN/H2 FORWARD	5′-TAGTATAATCAACTTTGAAAACCGCGATTCA-3′
OVALBUMIN/H2 REVERSE	5′-TGAATCGCAGTTTTTCAAAGTTGATTATACTA-3′
HEPB HELP/L1 FORWARD	5′-CTCTTGCACTCCTCCAGCTTATAGACCACCAAATGCCCCTATCCTATGGT-3′
HEPB HELP/L1 REVERSE	5′-ACCATAGGATAGGGGCATTTGGTGGTCTATAAGCTGGAGGAGTGCAAGAG-3′
CH1 STOP FORWARD	5′-CCAAGGTGGACAAGAAAGTTTGACCCAAATCTTGTGACAAAACTC-3′
CH1 STOP REVERSE	5′-GAGTTTTGTCACAAGATTTGGGTCAAACTTTCTTGTCCACCTTGG-3′
MURINE TRP2 FORWARD	5′-TTTCTAAGCTTATGGGCCTTGTGGGATGGGGGCTTC-3′
MURINE TRP2 REVERSE	5′-TTTCTGATATCTCAGGCTTCCTCCGTGTATCTCTTGC-3′
SIKB FORWARD	5′- ACCTCGCCCACATTCGCTGAGGTATTTCAAGAGAATACCTCAGAGAATGTGGGCTT-3′
SIKB REVERSE	5′-CAAAAAGCCCACATTCGCTGAGGTATTCTCTTGAAATACCTCAGAGAATGTGGGCG-3′

Generation of the human IgG1 construct lacking the Fc region

A stop codon was incorporated after the CH1 domain of the human IgG1 constant region within the human IgG1 construct containing TRP2 epitope in CDRH2 and the HepB helper epitope in CDRL1 using the Quik change site directed mutagenesis kit (Stratagene, USA) and the complementary oligonucleotides origstophuHeCH1 forward and OrigstophuHeCH1 reverse primers (Table 1) as instructed by the manufacturer.

Full length murine TRP2 plasmids

To construct pOrig murine TRP2, *c*DNA synthesized from total RNA isolated from the cell line B16F10 was used as a template for the amplification of full length murine TRP2 using the primers murine TRP2 forward and reverse (Table 1) with incorporation of a HindIII or EcoRV site, respectively. Full length TRP2 was ligated into the HindIII/EcoRV multiple cloning sites of the ImmunoBody^{TM} single heavy chain vector pOrigHIB.

Generation of murine IgG2a constructs

The human IgG1 and kappa constant regions within the double expression vector were replaced with murine IgG2a isotype and kappa equivalent, cloned in frame with the murine heavy and light variable region containing the TRP2 epitope in CDRH2 and the HepB helper epitope in CDRL1, as previously described [26].

Protein manufacture

CHO (Chinese hamster ovary cells, ECACC, UK) were transfected with DNA encoding human IgG1 Ab containing TRP2 epitope in CDRH3 using lipofectamine (Invitrogen, UK). Following 24 h incubation at 37°C, in 5% CO_2, cells were plated into media containing Zeocin at 300 μg/mL (Invivogen, USA). Resistant clones were screened for Ig secretion by capture ELISA and expanded. Human IgG1 protein was purified from supernatant using HiTrap protein G HP column (GE Healthcare).

Generation of murine DC

Bone marrow cells were flushed from limbs of C57BL/6 mice, washed and resuspended in RPMI 1640, 10% FBS, 2 mM glutamine, 20 mM HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin and 10^{-5} M 2-β mercapto-ethanol. Cells were plated into 6-well Costar dishes at 2×10^6 mL^{-1} (2 mL/well) in media supplemented with 20 ng/mL recombinant murine GM-CSF (Peprotech EC) and incubated at 37°C/5% CO_2. Half the media was replaced at day 4 with fresh media+GM-CSF and cells used for immunization on day 8.

Mice and immunizations

Animal work was carried out under a Home Office approved project license. Female C57BL/6 (Charles River, Kent, UK) or Fcγ chain-deficient (Taconic, USA) mice were used between 6 and 12 wk of age. Synthetic peptides (Department of Biomedical Sciences, Nottingham University, UK) TPPAYRPPNAPILAAAS-VYDFFVWL (HepB/TRP-2), TPPAYRPPNAPIL (HepB) and SIIN-FEKL (OVA) were emulsified with incomplete Freund's adjuvant. Human IgG1 protein was emulsified with CFA for the prime and incomplete Freund's adjuvant for subsequent boosts. Peptide or protein (50 μg/immunization) was injected *via* s.c. route at the base of the tail. DNA was coated onto 1.0-μm gold particles (BioRad, Hemel Hempstead, UK) using the manufacture's

instructions and administered intradermally by the Helios Gene Gun (BioRad). Each mouse received 1 μg DNA/immunization into the shaved abdomen. DC were labeled with 50 μg/mL of peptide for 90 min at 37°C, then washed and irradiated at 3000 rads prior to immunization s.c. at the base of the tail (5×10^5 DC/immunization). Mice were immunized at days 0, 7 and 14 and spleens removed at day 19 for analysis unless stated otherwise.

Re-stimulation *in vitro*

Five days following the final immunization, splenocytes (5×10^6 mL^{-1}) were co-cultured at 37°C with syngeneic, irradiated (3000 rads), peptide-pulsed LPS blasts (0.5 to 1×10^6 cells/mL). LPS blasts were obtained by activating splenocytes (1.5×10^6 cells/mL) with 25 μg/mL LPS (Sigma) and 7 μg/mL dextran sulfate (Pharmacia, Milton Keynes, UK) for 48 h at 37°C. Before use 2×10^7 LPS blasts were labeled with 10 μg/mL synthetic peptide for 1 h. Cultures were assayed for cytotoxic activity on day 6 in a ^{51}Cr-release assay.

^{51}Cr-release assay

Target cells were labeled for 90 min with 1.85MBq sodium (^{51}Cr) chromate (Amersham, Essex, UK) with or without 10 μg/mL peptide. Post incubation, they were washed three times in RPMI. 5×10^3 targets/well in 96-well V-bottomed plates were set up and co-incubated with different densities of effector cells in a final volume of 200 μL. After 4 h at 37°C, 50 μL of supernatants were removed from each well and transferred to a Lumaplate (Perkin Elmer, Wiesbaden, Germany). Plates were read on a Topcount Microplate Scintillation Counter (Packard). Percentage specific lysis was calculated using the following formula: specific lysis = 100 × [(experimental releasespontaneous release)/(maximum releasespontaneous release)].

Ex vivo ELISPOT assay

ELISPOT assays were performed using murine IFN-γ capture and detection reagents according to the manufacturer's instructions (Mabtech AB, Nacka Strand, Sweden). In brief, anti-IFN-γ Ab were coated onto wells of 96-well Immobilin-P plate and triplicate wells were seeded with 5×10^5 splenocytes. Synthetic peptides SIINFEKL (OVA), SVYDFFVWL (TRP2) and TPPAYRPPNAPIL (HepB) (at a variety of concentrations) were added to these wells and incubated for 40 h at 37°C. Following incubation, captured IFN-γ was detected by a biotinylated anti-IFN-γ Ab and development with a streptavidin alkaline phosphatase and chromogenic substrate. Spots were analyzed and counted using an automated plate reader (CTL Europe GmbH, Aalen, Germany). Functional avidity was calculated as the concentration mediating 50% maximal effector function using a graph of effector function *versus* peptide concentration. CD8$^+$ T cells were depleted using CD8 dynabeads (Invitrogen, UK) according to manufacturer's instructions.

Tumor studies

For the prophylactic lung metastases model, C57BL/6 mice were randomized into treatment groups and immunized at weekly intervals for 5 wk. Between the third and fourth immunization they were challenged by i.v. injection into the tail vein with 1×10^4 B16F10 IFN-α melanoma cells. At day 49 post tumor challenge, mice were euthanized and lungs analyzed for the presence of metastases. For the therapeutic subcutaneous model, 2.5×10^4 B16F10 melanoma cells were injected at day 0 followed by three immunizations at days 4, 11 and 18. Tumor growth was monitored at 3–4 day intervals and size of the tumor was measured using a calliper.

Statistical analysis

Comparative analysis of the ELISPOT results was performed by applying the Student's *t*-test with values of *p* calculated accordingly. Comparison of avidity curves was performed by applying the F test using the Graphpad Prism software.

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Conflict of interest: The authors wish to disclose that Lindy G. Durrant is a director of Scancell Ltd and V. A. P, R. L. M and B. G. are employees of Scancell Ltd.

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Abbreviations: **FcR:** Fc receptor · **HepB:** Hepatitis B · **TRP2:** Tyrosinase related protein 2

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