Scancell Investor Update

Tuesday 1 October, 2013

FTI Consulting
26 Southampton Buildings, London, WC2A 1PB

SCANCELL
USING IMMUNOLOGY TO FIGHT CANCER

Investor Update
Agenda

Location	FTI Consulting Holborn Gate 26 Southampton Buildings London, WC2A 1PB
10.45	Registration with refreshments
11.00	Introduction *David Evans, Scancell Non-Executive Chairman*
11.05	Cancer immunotherapy *Prof Peter Stern, Institute of Cancer Sciences, University of Manchester*
11.20	Scancell corporate update *Dr Richard Goodfellow, Joint CEO of Scancell*
11.30	SCIB1 trial update and introduction to Moditope™ *Prof Lindy Durrant, Joint CEO of Scancell*
11.45	Q&A
12.00	Interactive panel discussion *Led by Prof Karol Sikora, Dean of Medicine , University of Buckingham; Honorary Consultant Oncologist at Hammersmith Hospital* Panellists include: *Prof Lindy Durrant,* *Prof Peter Stern,* *Dr Steve Chan, Consultant Oncologist, City Hospital, Nottingham University* *and Prof Christian Ottensmeier, Dept. of Experimental Medicine, University of Southampton*
12.45	Buffet lunch
13.30	Close

1 October 2013

Scancell Holdings Plc

('Scancell')

Investor update showcases Moditope® platform and updates SCIB1 trial progress

Scancell Holdings Plc, (AIM:SCLP), the developer of novel immunotherapies for the treatment of cancer, will today hold an investor update. Following an introduction by Prof. Peter Stern, Institute of Cancer Sciences, University of Manchester, Dr Richard Goodfellow and Prof Lindy Durrant, Scancell's joint CEOs will present a business update as well an overview of the ImmunoBody® platform and SCIB1 clinical programme, including its current status. A detailed introduction to the new Moditope® platform will also be given for the first time.

Prof Durrant will review the SCIB1 clinical trial programme and confirm that the ongoing studies remain on track. Further results from this trial are expected by the end of 2013. SCIB1 is Scancell's first cancer immunotherapy and is a product of the Company's ImmunoBody® platform. It is being developed for the treatment of malignant melanoma and is currently in Phase 1/2 clinical trials. Encouraging results from Part 1 of the study have previously been presented and provide the first clinical evidence that Scancell's ImmunoBody® immunotherapy approach is producing an immune response in cancer patients which may also be associated with clinical benefit. Prof Durrant will today add that four out of the six evaluable patients treated with either the 2mg or 4mg dose of SCIB1 still remain alive. The mean survival time in this group of five Stage IV and one Stage IIIb patients is currently 21 months from trial entry.

In view of the positive results and minimal side effects seen with the 4mg dose (Part 1) of the SCIB1 Phase 1/2 trial, the Company has initiated evaluation of an 8mg dose in up to six patients with measurable tumours. Five patients have been recruited to date: one patient will no longer be evaluable due to delivery of an incomplete dose of SCIB1 and a further patient was not able to complete dosing within the required timeframe. A safety review of the data from the 8 mg cohort in Part 1 will be conducted and, if adequate safety is demonstrated, Scancell plans to recruit a further 10 patients with measurable disease into Part 2 of the study. An amendment has been submitted to the appropriate regulatory authorities to request approval to treat these additional patients at the 8 mg dose.

Prof Lindy Durrant, the inventor of the Moditope® platform, will present a detailed overview of the technology and its potential. She will describe how the technology was used to generate the lead product, SCMod1. Planning is underway for the preclinical and clinical development of SCMod1 as an immunotherapeutic, provisionally for the treatment of triple-negative breast cancer, ovarian and endometrial cancers. First-in-man clinical studies are scheduled to start in 2016. Moditope® harnesses CD4+ T cells to eradicate tumours and represents a new class of immunotherapeutic agents. The platform deploys citrullinated tumour-associated peptide epitopes to overcome self-tolerance and destroy tumour cells, with no requirement for blockade inhibitors (for example CTLA4 antibodies and PD-1 inhibitors). It can potentially be expanded to develop multiple immunotherapeutic agents for different cancers. A broad patent has been filed to protect this platform and covers the use of multiple tumour-associated modified epitopes for the treatment of cancer.

It will be recalled that the ImmunoBody® platform induces a high avidity CD8+ T cell response to tumour associated antigens. As the Moditope® platform stimulates a potent CD4+ T cell response to modified self-antigens both platforms are complementary relying on a response by different classes of T cell for their therapeutic effect. Thus, in principle, a combination of ImmunoBody® and Moditope® derived therapeutics may be a powerful approach to the treatment of both early and late stage cancers.

In the second part of the seminar, specialist guest speakers, led by Professor Karol Sikora, Dean of Medicine at University of Buckingham, Medical Director of CancerPartnersUK and honorary Consultant Oncologist at Hammersmith Hospital, will form an interactive panel to discuss the increasing importance of immunotherapy for the treatment of cancer and how Scancell's technology fits into this emerging landscape.

Dr. Richard Goodfellow, joint CEO of Scancell, commented: "We welcome this opportunity to update investors following the recent successful fundraising completed in August. The SCIB1 clinical programme remains on track and further data is expected by the end of 2013. Having now filed and exemplified the patents covering our Moditope® technology, we can provide more detail on how this second platform technology has the potential to generate a completely new class of potent and selective immunotherapy agents and which could have a profound effect on the way that cancer immunotherapies are developed. In particular, the technology may overcome the immune suppression induced by tumours themselves without the need for checkpoint blockade inhibitors, thereby allowing activated T cells seek out and kill tumour cells that would otherwise be hidden from the immune system.

"In view of the short to medium term licensing and partnership potential that both the Moditope® and Immunobody® programmes now bring to the Company, our strategy requires a more flexible approach. Whilst we are still fully focused on securing a sale of the business at the earliest opportunity, we will now consider technology validating and revenue generating deals on each individual platform when and where appropriate in order to enhance the value of the Company when it is sold."

-ENDS-

For Further Information:

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About Scancell

Scancell is developing novel immunotherapies for the treatment of cancer based on its ImmunoBody® and Moditope® technology platforms. Scancell's first ImmunoBody®, SCIB1 is being developed for the treatment of melanoma and is in Phase 1/2 clinical trials. Preliminary evidence from Part 1 of the study showing that SCIB1 produced an immune response which might be associated with clinical benefit in patients with malignant melanoma was released in December 2012.

Scancell's ImmunoBody® immunotherapies target dendritic cells and stimulate both parts of the cellular immune system; the helper cell system where inflammation is stimulated at the tumour site; and the cytotoxic T-lymphocyte or CTL response where immune system cells are primed to recognise and kill specific cells.

Scancell has also identified and patented a series of modified epitopes that stimulate the production of killer CD4+ T cells that destroy tumours without toxicity. The Directors believe that the Moditope® platform could play a major role in the development of safe and effective cancer immunotherapies in the future.

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Investor Update

Guest Speaker Biographies

Professor Peter Stern

Professor Peter Stern is at the Institute of Cancer Sciences, University of Manchester, UK.

He obtained his PhD at University College London, UK and previously held research positions as: a staff scientist at the Medical Research Council Molecular Biology Laboratory, Cambridge, UK; a European Molecular Biology Organization (EMBO) Fellow at the University of Uppsala, Sweden; a Cancer Research Campaign Fellow and Junior Research Fellow at Linacre College, University of Oxford, UK; a Lecturer at the Medical School, University of Liverpool, UK; a visiting Professor at the Free University of Amsterdam, The Netherlands and a Cancer Research UK Group Leader at the Paterson Institute for Cancer Research, Manchester, UK .

Professor Stern's work has involved the translation of knowledge of human papillomavirus driven carcinogenesis or the expression and function of oncofoetal molecules (e.g. 5T4 oncotrophoblast antigen) into new cancer immunotherapies using vaccine, antibody targeted drug or immune modulator based approaches. He has published extensively in this area.

Karol Sikora MA, MB.BChir, PhD, MD, FRCR, FRCP, FFPM

Karol Sikora is Medical Director of Cancer Partners UK a group he founded to create the largest independent UK cancer network. He was Professor and Chairman of the Department of Cancer Medicine at Imperial College School of Medicine and is still honorary Consultant Oncologist at Hammersmith Hospital, London. He is Dean and Professor of Medicine at Britain's first independent Medical School at the University of Buckingham and Fellow of Corpus Christi College, Cambridge.

He studied medical science and biochemistry at Cambridge, where he obtained a double first. After clinical training he became a house physician at The Middlesex Hospital and registrar in oncology at St Bartholomew's Hospital. He then became a research student at the MRC Laboratory for Molecular Biology in Cambridge working with Nobel Prize winner, Dr. Sydney Brenner. He obtained his PhD and became a clinical fellow at Stanford University, California before returning to direct the Ludwig Institute in Cambridge. He has been Clinical Director for Cancer Services at Hammersmith for 12 years and established a major cancer research laboratory there funded by the Imperial Cancer Research Fund. He chaired Help Hammer Cancer, an appeal that raised £8m towards the construction of the new Cancer Centre at Hammersmith. He became Deputy Director (Clinical Research) of the ICRF. From 1997 to 1999 he was Chief of the WHO Cancer Programme and from 1999 to 2002, Vice President, Global Clinical Research (Oncology) at Pharmacia Corporation.

He has published over 300 papers and written or edited 20 books including *Treatment of Cancer* - the standard British postgraduate textbook now going to its sixth edition and most recently *The Economics of Cancer Care*. He is on the editorial board of several journals and is the founding editor of *Gene Therapy* and *Cancer Strategy*. He was a member of the UK Health Department's Expert Advisory Group on Cancer (the Calman-Hine Committee), the Committee on Safety of Medicines and remains an adviser to the WHO.

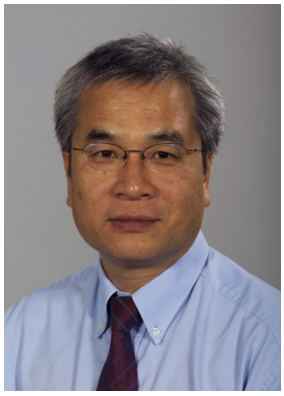
Prof Christian Ottensmeier

Christian Ottensmeier is Professor in Experimental Cancer Medicine. He graduated and began his training in Münster, Germany. After a 3 year training fellowship in the Dana Farber Cancer Institute in Boston, Massachusetts, he moved to Southampton where he completed his oncology training and undertook his PhD. He has been a consultant in medical oncology since 2000.

Christian leads the Experimental Cancer Medicine Centre in Southampton and his core academic interest is the early translation of immunotherapeutic strategies into the clinic. His clinical interests are thoracic malignancies and melanoma, and he has co-developed a number of national NCRI studies in lung cancer. He manages a broad and active portfolio of clinical trials in both lung cancer and melanoma.

The overarching aim of his laboratory group is the preclinical development and early phase clinical testing of strategies to induce anti-tumour immune responses in patients. His work focuses on three linked but distinct areas of investigation: Detailed immunological evaluation of the effect of immunological intervention in patients, assay development and validation, and mechanistic studies in murine models as well as human modelling of immune responses to vaccination for further preclinical cancer vaccine development.

He has served on a number of industry advisory boards and DSMBs for industry led studies. He is a member of a number of national peer reviewed funding committees.



Stephen Chan

Dr Chan qualified in Nottingham University medical school. He started his training in Oncology at Oxford and was a Research Fellow at the Ludwig Institute at Cambridge MRC and Imperial College London. He returned to Nottingham after working at the Christie Hospital Manchester.

He took up his current post in 1989. Between 1995-2000 he chaired the Mid Trent postgraduate educational board. He is a member of national and international research groups including ASCO, EORTC (Gynae group) and the British Breast Group and was made a Visiting Professor at Nottingham Trent University in 2011.

He is the author of over 80 peer reviewed scientific papers and principal author of studies in the development of new treatments including Docetaxol and targeting therapy. His current research interests include predictor markers as well as specific antigens in Breast and Ovarian cancers.

Dr Chan is a member of numerous national and international steering committees for clinical trials.

He is currently the Director of Clinical Trials in Breast and Gynaecological research at Nottingham University Hospitals where he works closely with his Radiology, Pathology and Surgical colleagues.

Immunotherapy

Based on premise of Immunosurveillence of cancer

- Not believed or properly considered for decades but now the evidence is overwhelming for immune control in cancer
  - Animal models showing importance of cellular immunity in controlling experimental & spontaneous tumours
  - Demonstration of immunity to tumour associated antigens in patients – identification of targets and effectors
  - Positive association of the degree & type of immune infiltration into a tumour with improved survival of patients in several types of cancer & independent of tumour molecular features.
  - A comprehensive assessment of host immune response, disease staging, node count, and tumour molecular variables is necessary to evaluate the clinical utility of host immune response evaluation*

*Cancer immunology—analysis of host and tumor factors for personalized medicine Shuji Ogino, Jérôme Galon, Charles S. Fuchs & Glenn Dranoff Nat. Rev. Clin. Oncol. 8, 711–719 (2011); published online 9 August 2011; doi:10.1038/nrclinonc.2011.122

MANCHESTER 1824
The University of Manchester
PLS 2013

The components of immunity

- Innate immunity
  - First line of defence, is non- specific provides the critical means for activating adaptive immunity
  - Control required to needs to discern “danger”
- Adaptive immunity
  - Specific (antibodies & T cells), expands to deal with problem & retains memory
  - Depends on randomly generated repertoire which in development needs to eliminate or provide for control of any potential autoimmunity
- Immune control mechanisms
  - Checkpoint control mechanisms (CTLA-4; PD-1 etc)
  - Natural Tregs
  - Tumour induced Tregs
  - Inflammation promotion of cancer

Immunotherapy

- Natural history of carcinogenesis de facto involves the immune response in the multistep process
- Each tumour has its own unique set of genomic & epigenomic changes which can influence the host immune response to tumour positively or negatively
  - Drivers of cancer (oncogenes) & associated genomic instability
  - Provide targets for adaptive immunity
  - Provide for immuno-suppressive or -evasive tumour evolution
- Human tumour cells evolve in the face of an immune response which is itself altered by the experience

What are the tumour targets of the adaptive immune response ?

- Viral Antigens (HPV, EBV etc)
- Mutated oncogenes: ras, p53 etc
- Autologous CTL defined
  - Cancer-Testes family, MAGE etc
  - Differentiation antigens, Tyrosinase etc
  - Mutated genes in cancer, CDK4, β-catenin, caspase 8, MUM-1
- Serex defined human tumour Ags
  - Includes tumour suppressor, mutated, translocated, amplified, retroviral Ags

Anything for which there is the potential
(repertoire) for a useful anti-tumour respose

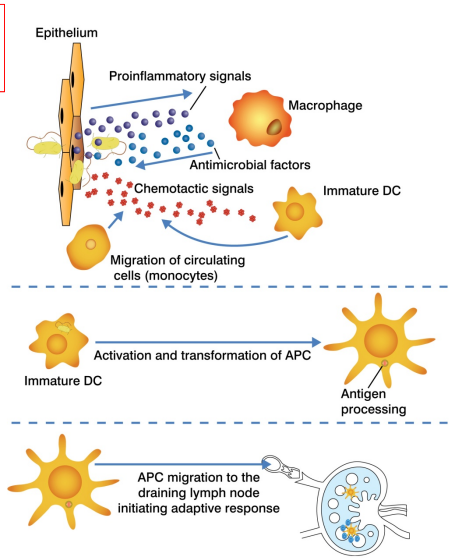
Surveillance and detection of "danger"

Danger signals generated by pathogens, tissue damage or stress & detected by specific receptors including TLRs

Innate Immunity is the first line of defence

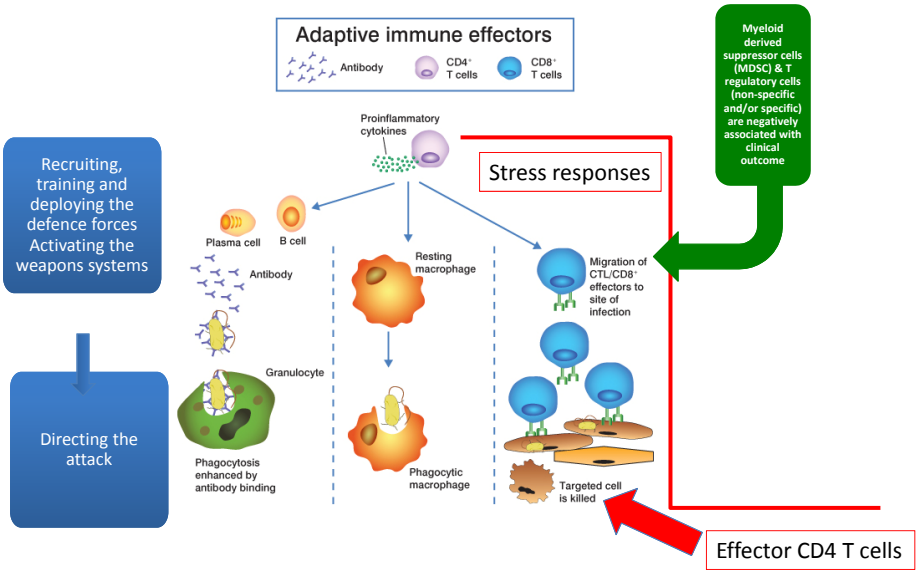
Communicating information about the threat

Calling up/mobilising the troops of Adaptive Immunity



Garcon N, Stern P, Cunningham T and Stanberry L. (2011) Understanding modern vaccines. Elsevier http://www.sciencedirect.com/science/journal/22107622.

Adaptive immune responses



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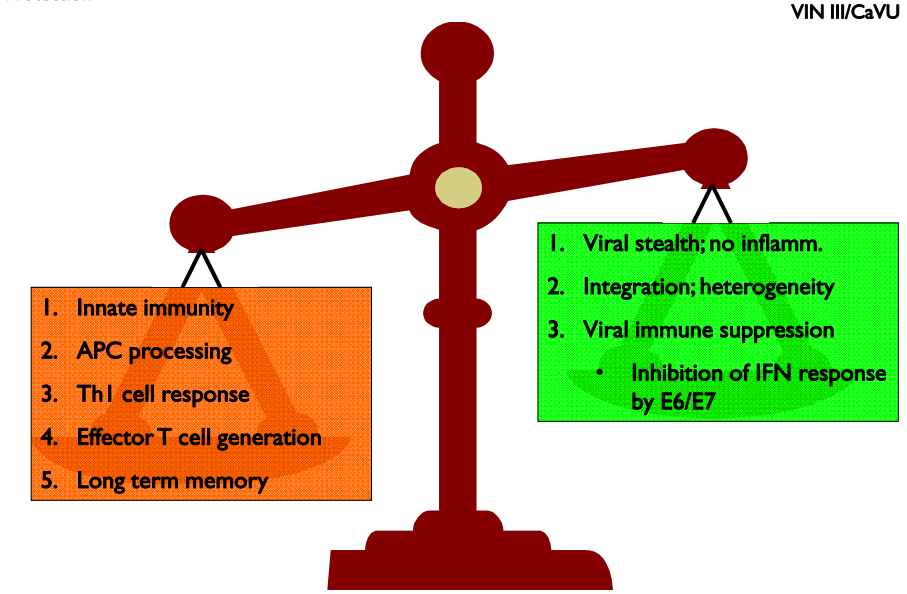

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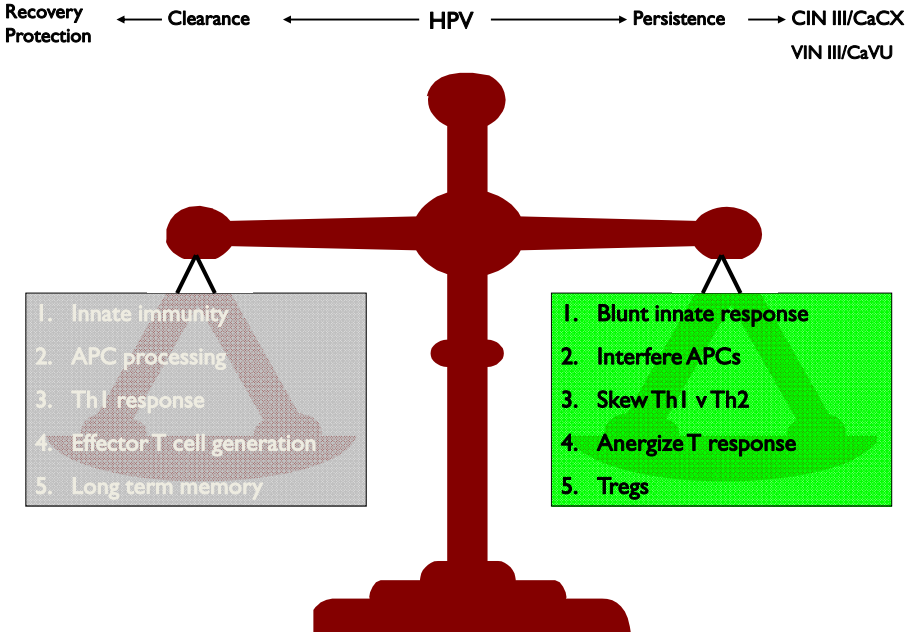

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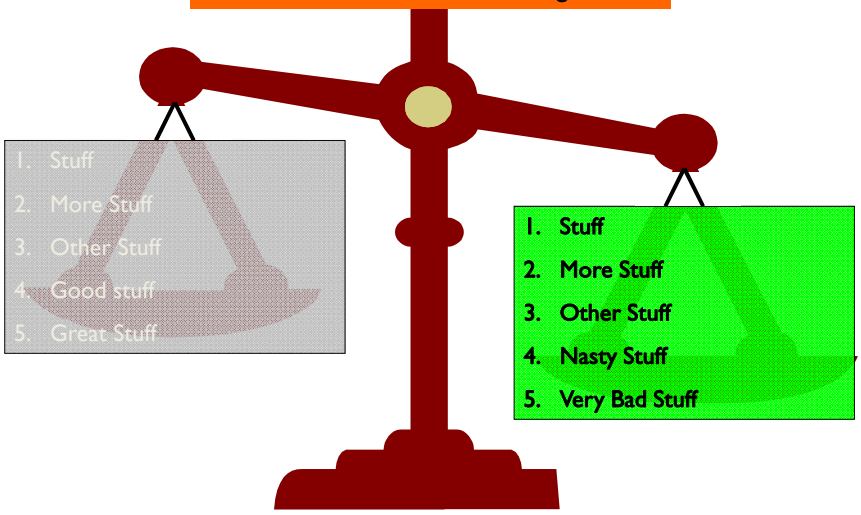
Recovery Protection ← Clearance ← HPV → Persistence → CIN III/CaCX
VIN III/CaVU

There is not enough of good things
There is to much of bad things



Meeting the challenges for Immunotherapy

- How can we change the balance in favour of useful tumour immunity
  - Stimulate the good things
  - Inhibit or remove the bad things
- Heterogeneity of individual tumours
  - Emerging tumours may be only a subset of tumour potential ; evolution avoiding immune attack
- Immunotherapy aims to recover immune control & eliminate all the tumour cells
  - Challenge is to activate the "residual" immune repertoire of the patient with cancer & reset the balance between useful anti-tumour immunity (natural or exogenously produced)& immune control or suppression
- Cancer Vaccine immunotherapy requires:-
  - Suitable target antigen(s)
  - Generation of immunogenic, appropriate and effective immunity
- New ScanCell Technology "Moditope"
  - A generic set of TAA
  - *Adjuvanted vaccination based on knowledge of " danger signals"
  - Immunobodies

* Garcon N, Stern P, Cunningham T and Stanberry L. (2011) Understanding modern vaccines. Elsevier http://www.sciencedirect.com/science/journal/22107622.

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AIM-listed clinical stage biotech company



Investor day October 1st 2013

Clinical Stage Cancer Immunotherapy

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THE POTENTIAL OF CANCER IMMUNOTHERAPY

- Oncology market worth $68 billion in 2012 (9.1% of all pharma sales)*
- By 2018 four of the top five cancer products are expected to be immunotherapies *
- Immunotherapy market expected to generate sales of up to $35 billion per year within the next 10 years**
- FDA committed to fast track approvals via new “breakthrough” drug designation
- Traditional 10 year time lines could be dramatically cut redefining value of oncology immunotherapy assets

**Evaluate Pharma*
***Andrew Baum, Citigroup Analyst*

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CURRENT LIMITATIONS OF CANCER IMMUNOTHERAPY

- Poorly immunogenic or generate the “wrong” kind of immune response
- Emerging treatments not yet administered as first-line therapy and used when immune system is weakened by chemo, surgery and radiation
- Autologous immunotherapy (eg Provenge) present manufacturing challenges and are expensive
- Toxicity can be significant

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OVERCOMING THE LIMITATIONS

ImmunoBody®

- Avidity and frequency of the immune response superior to peptides, peptide pulsed DC (eg Dendreon), DNA vaccines without electroporation and protein vaccines
- Clinical data from Part 1 of Phase 1/2 melanoma study confirms immune response and potential clinical benefit
- Safe to use, with potential for use in early stage patients and over long periods
- First validating deal with DNA vaccine/electroporation technology done in Sept 2013 (Inovio/Roche, Headline value $412.5m)

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OVERCOMING THE LIMITATIONS

Moditope®

- New powerful class of immunotherapeutic agents
- Early data indicates potent anti-tumour activity, even against advanced cancer
- Mode of action elucidated. No “checkpoint Inhibitors” required
- Broad utility. Multiple products against multiple targets
- Minimal toxicity. Good safety profile anticipated
- Simple and cheap to manufacture

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BUSINESS STRATEGY (1)

ImmunoBody®

- **Validate** ImmunoBody® platform by generating immune response and clinical data on SCIB1 in patients with metastatic melanoma (Phase 1/2 trial) and pre-clinical data on SCIB2 (for lung and other epithelial cancers)
- Phase 1/2 study **results** scheduled to be announced before the end of 2013
- Gather additional data on 8mg dose of SCIB1 in M1b patients (Stage 4 with lung mets) to **confirm anti-tumour activity in patients with tumour load**
- Continue to monitor patients treated with SCIB1 **long term** in terms of disease progression and survival
- Actively seek co-development partners for SCIB1 and **licensing partners** for SCIB2 and the ImmunoBody® platform

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BUSINESS STRATEGY (2)

Moditope®

- **Prepare SCMod1 for clinical trials** (manufacture, pre-clinical testing, submit CTA) . Target indications provisionally TNBC, ovarian and endometrial cancer
- **Identify new Moditope® targets and products** for further development
- Actively **seek licensing partners** for development of products derived from Moditope® platform

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BUSINESS STRATEGY (3)

Funding and Trade Sale

- **Placing and Open Offer** of up to £6.5m announced on July 9th 2013 to fund completion of SCIB1 clinical trials and commence development of first Moditope® product. Funds expected to be sufficient for planned operations until at least end 2015
- **Seek trade sale** at an optimal point at which significant shareholder value has been achieved

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WHY PARTNER NOW?

- **Cancer immunotherapy market has become very hot** recently, opening up new opportunities for partnerships
- **SCIB1 close to reaching a value inflection point** validating ImmunoBody® platform (ie Phase 2 results)
- First DNA vaccine plus electroporation deal concluded between **Roche and Inovio** in September
- Already **substantial interest in Moditope®** and the potential that it offers despite its early stage of development
- **Change in business strategy** allows time for technology validating and value enhancing deals to be concluded prior to trade sale

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A YEAR OF OUTSTANDING PROGRESS

- Preliminary results from Phase 1/2 trial of SCIB1 in melanoma show immune response associated with clinical benefit
- Follow up ImmunoBody® for lung and other cancers (SCIB2) produces outstanding results in animal studies. SCIB2 is ready for clinical development
- Announcement of Moditope® technology platform. New powerful class of immunotherapeutic agents that destroy tumours without toxicity
- Steady appreciation in value as clinical data builds
- Placing and Open Offer to raise £6.5m to fund completion of SCIB1 Phase 1/2 programme and start development of SCMod1 oversubscribed

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SHORT AND MEDIUM TERM NEWSFLOW

- **Full results from Phase 1/2 trial** of SCIB1 in melanoma by year end 2013
- Progress on **8mg study in Stage 4 (M1b) patients with lung metastases** during 2014
- Updates on patients receiving **long term treatment** with SCIB1
- Continued progress on development of **SCMod1 and Moditope®** technology platform.
- Building and strengthening **IP position**
- **Partnership agreements** on Scancell's ImmunoBody® and Moditope® platforms and products

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IMMUNOBODY AND MODITOPE

INVESTOR DAY

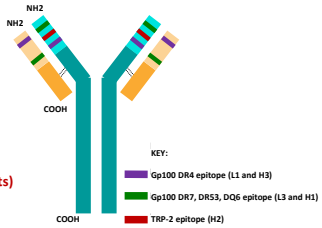
TUESDAY 1 OCTOBER 2013

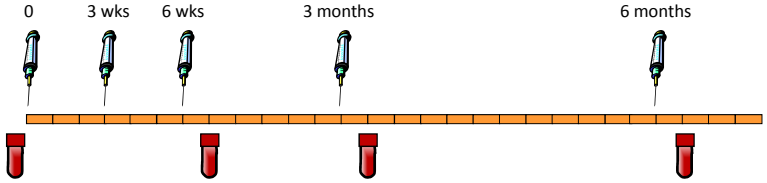
PHASE 1/2 TRIAL IN STAGE IV/III MELANOMA

- **Primary Objective:**
  - To demonstrate safety and tolerability
- **Secondary Objectives:**
  - To demonstrate cellular immune response (high avidity T-cells)
  - To assess tumour response

PART 1
- 9 patients
- 3 subjects per cohort (0.4, 2.0 or 4.0 mg)
- Progression only if adequate safety demonstrated at previous dose
- 2 mg patients allowed to progress to 4mg
- **New 8 mg cohort**

PART 2
- 14 patients (4 mg)
- 6 on continuation therapy
- **New cohort planned (10 patients)**





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CLINICAL RESPONSE – PART 1 SUMMARY

Patient Number	CLINICOPATHOLOGY							
	Dose	No of doses	Stage	Survival from trial entry	Survival from diagnosis of mets	Other treatments post SCIB1	Alive	Disease
01-01	0.4 mg	4	IV	7	34	no	-	
02-01	0.4 mg	4	IV	16	67	Ipilimumab (non-responder)	-	
02-04	0.4 mg	3	IV	7	20	no	-	
01-16	2/4 mg	3 + 2	IIIb	14	19	no	-	
01-19	2/4 mg	4 + 1	IV	30	68	Verumafenib (non-responder)	alive	PD
01-24	2/4 mg	3 + 2	IV	27	51	no	alive	ND
04-03	4 mg	5	IV	24	29	no	alive	PD
05-05	4 mg	5	IV	13	16	no	-	
04-16	4 mg	5	IV	19	64	no	alive	DR
	Mean months survival (cohorts 2 and 3):			21	42			

ND = no disease progression; PD = disease progression; DR = differential response

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PATIENT OF SPECIAL INTEREST: 04-16

- 49 yo female, stage IV
- Diagnosis 2003 - skin lesions excised 2003 and 2009
- Pulmonary mets since 2010
- At screening, mets in node, subcutaneous tissue and lung (several)
- SCIB1 dosed 5 x 4 mg
- Immune response seen from D42
- 3 target lesions and several pulmonary mets decreased in size, reaching PR in target lesions by Week 28 but one new subcutaneous lesion grew from Week 18 (present as speck on screening scan) = differential response
- New lesion excised; patient received further dosing
- Further lesions

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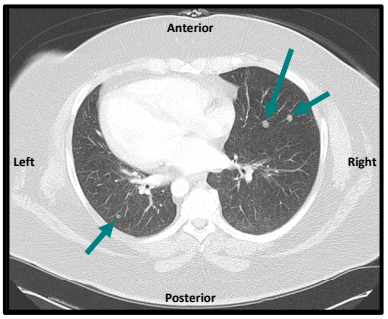
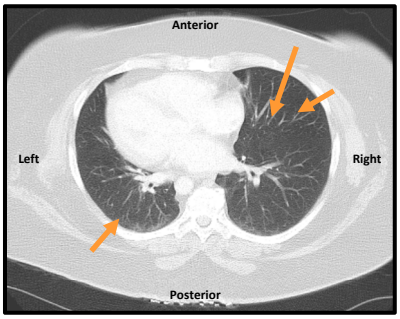
PATIENT 04-16 NON-TARGET LUNG LESIONS

17 Jan 2012



3 non-target lesions
Slice image 30

31 Oct 2012



No lesions visible
Slice image 33

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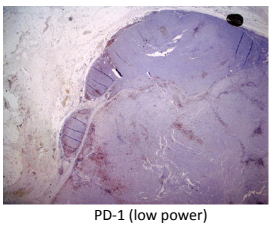
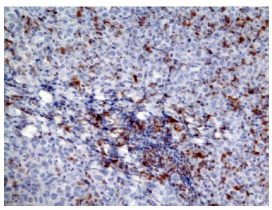
PATIENT 04-16 LESION: EXPRESSION OF PD-1 AND PDL-1

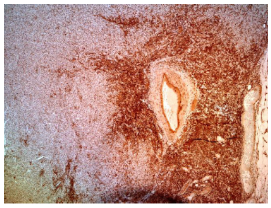
- PD-1 and its ligand PDL-1 highly expressed (immunosuppressive environment)
- Potential to combine SCIB1 with anti-PD-1 monoclonal antibody



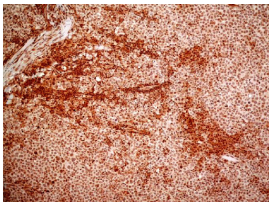
PD-1 (low power)



PD-1 (high power)



PDL-1 (low power)



PDL-1 (high power)

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SCIB1-001 STUDY – CURRENT STATUS

Part 2 (4 mg):

- 13/14 patients completed main study dosing
- One patient unable to tolerate more than one electroporation per visit due to discomfort so only received three x 2 mg doses
- Patients all stage III/IV; post-resection with no disease present at screening
- Continued dosing allowed if requested – 6 patients currently continuing, one more planning to start continuation
- Follow-up on-going for time-to-progression and survival (up to 5 years)

Part 1 cohort 4 (8 mg):

- Patients with stage IV disease present (excluding M1c)
- 5 (of 6 planned) patients on study (one only received 2 mg doses)

Part 2 (8 mg) – planned expansion:

- Patients with stage IV disease present (excluding M1c)
- Approval submitted for another 10 patients

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THE TUMOUR CELL ENVIRONMENT

- Tumour cells promote an anti-inflammatory environment (PD-1, PDL-1)
- Although anti-tumour CD8+ T cell responses have been reported, very few CD4+ T cell responses have been observed due to the phenomenon of self-tolerance against CD4+ T cell epitopes
- In general, tumours do not express MHC class II antigens and do not present tumour-associated antigens to CD4+ T cells

THE ABILITY TO INDUCE CD4+ T CELLS AGAINST TUMOUR-ASSOCIATED EPITOPES WOULD ADD A NEW DIMENSION TO CANCER IMMUNOTHERAPY

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CD4+ T CELLS – KEY PLAYERS IN THE IMMUNE RESPONSE

- Provide vital help for CD8+ T cells
- Locate to the tumour site and release IFNγ which induces a plethora of immune responses resulting in tumour eradication
- Directly inhibit tumour growth
- Promote tumour cell apoptosis
- Induce cascade of of events at tumour site including activation of dendritic cells, CD8+ T cells, natural killer cells, macrophages
- IFNγ facilitates a pro-inflammatory environment by inhibiting regulatory T cells (Tregs) and promoting expression of other cytokines to attract T cells to the tumour site
- IFNγ also up-regulates MHC class I and class II antigens on tumour cells that aid their recognition by cytolytic CD8+ and CD4+ T cells

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MODITOPE® - ADDRESSING THE LIMITATIONS

- Moditope® is a new and proprietary platform for activating the immune system against tumour cells
- Moditope® exploits the normal immune response to stressed cells, which is largely mediated by cytotoxic CD4+ T cells
- When normal cells are stressed or dying, they start to digest their own internal proteins (autophagy)
- Activated enzymes modify the digested protein fragments within autophagosomes and convert certain arginine amino acids to citrulline
- These **MODIf**ied epi**TOPES** are targets for CD4+ T cells in autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS)

CAN THESE T CELLS BE HARNESSED TO KILL TUMOUR CELLS?

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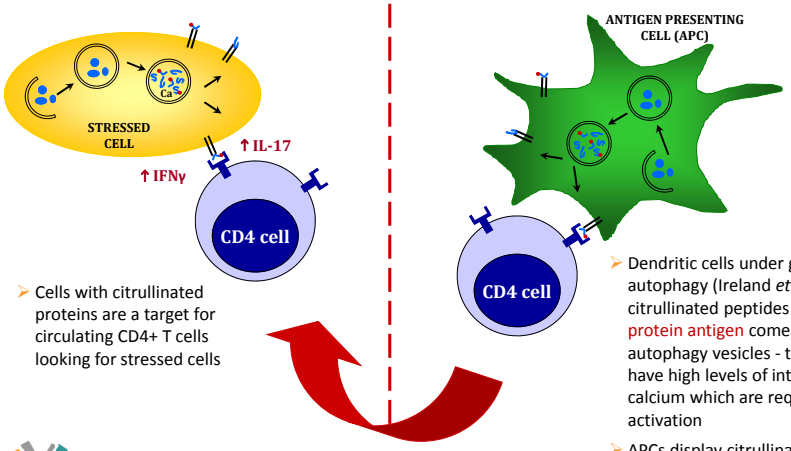
CITRULLINATION TRIGGERS IMMUNE RESPONSE

- Stressed cells induce citrullination of proteins within autophagosomes
- Expressed at cell surface by MHC Class II antigens



- Cells with citrullinated proteins are a target for circulating CD4+ T cells looking for stressed cells
- Dendritic cells under go constitutive autophagy (Ireland *et al*) and present citrullinated peptides after the protein antigen comes in contact with autophagy vesicles - these vesicles have high levels of intracellular calcium which are required for PAD activation
- APCs display citrullinated peptides
- Activates killer CD4+ T cells

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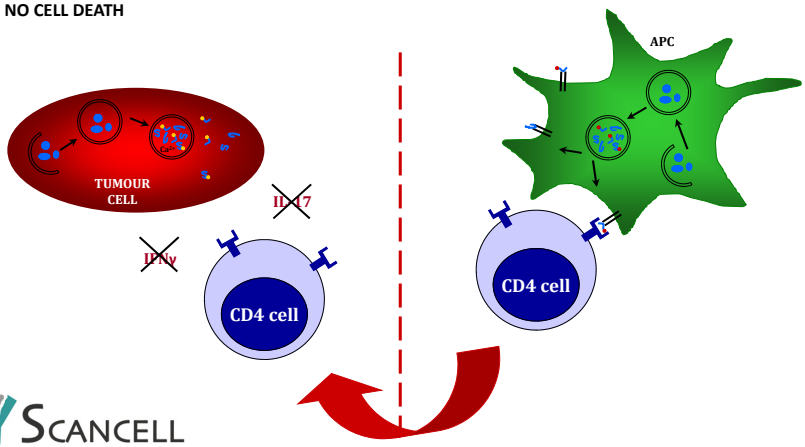
CITRULLINATED TUMOUR CELLS EVADE IMMUNE SURVEILLANCE

- Fast growing tumour cells often have insufficient nutrients and oxygen → stress
- Triggers citrullination of intracellular proteins in autophagosomes
- **BUT most tumours do not express MHC Class II**
- **Tumour cells do induce regulatory T cells (iTregs) to suppress immune responses**
- Therefore citrullinated peptides are not recognised by killer CD4+ T cells
- **NO CELL DEATH**



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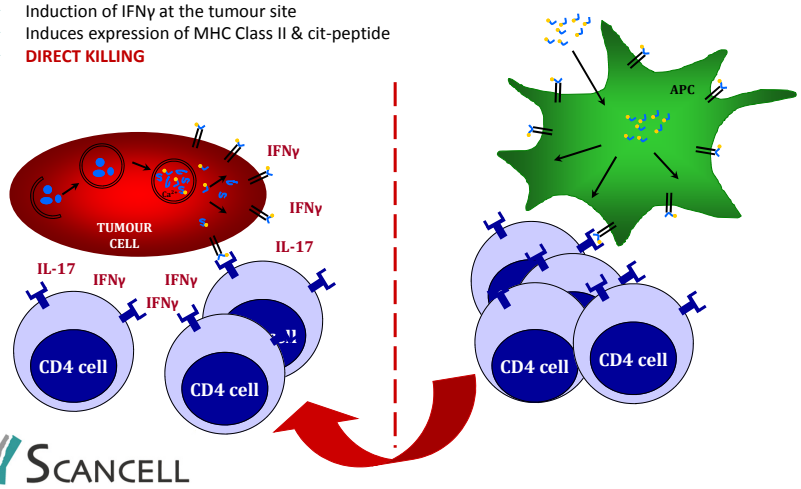
THE MODITOPE CONCEPT

- Moditopes taken up by Antigen Presenting Cells (APC)
- Presented on APC cell surface
- Activation and expansion of CD4+ killer T cells
- Induction of IFNγ at the tumour site
- Induces expression of MHC Class II & cit-peptide
- **DIRECT KILLING**



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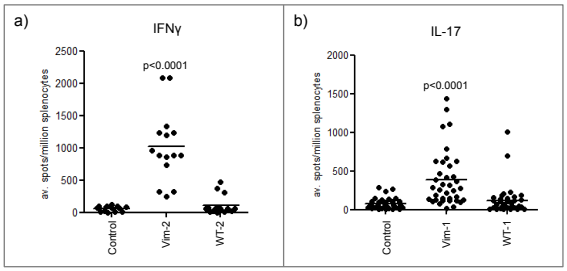
SCMOD1 INDUCES T CELL RESPONSES

- Single immunisation with Vim-1 or Vim-2
- Frequency of IFNγ (a) or IL-17 (b) secreting cells measured in response to WT or citrullinated peptides
- Responses are specific for citrullinated peptides



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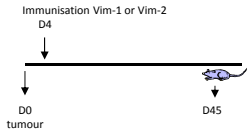
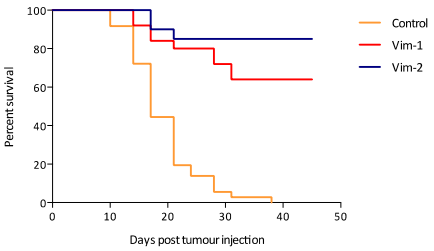
MODITOPE® INDUCES POTENT ANTI-TUMOUR RESPONSES

- B16-DR4 tumour established in DR4 transgenic mice (Day 0)
- Vim-1 or Vim-2 administered on Day 4
- All control animals dead 38 days after tumour challenge
- Up to 85% of animals treated with citrullinated vimentin peptides survived





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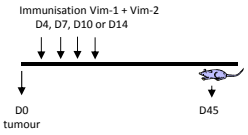
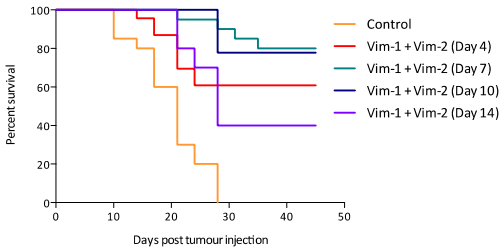
MODITOPE® INDUCES RESPONSES AGAINST ESTABLISHED TUMOURS

- B16-DR4 tumour established in DR4 transgenic mice (Day 0)
- Vim-1 + Vim-2 administered on Day 4, Day 7, Day 10 or Day 14
- All control animals dead 28 days after tumour challenge
- More animals receiving SCMod1 after tumours had been established for 7 or 10 days were still alive 45 days after tumour challenge than animals that were treated 4 days after challenge
- 40% of animals receiving SCMod1 14 days after tumour challenge still survived





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MODITOPE® - A BROAD PLATFORM

- Many proteins can be citrullinated
  - Adenylcyclase associated protein, α-enolase, aggregan, albumin, aldolase, anti-thrombin, asporin, β-actin, B23, BiP, calreticulin, capping protein α-1 subunit, cartilage intermediate layer protein, cathepsin D, co-activator complex, collagen, elongation factor 1α, F-actin, far upstream element-binding proteins 1 and 2, fibrinogen, filaggrin, glucose regulated protein, heparin binding protein, histamine receptor, histone, HSP60, HSP90, mitochondrial aldehyde dehydrogenase, nucleophosmin, phosphoglycerate kinase 1, protein disulphide-isomerase ER60 precursor, vimentin
- Any of these could be targets for incorporation into Moditope® immunotherapies
- Other post-translational modifications also occur
  - Nitration of tyrosine
  - Oxidation of tryptophan
  - Demination of glutamine or asparagine
- Many such modified amino acids have been found within MHC-bound peptides, indicating they may also be targets for immunotherapy

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SELECTION OF SCANCELL'S LEAD PRODUCT

- Cytoskeletal (structural) proteins preferentially digested in stressed cells
  - **Vimentin**
  - Fibrinogen
  - Collagen
- **Vimentin** is the major cytoskeletal protein found in mesenchymal cells
- All mesenchymal tumours (endometrial, renal, lung, sarcomas, lymphomas) express **vimentin**
- Many epithelial tumours (e.g., thyroid, nasopharangeal, melanoma) switch from cytokeratin to **vimentin** expression during metastasis (EMT transition) or on stem cells
- Most solid tumours (breast, ovarian, gastrointestinal, prostate) also switch to **vimentin** expression during metastasis (>50% of triple-negative breast cancer tumours express vimentin)

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SCMOD1

- Incorporation of citrullinated vimentin peptides that can be recognised by CD4+ T cells into a Moditope® immunotherapeutic has the potential to treat many different forms of cancer
- **SCMod1** is Scancell's lead Moditope® product
- SCMod1 comprises two citrullinated vimentin peptides
  - Vim-1
  - Vim-2
- Patients with RA will not be treated with SCMod1

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FUTURE POSSIBLE INDICATIONS

- Potential for treating any tumour expressing modified peptides
- From antibody studies, vimentin expressed in all of the following tumour types:

  - Breast
  - Prostate
  - Ovarian
  - Cervical
  - Endometrial
  - Head and neck
  - Thyroid
  - Glioma
  - Lung
  - Melanoma
  - Skin
  - Testis
  - Urothelial
  - Renal
  - Stomach
  - Pancreatic
  - Liver

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PROVISIONAL PHASE 1/2 PLAN

- Open label
- Tumour types
  - Triple negative breast cancer
  - Ovarian cancer
  - Endometrial cancer
- 20 patients with each tumor type (60 total)
- 2 mg (per peptide) per administration
- Given at weeks 0, 6, 12, and 24
- Administered by injection

- Primary endpoint: Safety
- Secondary endpoints
  - Clinical responses
  - Immunogenicity

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KEY BENEFITS OF MODITOPE® PLATFORM

- Proprietary immunotherapy platform that harnesses CD4+ T cells to eradicate tumours
- Mode of action elucidated: the Moditope® platform deploys certain tumour-associated peptide epitopes as immunotherapeutic agents to overcome self-tolerance and eradicate tumour cells, with no requirement for blockade inhibitors
- Broad application: potential to expand the Moditope® platform broadly to develop multiple immunotherapeutic agents for different cancers, with the prospect of developing multiple cancer immunotherapeutic drugs
- Moditope® platform exemplified by lead product SCMod-1, which exhibits potent anti-tumour efficacy in in vivo therapeutic models; efficacy observed even against aggressive, established tumours, improving survival rates

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POTENTIAL SYNERGY BETWEEN IMMUNOBODY® AND MODITOPE®

ImmunoBody®	Moditope®
High avidity CD8+ T cell responses	Potent CD4+ T cell responses
DNA product	Peptide or DNA product
Eradicates small primary and metastatic tumours	Eradicates large bulky tumours
Checkpoint inhibitors may enhance responses	No requirement for checkpoint inhibitors
Potent killer cells induced	Reverses immunosuppressive tumour environment
Targets tumour-associated antigens	Targets modified self-antigens

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Q&A

Followed by

Interactive panel discussion led by Karol Sikora

Panellists include:

Prof Lindy Durrant
Prof Peter Stern
Dr Steve Chan
Prof Christian Ottensmeier

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PLoS one

High Avidity Cytotoxic T Lymphocytes Can Be Selected into the Memory Pool but They Are Exquisitely Sensitive to Functional Impairment

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Abstract

High avidity cytotoxic T lymphocytes (CTL) are important in viral clearance and anti-tumor immunity, however, mechanisms for their optimal generation and maintenance *in vivo* remain unclear. Immunizing mice with an antibody-DNA vaccine encoding a single CTL epitope, induces a 100 fold higher avidity response than peptide vaccination with the identical epitope. The high avidity response is retained into memory and can be efficiently reactivated with an antibody-DNA boost. In contrast, reactivation of high avidity CTL with peptide, stimulated responses with a significant drop in avidity, suggesting loss or conversion of the high avidity CTL to lower avidity. Similarly, high avidity T cells maintained *ex vivo* were exquisitely sensitive to signaling with low doses of peptide (1 ng/ml) giving optimal TCR stimulation and resulting in retained avidity, proliferation and ability to kill specific targets. In contrast, high avidity T cells maintained *ex vivo* with supraoptimal TCR stimulation (10 μg/ml peptide) resulted in reduced avidity and failure to kill tumor cells. They also failed to proliferate, showed a significant increase in apoptosis and expressed high levels of the exhaustion marker programmed death-1 (PD-1) and low levels of the lymphocyte-activation gene 3 (LAG-3). This suggests high avidity T cells are recruited to the memory pool but can be lost by supraoptimal stimulation *in vitro* and *in vivo*. This is characterized by loss of function and an increase in cell death. The remaining CTL, exhibit low functional avidity that is reflected in reduced anti-tumor activity. This could contribute to failure of the immune system to control the growth of tumors and has implications for vaccination strategies and adoptive transfer of T cells.

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Competing Interests: The authors wish to declare that LG Durrant is also a director of Scancell Holdings plc and that V.A.B, R.L.M and B.G are employees of Scancell Holdings plc. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

It is widely accepted that the generation of high frequency T cell responses is not necessarily an indication of the induction of an effective immune response. It is apparent from previous published work that T cell functional avidity is a better indicator of clinical response [1,2,3,4,5]. The term functional avidity is often confused with affinity. Affinity is most often classified as a measure of the strength of binding of the peptide MHC molecule to the T cell receptor (TCR) whereas functional avidity is a measure of the combination of stimulation via TCR, co stimulatory molecules, adhesion molecules and cytokines and is indicative of the overall strength of interaction between T cell and target [6]. In both viral infection and tumor models, only high avidity cytotoxic T lymphocytes (CTL) mediate viral clearance and tumor eradication [1,3,7,8,9]. During the generation of an immune response *in vivo* CTL can show a range of functional avidities both at the clonal and polyclonal level. Although avidity has been shown to be important in both viral and tumor settings, the mechanisms by which high and low avidity CTL are generated *in vivo* remains unclear as the TCR cannot undergo somatic hypermutation. It has been demonstrated *in vitro* that culturing of TCR transgenic CTL in the presence of high or low dose of antigen leads to polarization of low and high avidity responses respectively [1,3]. Evidence for polarisation of polyclonal immune responses is becoming more apparent *in vivo*. There is a growing body of information suggesting that CTL undergo clonal exhaustion *in vivo* leading to the anergy and deletion of vital antigen specific CTL [10]. This is especially common in chronic viral infections where antigen is often expressed for prolonged time periods [11,12]. This clonal exhaustion is believed to be a result of antigen-dependent apoptosis of CTL [13]. High avidity CTL have been shown to be more sensitive to antigen dose and therefore could be subject to negative regulation by supraoptimal antigen levels and persistence of antigen *in vivo*.

We have previously shown that immunization with a DNA vaccine, that encodes tumor peptides within the complementarity determining regions (CDRs) of an antibody, results in high avidity T cells to a range of encoded peptides whereas peptide immunization results in lower avidity responses [14]. This has enabled us to use this model to further study the role of high avidity response in tumor immunity. In this study we hypothesize that high avidity anti-tumor CTL can be generated and efficiently

recruited to the memory pool but that they can be subsequently impaired by supraoptimal TCR signaling.

Materials and Methods

Ethics Statement

Animal work was carried out under a Home Office approved project license.

Reagents

RPMI-1640, fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin-streptomycin, HEPES, glutamine, 2-mercaptoethanol, lipopolysaccharide (LPS), Carboxyfluorescein succinimidyl ester (CFSE) and complete/incomplete Freund's adjuvant (FA) were obtained from Sigma (Poole, UK). Murine cytokines were obtained from Peprotech EC (London, UK). Dextran sulphate was obtained from Pharmacia (Milton Keynes, UK). Fluorochrome conjugated antibodies targeting CD62L (clone MEL-14), CD127 (clone A7R34), CD86 (clone GL1), CD80 (clone 16-10A1), CD11c (clone N418), PD-1 (clone J43) and MHC class I (clone 28-14-8) were obtained from eBiosciences (San Diego, USA). Unconjugated antibody to LAG-3 (clone C9B7W) was obtained from eBioscience and used in conjunction with an anti-Rat IgG FITC conjugated secondary antibody also from eBioscience. PE-Alexa-647 or FITC labeled antibody targeting CD8 (clone KT15) was obtained from AbD Serotec (Oxford, UK) and PE labeled H-2 Kb SVYDFFVWL pentamer from ProImmune (Oxford, UK). Synthetic peptides were obtained from Peptide Synthetics (Cambridge, UK).

Cell Lines

B16F1, MeWo and RMAS cell lines were obtained from the ATCC and maintained in RPMI with 10% FBS. Media used for splenocyte culture was RPMI-1640 with 10% FBS, 2 mM glutamine, 20 mM HEPES buffer, 100 units/ml penicillin, 100 μg/ml streptomycin (complete media) and 10^{-5}M 2-mercaptoethanol.

Mice and Immunizations

Female C57Bl/6 (Charles River, Kent, UK) mice were used between 6 and 12 weeks of age. Synthetic peptide SVYDFFVWL (Tyrosinase relate protein (TRP2) 180–188) was emulsified with complete (CFA for prime) or incomplete Freund's adjuvant (IFA for subsequent boosts) and delivered subcutaneously (s.c.) at 25 μg/immunization. Human IgG1 antibody DNA encoding a TRP2 epitope within CDRH2 was coated onto 1.0 μm gold particles (BioRad, Hemel Hempstead, UK) using the manufacturer's instructions and administered intradermally by the Helios Gene Gun (BioRad). Each mouse received 1 μg DNA/immunization into the shaved abdomen. Mice were immunized at days 0, 7 and 14 with DNA or peptide boosts where stated. Spleens were removed at day 20, 48 or 70 for analysis. For tumor challenge studies mice were immunized at days 0, 7, and 14 with DNA and boosted at day 64 with DNA or peptide. At day 70, 2.5×10^4 B16F1 cells were implanted s.c. and growth monitored at 3–4 day intervals using a caliper.

Elispot and Elisa Assays

Ex vivo elispot assays were performed using murine IFNγ capture and detection reagents according to the manufacturer's instructions (Mabtech AB, Nacka Strand, Sweden). In brief, anti-IFNγ antibodies were coated onto wells of 96-well Immobilin-P plate and triplicate wells were seeded with 5×10^5 splenocytes in complete media plus 2-mercaptoethanol. Synthetic peptide SVYDFFVWL (TRP2) at a variety of concentrations or tumor cells at 5×10^4/well in complete media plus 2-mercaptoethanol were added to these wells and incubated for 40 hrs at 37°C. Following incubation, captured IFNγ was detected by a biotinylated anti-IFNγ antibody 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). For elispot assays on CTL lines, triplicate wells were seeded with 5×10^4 CTL lines in complete media. Synthetic TRP2 peptide (at a variety of concentrations) was pulsed onto RMA-S cells for 1½ hrs at 37°C and added to wells at 5×10^3/well in complete media. Tumor cells at 5×10^3/well were added to appropriate wells and plates incubated for 20 hrs at 37°C prior to development as detailed above. Anti-CD3 stimulation of CTL lines (5×10^4/well) was performed in 96 well plates previously coated with anti-CD3 antibody at different concentrations and supernatant analyzed for presence of IFNγ by elisa assay after 20 hrs at 37°C. Functional avidity was calculated as the concentration mediating 50% maximal effector function using a graph of effector function versus peptide concentration.

CTL Stimulation *in vitro* and Proliferation

Six days following the final immunization, splenocytes (5×10^6/ml) were isolated and co-cultured at 37°C with syngeneic, irradiated (3000rads), peptide-pulsed LPS blasts in complete media plus 2-mercaptomethanol. Before use stimulator cells were labeled with relevant concentration of synthetic peptide at concentration of 2×10^7/ml for 1 hr at 37°C in RPMI-1640. For proliferation assays red blood cells (RBCs) were lysed and splenocytes were labeled with 0.5 μM CFSE prior to culture. LPS blasts were obtained by activating splenocytes (1.5×10^6 cells/ml) with 25 μg/ml LPS and 7 μg/ml dextran sulphate in complete media for 48 hrs at 37°C. Cultures were assayed for cytotoxic activity, IFNγ release or proliferation on day 6 in a ^{51}Cr-release assay, elispot/elisa assay or by flow cytometry.

^{51}Cr-release Assay

Target cells were labeled for 90 mins with 1.85 MBq sodium (^{51}Cr) chromate (Amersham, Essex, UK) and plated at5×10^3 targets/well in 96-well V-bottomed plates. These were co-incubated with different densities of effector cells in a final volume of 200 μl complete media. After 4 hrs 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 $=100\times$[(experimental release-spontaneous release)/(maximum release-spontaneous release)].

Flow Cytometry

Staining of splenocytes *ex vivo* was performed by lysing RBCs using RBC lysing solution (Sigma) according to manufacturers guide lines. No RBC lysis was performed before staining of *in vitro* cultured CTL. Cells were subsequently stained with Phycoerythrin (PE) labeled H-2 Kb SVYDFFVWL (TRP2 180–188) pentamer at room temperature in the dark for 15 mins followed by incubation with other cell surface markers CD8-PE-Alexa-647 or FITC, CD62L-FITC, CD127-PECy7, PD-1 PE-Cy7 or LAG-3 for 30 mins on ice. Viability stain 7-AAD (eBioscience) was added at room temperature in the dark for 10mins immediately prior to analysis. For staining of LPS blasts, cells were stained with CD11c-PECy5, MHC I FITC, CD80 PE or CD86 PE for 30mins on ice. Data acquisition and analysis was performed on a FC500 flow cytometer (Beckman Coulter).

Statistics

Comparative analysis of the Elispot, flow cytometry, tumor volumes and CTL lysis data 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 and survival analysis by applying the Log-rank test using Graphpad Prism software.

Results

We have previously demonstrated that immunization with epitopes engineered within a human antibody framework induces high avidity CTL [14]. We have used this model to investigate if high avidity T cells are recruited to the memory pool and if they are acutely sensitive to TCR signaling.

DNA Immunization Induces CTL with High Functional Avidity whereas Peptide Immunization Elicits Low Avidity, Functionally Impaired CTL

C57Bl/6 mice were immunized with either a DNA construct containing the H-2 Kb restricted SVYDFFVWL epitope from TRP2 antigen within the CDRH2 region of an antibody or the same epitope as a peptide. Peptide immunized mice showed similar frequencies of antigen specific CD8 cells to DNA immunized mice by pentamer staining (Figure 1a) and by IFNγ elispot assay (Figure 1b). In contrast, mice immunized with antibody-DNA induced over a 100 fold higher avidity response than mice immunized with peptide ($p = 0.0008$) (Figure 1c,d). This difference in functional avidity was reflected in tumor cell killing. CTL were tested for lysis of B16F1 tumor cells, those derived from antibody-DNA immunized mice were capable of efficient killing whereas those derived from peptide immunized mice were not (Figure 1e).

High Avidity Responses are Efficiently Maintained into Memory

To determine if high avidity responses induced by antibody-DNA immunization were maintained into memory, splenocytes from immunized mice were analyzed for the frequency and avidity of functional epitope specific immune responses by IFNγ elispot assay, 48 and 70 days post immunization. Parallel groups of mice were boosted at 42 or 64 days post immunization with a single dose of antibody-DNA to determine the effect of booster immunization (Figure 2a). At 48 days following immunization low level TRP2 epitope specific responses (three fold over background) were observed which significantly expanded six fold over background upon booster immunization (Figure 2b). Responses detectable at 48 days were of high functional avidity (10^{-10} M peptide) (Figure 2c). Analysis of immune responses at 70 days revealed a similar frequency as at 48 days. However, these responses showed higher functional avidity ($<10^{-12}$ M) than those analyzed at earlier time points ($p<0.0001$), suggesting that the progression into memory selects for and retains the higher avidity T cells. This response was boosted into even higher avidity ($<10^{-13}$M) by DNA immunisation.

To assess the memory phenotype of these high avidity responses, splenocytes from mice taken at 70 days post immunization, were also analyzed for the expression of the memory markers, CD62L and CD127 (IL-7Rα) on TRP2 pentamer stained CD8 T cells. Combined analysis of CD62L and CD127 expression on TRP2 specific CD8 cells shows cells are mainly of the central memory phenotype (CD62L+ CD127+) with a smaller proportion of effector memory (CD62L- CD127+) and effector cells (CD62L- CD127-) (Figure 2e). Following booster immunization at day 64 a higher frequency of effector and effector memory phenotype are observed although a significant central memory population is retained.

High Avidity Memory Responses are Lost by Booster with Peptide Immunogen *in vivo*

Since peptide immunogen has been shown to induce low avidity responses *in vivo*. It was examined if high avidity responses induced by antibody-DNA could be influenced by subsequent exposure to peptide immunogen. Mice were immunized with antibody-DNA and responses left to establish into memory. At day 64 without any boost they had a functional avidity of $1E^{-10}$M they were then boosted *in vivo* with peptide (Figure 3a). Boosting of high avidity memory CTL responses using peptide immunogen revealed an increase in the frequency of the response compared to no boost (Figure 3b) with a 100 fold reduction in functional avidity ($2E^{-08}$M, $p<0.0001$) (Figure 3c,d) compared to no boost and a 5,000 fold reduction in avidity when compared to a DNA boost ($2E^{-11}$M, $p<0.0001$) (Figure 3c,d). Pentamer staining of splenocytes from these mice reveals similar numbers of antigen specific CD8s in each group (Figure 3e). Antigen specific CD8s also show similar frequency of effector and memory phenotypes upon booster immunization (Figure 3f).

To determine if this lower avidity response could be reactivated to high avidity, mice were further boosted with antibody-DNA (Figure 3c,d). Boost, with antibody-DNA following the peptide challenge showed restimulation of high frequency epitope specific CTL (Figure 3b). However, this response remained of similar functional avidity when compared to mice that had not received a peptide boost suggesting that the DNA could still selectively stimulate any remaining higher avidity T cells.

Low Avidity Responses Resulting from Peptide Boost Show Limited Anti-tumor Efficacy *in vitro* and *in vivo*

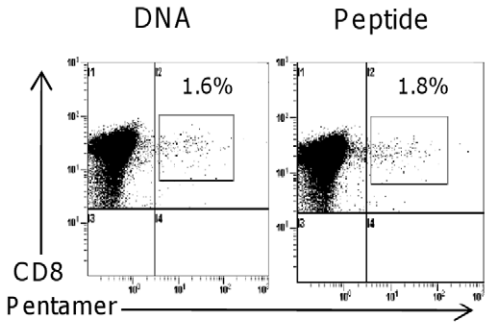
This low avidity response induced as a result of boosting high avidity memory responses with peptide was analyzed for its ability to recognize the syngeneic B16F1 melanoma cell line expressing TRP2 *in vitro* compared to the HLA mismatched MeWo control. Responses from mice boosted with antibody-DNA that retain high avidity show recognition of B16F1 cells whereas low avidity responses from those boosted with peptide are incapable of specific tumor cell recognition (Figure 4a). To analyse the effect of low avidity responses *in vivo* mice boosted with antibody-DNA or peptide were challenged with B16F1 tumor (Figure 4b). Mice with high avidity responses (10^{-12} M) that were boosted with antibody-DNA show significantly delayed tumor growth ($p = 0.03$) at day 16 post tumor implant compared to control. This is reflected in significantly enhanced survival ($p = 0.037$) in this group compared to control (Figure 4d).

Although the peptide boosted mice did show some anti-tumour effects this did not reach significance due to the lower avidity (Figure 4c).

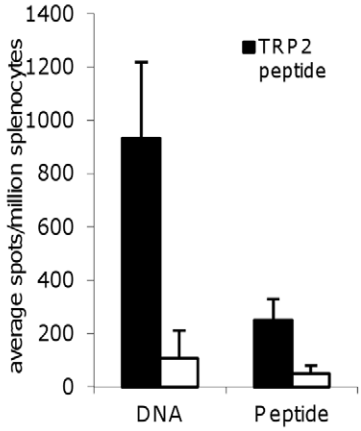
Supraoptimal TCR Stimulus Results in Loss of High Avidity Response *in vitro*

To assess if high avidity responses were differentially affected by TCR signaling, high avidity CTL responses were subjected to a short *in vitro* culture with supraoptimal (10 μg/ml) and optimal (1 ng/ml; Ic50 of high avidity T cells) dose of peptide pulsed on LPS blasts. These stimulated antigen presenting cells (APCs) express high levels of costimulatory molecules and therefore provide optimal costimulation for CTL responses (Figure 5a).

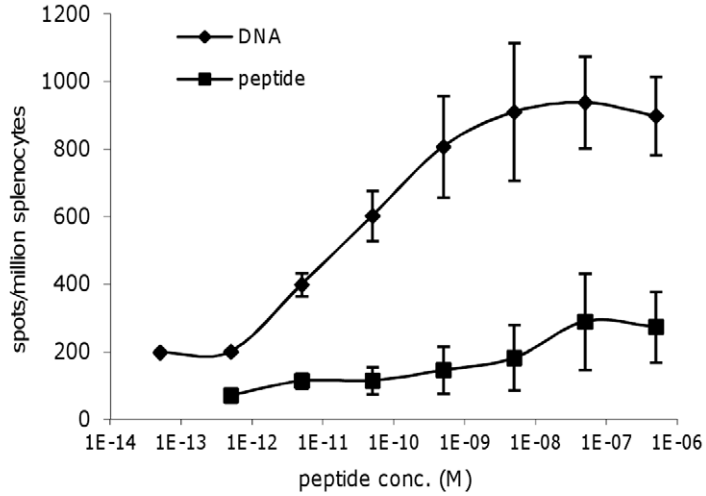
A.


DNA
Peptide
1.6%
1.8%
CD8
Pentamer

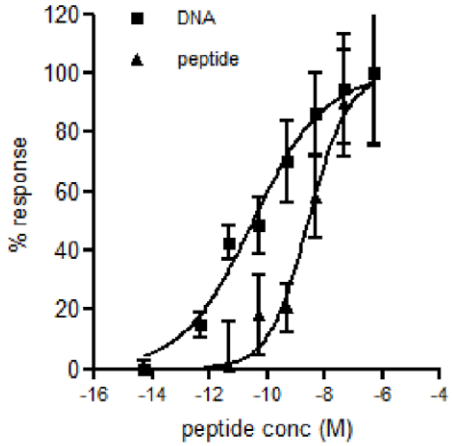
B.


1400
1200
1000
800
600
400
200
0
average spots/million splenocytes
TRP2 peptide
DNA
Peptide

C.


1200
1000
800
600
400
200
0
spots/million splenocytes
DNA
peptide
1E-14
1E-13
1E-12
1E-11
1E-10
1E-09
1E-08
1E-07
1E-06
peptide conc. (M)

D.


120
100
80
60
40
20
0
% response
DNA
peptide
-16
-14
-12
-10
-8
-6
-4
peptide conc (M)

E.

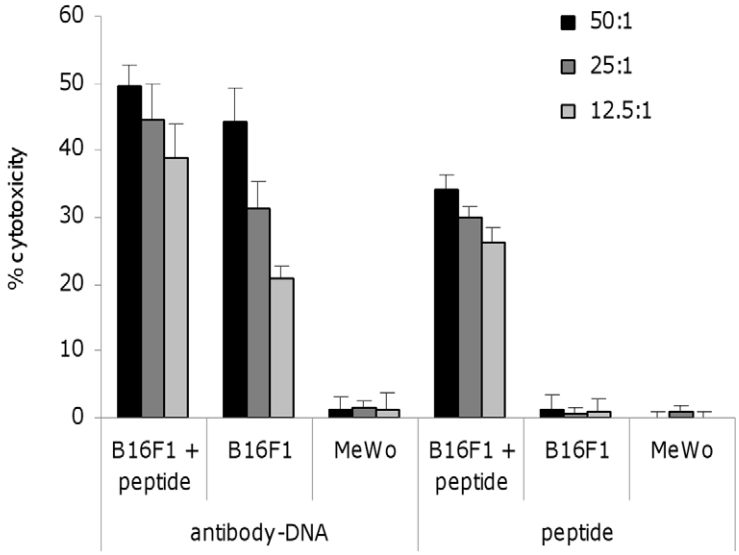

60
50
40
30
20
10
0
% cytotoxicity
50:1
25:1
12.5:1
B16F1 + peptide
B16F1
MeWo
B16F1 + peptide
B16F1
MeWo
antibody-DNA
peptide

Figure 1. Peptide immunization induces low avidity functionally impaired CTL. Splenocytes from mice immunized with peptide or DNA were analyzed at day 20 for A, Presence of antigen specific CTL by pentamer and CD8 staining. B, Frequency of epitope specific responses in IFNγ elispot assay C, Avidity and frequency of epitope specific responses by measuring responses to increasing peptide concentration in IFNγ elispot assay, D, Analysis of the avidity by normalization of responses. E, The ability of CTL lines to lyse tumor cells in chromium release assay. Data is representative of at least three independent experiments.
doi:10.1371/journal.pone.0041112.g001

After 6 days stimulation, resulting CTL lines were tested for functional epitope specific response and tumor cell recognition in elispot assay. Figure 5b shows stimulation of the high avidity response *in vitro* with high or low dose peptide maintained a similar frequency of peptide specific responses. In contrast, analysis of functional avidity revealed that optimal stimulation (low dose) retained avidity whereas supraoptimal stimulation (high dose) resulted in significantly lower avidity ($p = 0.0018$) (Figure 5c). The higher functional avidity of the optimally stimulated CTL was reflected in the ability of these cells to recognize and kill the B16F1

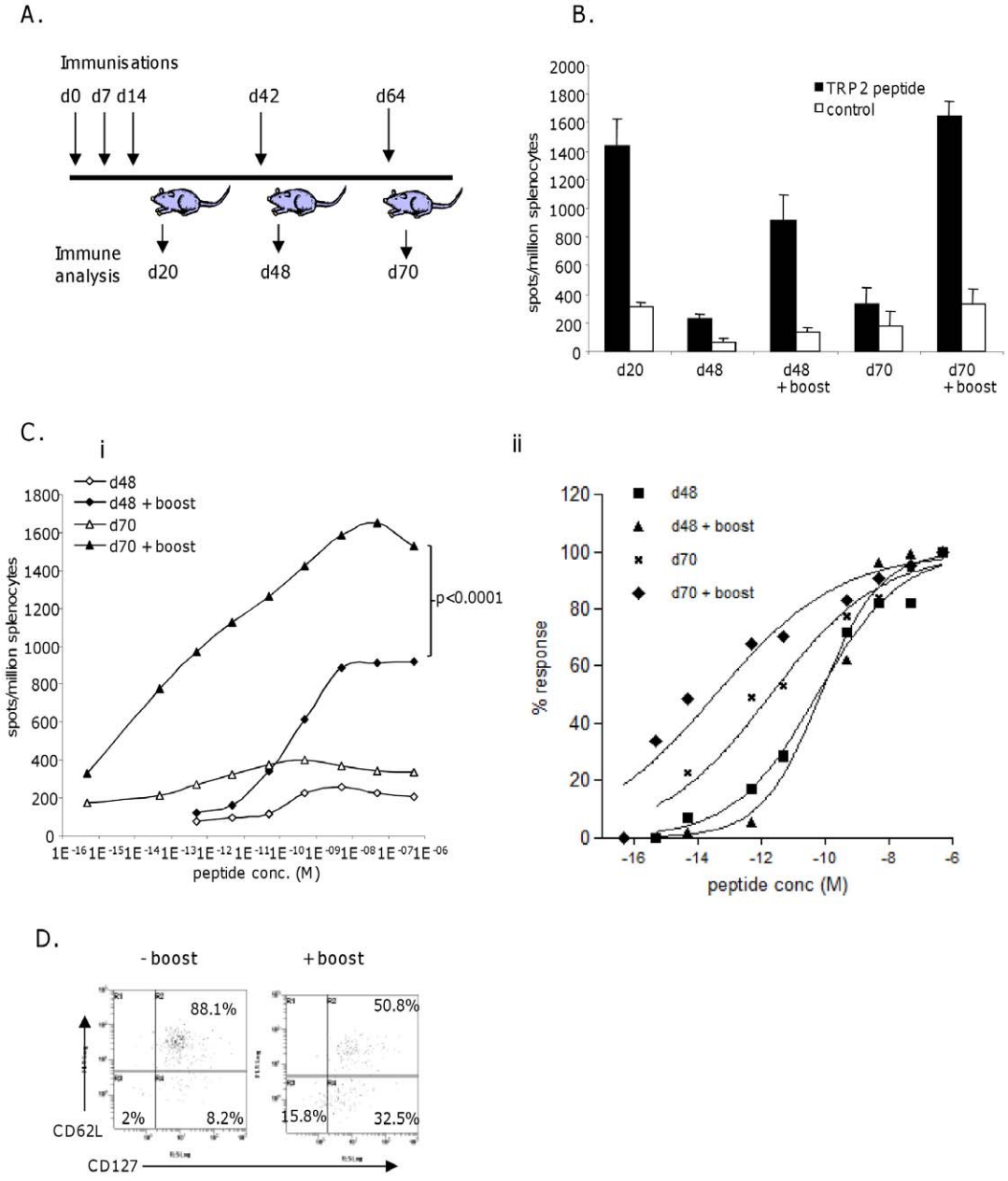
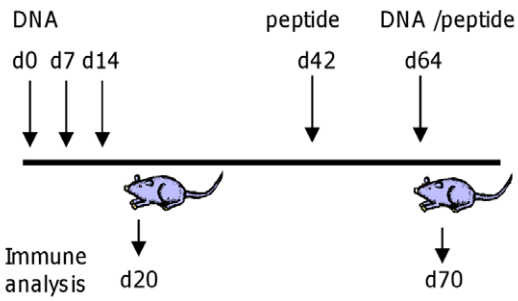
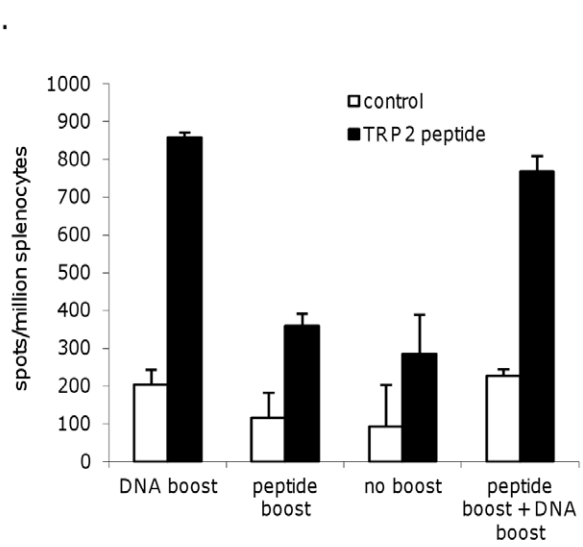
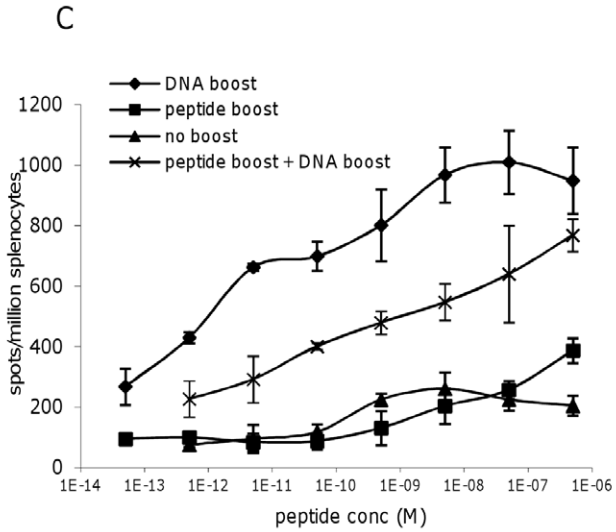
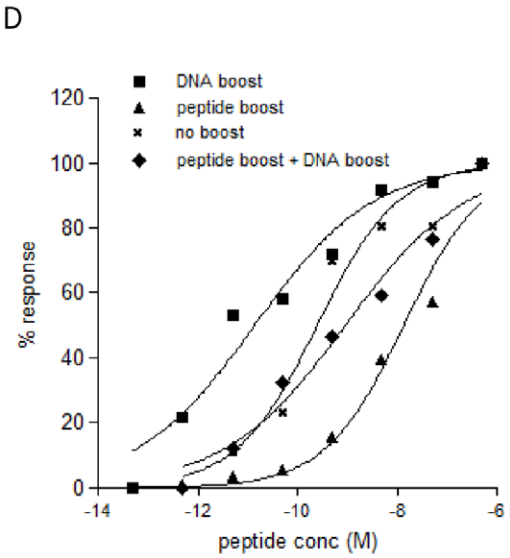


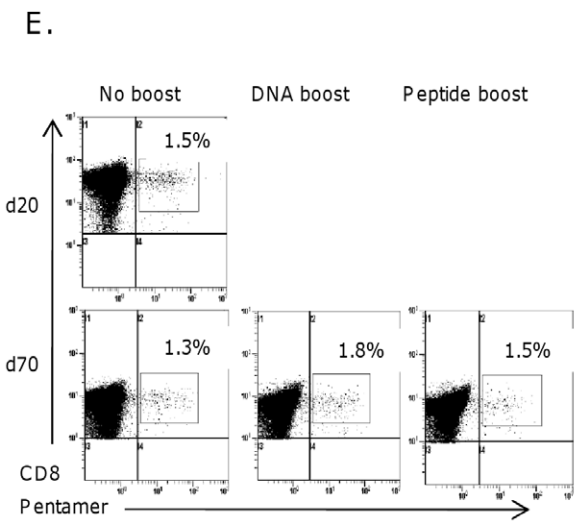
Figure 2. High avidity responses induced by DNA immunization are efficiently maintained into memory. A, schematic of immunization regime. B, Mice immunized with DNA were boosted at days 42 or 64 and frequency of immune responses analysed in IFNγ elispot assay at days 20, 48 and 70. C, Analysis of avidity of responses by peptide titration in IFNγ elispot assay, D, normalization of responses to increasing peptide concentration. E, Analysis of memory phenotype of antigen specific CTL by combination staining for CD62L and CD127 markers. Data is representative of at least three independent experiments.
doi:10.1371/journal.pone.0041112.g002


A.
DNA
d0 d7 d14
peptide
d42
DNA /peptide
d64
Immune analysis
d20
d70


B.
control
TRP2 peptide
spots/million splenocytes
DNA boost
peptide boost
no boost
peptide boost + DNA boost


C
DNA boost
peptide boost
no boost
peptide boost + DNA boost
spots/million splenocytes
peptide conc (M)


D
DNA boost
peptide boost
no boost
peptide boost + DNA boost
% response
peptide conc (M)


E.
No boost
DNA boost
Peptide boost
d20
d70
1.5%
1.3%
1.8%
1.5%
CD8
Pentamer

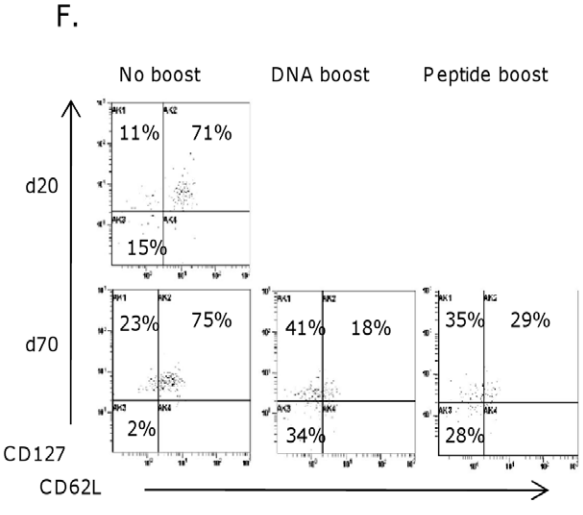

F.
No boost
DNA boost
Peptide boost
d20
d70
11%
71%
15%
23%
75%
2%
41%
18%
34%
35%
29%
28%
CD127
CD62L

Figure 3. Peptide boost leads to loss of high avidity responses *in vivo*. A, schematic of immunization regime. Mice immunized with DNA were boosted at day 63 with DNA or peptide, or with peptide at day 42 followed by DNA at day 63. Responses were analyzed at day 70 for frequency (B) and avidity (C) by measuring responses to increasing peptide concentration in IFNγ elispot assay, D) normalization of responses to increasing peptide concentration in IFNγ elispot assay. E, Analysis of antigen specific CTL by pentamer and CD8 staining of immunized splenocytes. F, Analysis of memory phenotype of antigen specific CTL by combination staining for CD62L and CD127 markers. Data is representative of at least three independent experiments.
doi:10.1371/journal.pone.0041112.g003

tumor cells ($p = 0.022$) (Figure 5b and d). The activity of the high avidity T cells suggests higher sensitivity to TCR triggering than seen in low avidity T cells. This was supported by measuring responses of these cells to CD3 stimulation. High avidity CTL were more sensitive to lower doses of anti-CD3 compared to low avidity T cells ($p = 0.008$) (Figure 5e).

High dose TCR Stimulus Promotes T Cell Impairment and Death *in vitro*

The loss of functional avidity with supraoptimal stimulation may suggest that overstimulation of TCR can lead to cellular exhaustion, cell death or a combination of both. Analysis of proliferation of high avidity CTL stimulated with high and low dose peptide demonstrated that the low, optimal dose of peptide induced better proliferation than the higher dose of peptide (Figure 6a) resulting in a higher percentage of pentamer positive cells (14.5%) compared to cells stimulated with supraoptiomal doses (5%; Figure 6a ii). Supraoptimal, high dose peptide shows CFSE dilution in only 5.2% of pentamer positive CD8s as opposed to 62.5% dilution with optimaldose peptide. Unstimulated cells show 2.6% of pentamer positive CD8s with dilution in CFSE staining intensity. High avidity CTL lines exposed to optimal and supraoptimal doses of peptide were also were examined for the extent of cell death by 7-AAD uptake. Figure 6b shows that stimulation of the CTL with high supraoptimal dose of peptide leads to more 7-AAD positive antigen specific CTL than lines stimulated with low optimal dose peptide ($p = 0.0093$). This suggested a higher rate of cell death amongst those stimulated with high supraoptimal dose peptide. To examine the possibility of exhaustion cell lines were analyzed for the markers PD-1 and

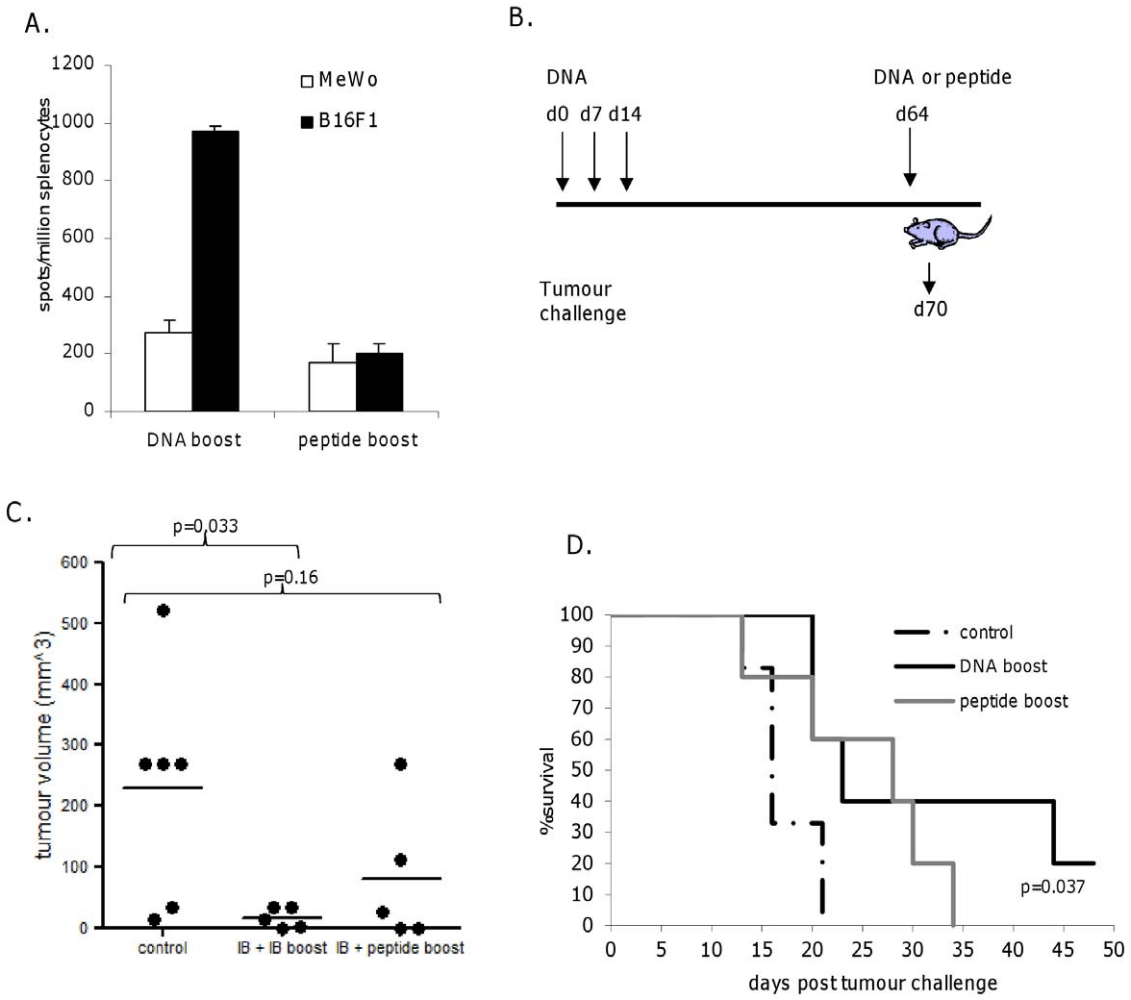
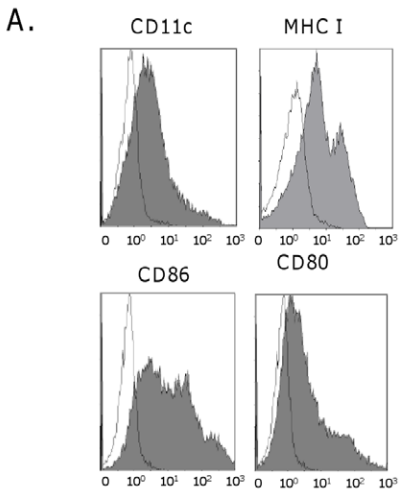
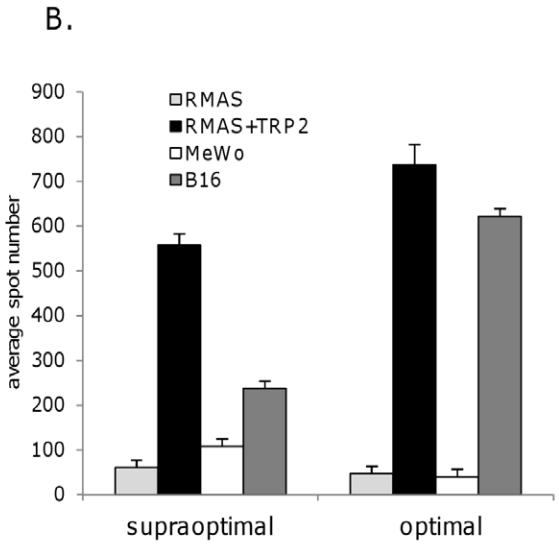
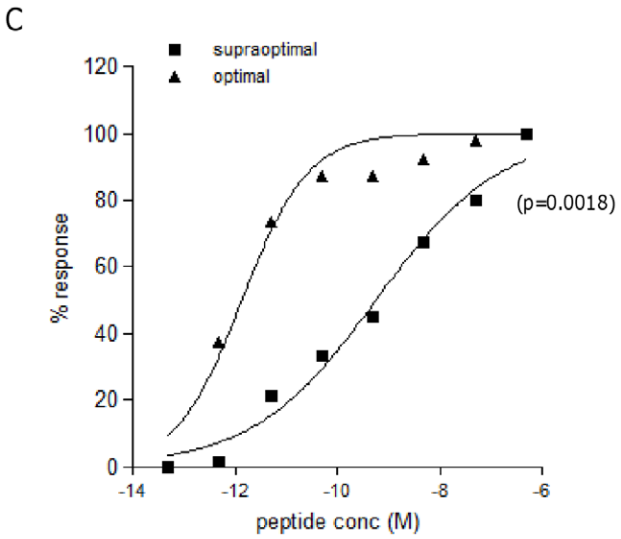
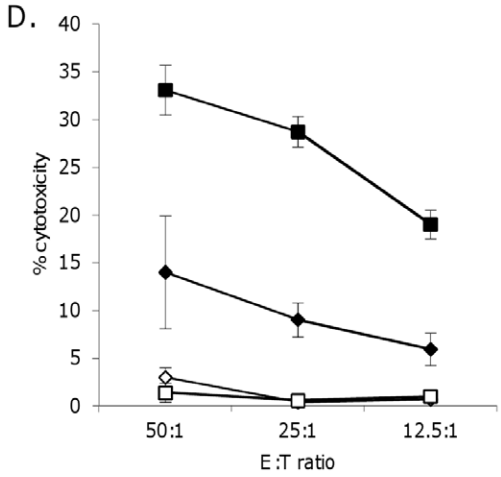


Figure 4. Low avidity responses show limited anti-tumor efficacy. A, Recognition of B16F1 and MeWo cells by immunized splenocytes in IFNγ elispot assay. B, schematic of immunization regime. C, Tumor volume at day 16 post tumor challenge. D, Survival analysis of mice immunized with DNA and boosted with DNA or peptide followed by challenge with B16F1 tumor.
doi:10.1371/journal.pone.0041112.g004


A.
CD11c
MHC I
CD86
CD80


B.
RMAS
RMAS+TRP2
MeWo
B16
average spot number
supraoptimal
optimal


C
supraoptimal
optimal
(p=0.0018)
% response
peptide conc (M)


D.
% cytotoxicity
50:1
25:1
12.5:1
E:T ratio

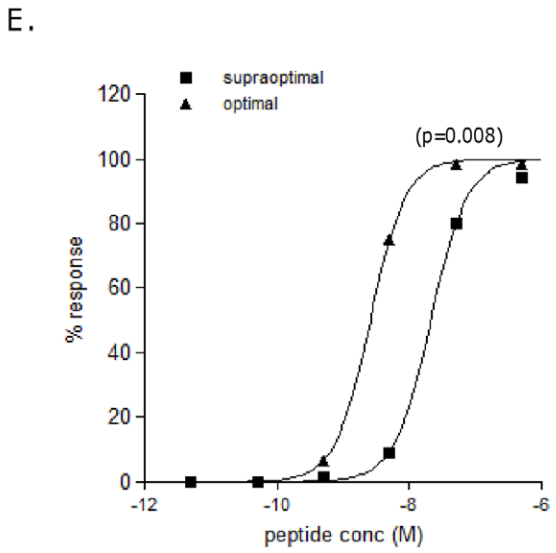

E.
supraoptimal
optimal
(p=0.008)
% response
peptide conc (M)

Figure 5. High avidity CTL responses can be modulated by the doses of TCR stimulus. A, LPS blasts were stained for expression of CD11c, MHC class I, CD80, CD86 (filled histograms) compared to control (open histograms). B, High avidity CTL from DNA immunization were stimulated *in vitro* with supraoptimal (100 μg/ml) and optimal (10 ng/ml) dose peptide pulsed LPS blasts. After 6 days *in vitro* cultures were assessed for peptide specific responses against peptide (1 μg/ml) pulsed RMAS cells and tumor cell recognition. C, i) CTL cultures were analyzed for avidity of epitope specific responses by measuring responses to increasing peptide concentration on RMAS cells in IFNγ elispot assay, ii) normalization of responses. D, supraoptimal (diamonds) and optimal (squares) dose peptide stimulated CTL were assayed for cytotoxicity against B16F1 (closed symbols) or MeWo (open symbols) by chromium release assay at 50:1, 25:1 and 12.5:1 effector:target ratios. Data is representative of at least three independent experiments. E, CTL cultures stimulated with supraoptimal and optimal dose peptide pulsed LPS blasts were analyzed for sensitivity to CD3 stimulation in IFNγ elisa assay (normalized data is shown). Data is representative of at least three independent experiments.
doi:10.1371/journal.pone.0041112.g005

LAG-3 after optimal and supraoptimal peptide stimulation. Expression of PD-1 is known to be upregulated upon activation and it has been suggested that predominantly CD8 cells expressing high levels of PD-1 are those that are functionally inert. Cells lines stimulated supraoptimally showed larger numbers of antigen specific CD8s expressing high levels of PD-1 than those with optimal stimulation (Figure 6c,d). More recently cells expressing intermediate levels of PD-1 have been shown to retain functional capability with those that also express the marker LAG-3 being most responsive [15]. Staining of optimal and supraoptimal stimulated cultures for PD-1 and LAG-3 reveals more of PD-1 intermediate cells in optimally stimulated cultures to express LAG-3 (Figure 6e,f).

Discussion

High avidity T cells have been shown to be vital for both anti-tumor immunity and viral clearance. Using the TRP2, CD8 epitope SVYDFFVWL, as a model self-epitope in mice and the ability to induce high avidity CTL by encoding this epitope within an antibody-DNA construct, we have shown that high avidity CTL are predominantly recruited to the memory response. Analysis of immune responses at 70 days revealed a similar frequency as at 48 days. However, the day 70 responses showed remarkable picomolar avidity to a self-antigen which was 100 fold higher than those seen at earlier time points. Others have suggested that the progression into memory selects for and retains the higher avidity T cells [16,17]. In our study we generated CTL responses with picomolar avidities which caused vitiligo and tumor rejection. One possible mechanism for progressive selection of high avidity T cells is that high avidity T cells are more sensitive to IL-15 driven homeostatic proliferation in the absence of antigen [18]. This may explain the increase in avidity seen in our study following a boost at d70, as the antigen from our DNA vaccine persists at the site of injection for 35 days but is undetectable even by PCR by day 90 (unpublished results). These highly functional CTL were able to reject a tumor challenge in 20% of animals. There is some controversy in the literature as to whether high avidity T cells are preferentially selected into memory. Higher avidity responses in memory than during the primary response have been shown [19]. However, these were in response to a viral prime and challenge and it was unclear if the avidity was based upon epitope selection. In our study, we focused on the avidity of the response to a single, self epitope and showed a remarkable increase in avidity. Turner *et al.* showed that whereas the avidity of the memory response to OVA encoded within viruses increased in wild type mice, avidity maturation was self limited in mice that express OVA as a self antigen [20]. They also failed to see autoimmunity.

The high avidity CTL memory responses could be efficiently boosted by DNA immunization. In contrast, the functional capability of the high avidity memory response was dramatically reduced upon boosting *in vivo* with peptide immunogen. If this was a result of the peptide attenuating the high avidity memory response, then a subsequent boost with DNA should not recover the avidity. This was indeed the case as antibody-DNA, restimulated the low avidity T cells to a higher frequency but could only partially recover the avidity of the original DNA prime and was very poor in comparison to a DNA boost in the absence of peptide immunisation. This suggests that the peptide boost had deleted/impaired the original high avidity memory response. It has been suggested that CD8 tolerance/anergy can be induced by peptide immunization specifically when multiple doses are given [21,22,23,24]. It is thought that this is due to the presentation of these peptides on non professional APCs or long term systemic presentation of the epitope [25]. A report from Rezvani *et al.* highlights this in a clinical setting where patients receiving repeated immunizations with peptide in montanide adjuvant, showed loss of high avidity CTL responses correlating with lack of anti-tumor efficacy [26]. However the fact that the DNA boost appears to correct, at least in part, the low avidity effect of the peptide boost is an exciting result, suggesting that even in the face of a very low avidity population, the DNA vaccination can still move the response towards higher avidity.

It has previously been suggested that high avidity T cells are highly susceptible to signaling via MHC:peptide due to the assimilation of the TCR into preformed signaling rafts which can rapidly amplify signal [27,28]. The functional avidity of CTL has been shown to be linked to the surface expression of CD8, engagement of CD3 and signal transduction following TCR engagement with peptide MHC. High avidity CD8 cells are known to express higher levels of CD8 and show clustering of signaling molecules into lipid rafts resulting in lower activation thresholds and stronger stimulation signals from TCR:peptide MHC complexes [28,29,30]. To determine if the high avidity T cells were attenuated by supraoptimal signaling, the high avidity T cells were stimulated *in vitro* with different doses of TCR signaling. The use of LPS blasts provided high levels of costimulation so as to solely examine the effect of TCR signal strength on restimulation of responses.

Stimulation of a high avidity response *ex vivo* with optimal doses of peptide, induces proliferation and maintains their potent avidity and killing function. Thus high avidity memory T cells will be acutely sensitive to further encounter with low dose antigen, either low viral infection or early tumor development. In contrast, stimulation of a high avidity response *ex vivo* with high supraoptimal dose of peptide immunogen resulted in low avidity responses and loss of functional capability *in vitro*. This suggests that the strength of the TCR signal received plays a major role in restimulation of responses. High dose peptide stimulus *in vitro* appeared to induce less proliferation and increased cell death which is consistent with reports of T cells pushed to exhaustion. This is consistent with the hypothesis of others that over stimulation through TCR:peptide MHC complex pushes high avidity CTL towards apoptosis [13,31,32]. Interestingly, a recent study by Muraoka *et al.* demonstrated the apoptosis of epitope specific CTL upon repeat peptide vaccination [24].

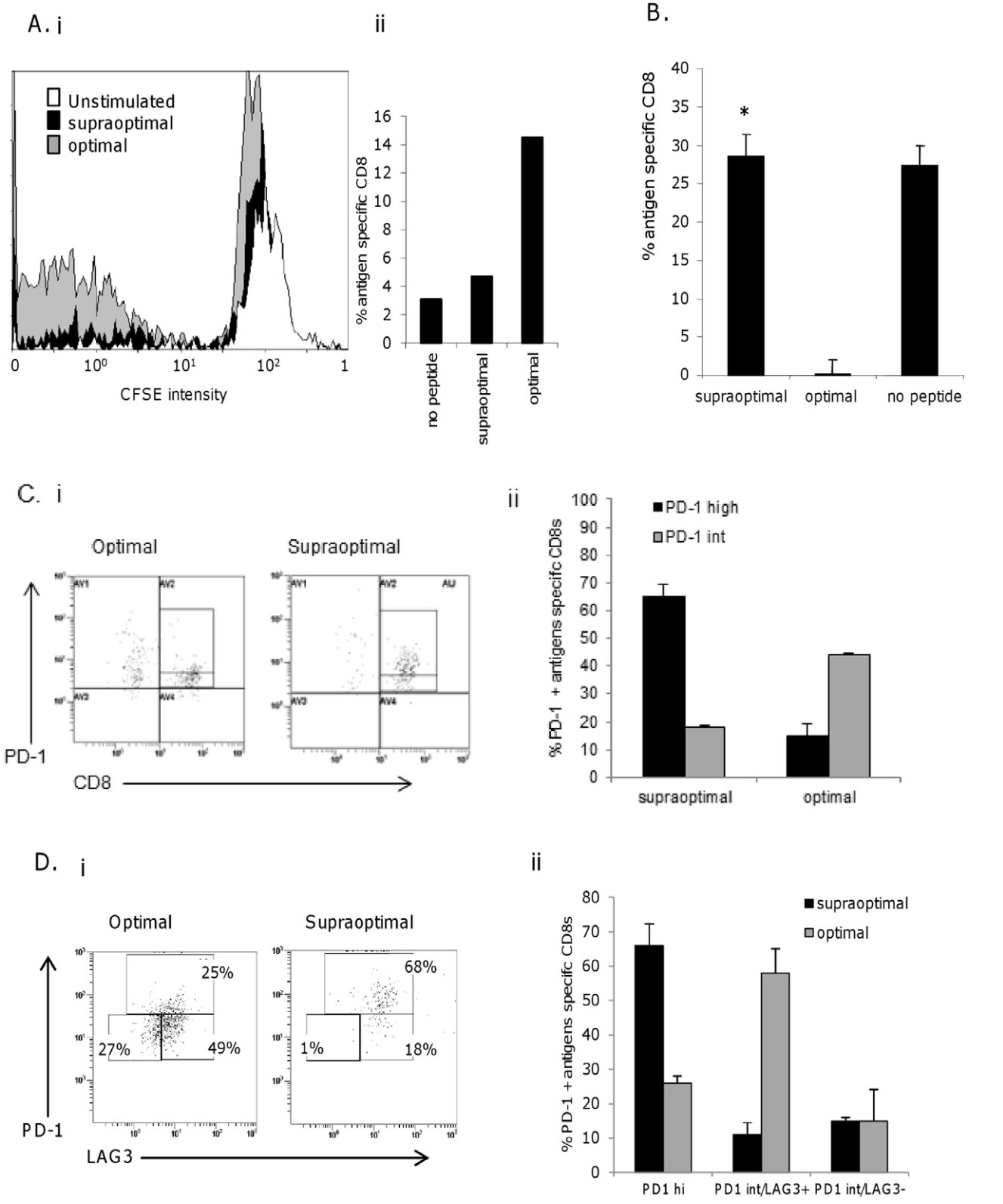


Figure 6. Supraoptimal dose antigen promotes T cell impairment and death. Splenocytes from DNA immunization were stimulated *ex vivo* with supraoptimal (100 μg/ml) and optimal (10 ng/ml) dose peptide pulsed LPS blasts. A, CFSE labeled splenocytes were assayed for proliferation of antigen specific CTL after 6 days *in vitro* culture. CFSE intensity is shown gated on pentamer positive CD8 cells. CTL were stained for B, the uptake of 7-AAD (plots gated on Pentamer+ CD8+ cells) * $p = 0.0093$, C, the expression of PD-1 (plots gated on Pentamer+ cells), D) average data and E, expression of PD-1 and LAG-3 (plots gated on Pentamer+ CD8+ cells), F) average data. Data is representative of at least three independent experiments.

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The inhibitory receptor PD-1 is known to be upregulated upon T cell activation and the extent of engagement of PD-1 by its ligands is known to regulate the threshold for T cell activation [33,34,35,36]. It is also a marker that has been associated with functional exhaustion of CD8 T cells. CTL cultures in this study stimulated with optimal and supraoptimal peptide dose both show expression of PD-1. However, the level of PD-1 expression differs dramatically with supraoptimal stimulated CTL expressing higher levels of the marker. Increased expression of PD-1 has been demonstrated on antigen specific CD8 cells induced by peptide immunization which exhibited low *in vivo* cytotoxicity and on exhausted CTL [37,38]. More recently it has also been documented that expression of high levels of PD-1 by tumor infiltrating lymphocytes correlates with functional impairment and suggests a role for PD-1 ligands in combination with prolonged antigen expression by tumors in establishment of T cell anergy [39,40,41]. Supraoptimal TCR stimulus and high level of PD-1 expression are therefore likely to lead to cell death and exhaustion and would help explain the loss of high avidity responses. Future studies to assess if blockade of the PD-1 pathway restores proliferation and prevents cell death will be undertaken. It has been suggested that expression of PD-1 alone cannot be taken as a marker of functionally exhausted cells. Other markers such as LAG-3 are also upregulated upon T cell activation and associated with negative regulation [42]. A study by Grosso *et al.* on chronically stimulated CD8 T cells interestingly discovered that the presence of LAG-3 does not always correlate with a decrease in function. Those cells expressing low levels of PD-1 in combination with LAG-3 correlated with increased functional ability which is a phenotype observed in the majority of optimally stimulated CTL cultures in this study [15]. However, the functional ability of cells expressing high levels of PD-1 was impaired independent of the LAG-3 status.

This study highlights the importance of optimal stimulation for the *in vivo* induction and maintenance of high avidity CTL responses. In contrast, supraoptimal stimulation can lead to non productive immune responses. This has implications for tumor therapy as high dose sustained TCR stimulation either by inappropriate vaccination or by tumor cells presenting cognate peptide:MHC in the absence of costimulation could lead to selection of low avidity T cells that fail to control tumor growth.

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Author Contributions

Conceived and designed the experiments: LGD VAB RLM. Performed the experiments: VAB BG. Analyzed the data: VAB BG LGD. Contributed reagents/materials/analysis tools: RLM VAB BG. Wrote the paper: LGD VAB.

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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.

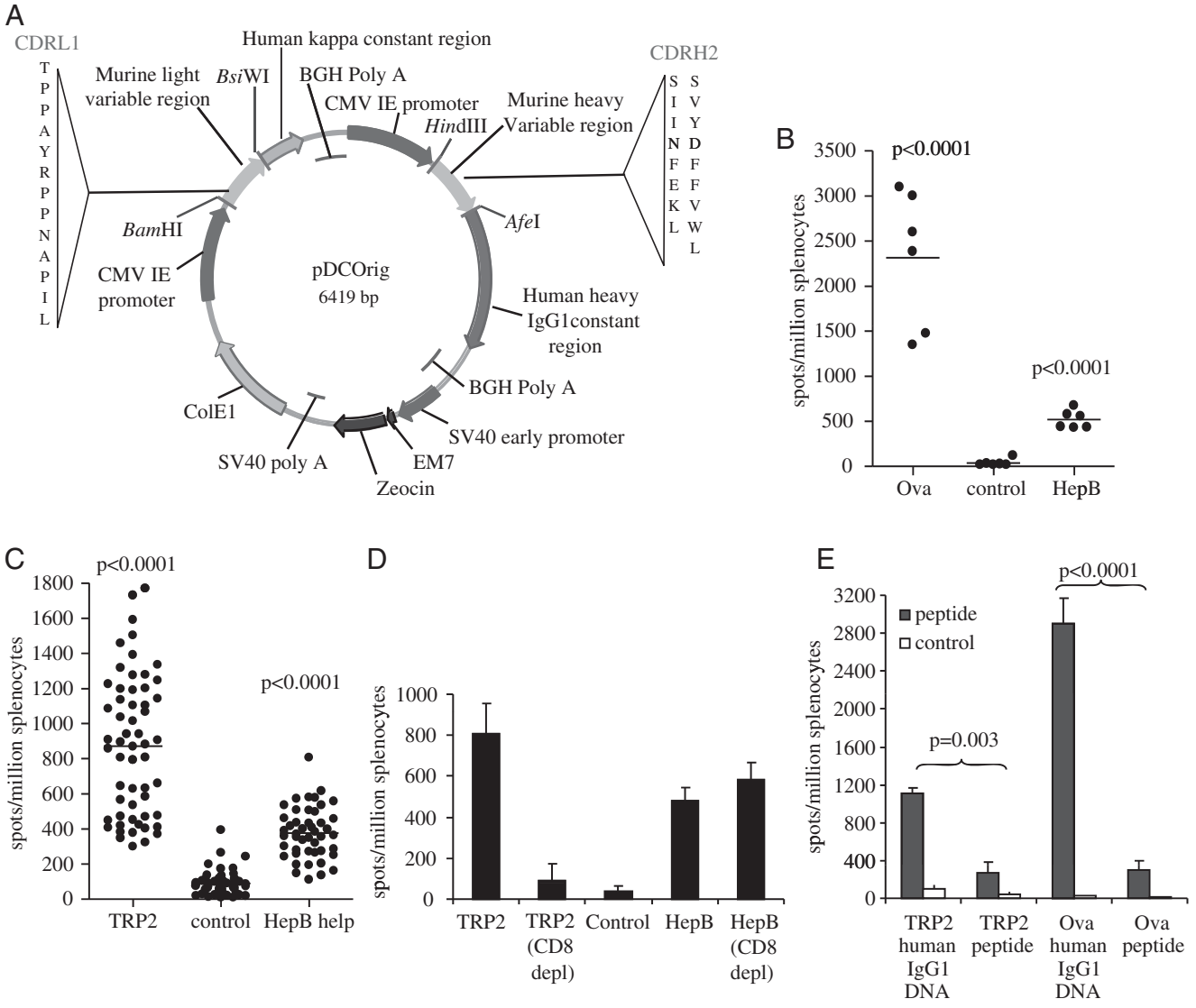

Figure 1. Foreign and self CTL and helper epitopes incorporated into human IgG1 framework are processed and presented to elicit immune responses *in vivo*. (A) Schematic diagram depicting features of the double expression vector pDCOrig. C57BL/6 mice were immunized with human IgG1 constructs. On day 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

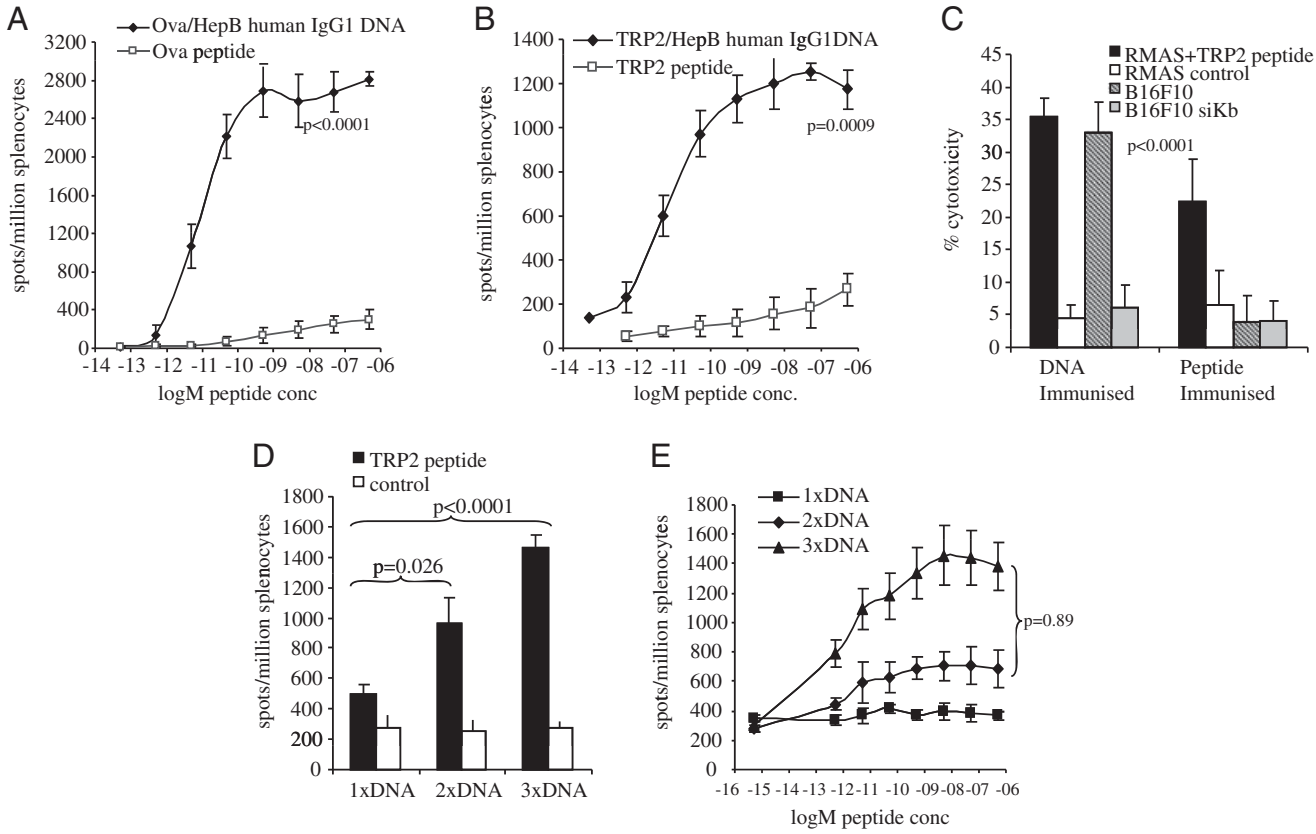

Figure 2. Prime boost immunization regime is essential for the induction of high-avidity epitope-specific responses. (A) Splenocytes from mice immunized with OVA/HepB human IgG1 DNA construct or synthetic peptide were assayed for avidity to the SIINFEKL epitope by measuring responses to increasing peptide 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.

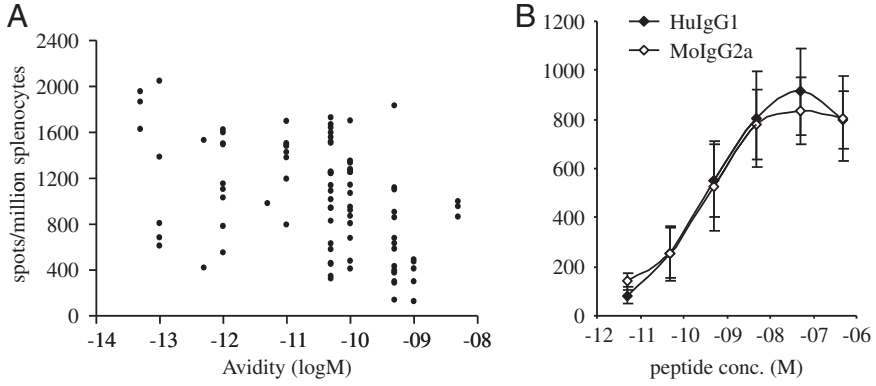

Figure 3. High-avidity responses do not correlate with high frequency and are not a result of influence from xenogeneic human Fc. (A) Correlation of frequency and avidity of responses generated in C57BL/6 mice with the TRP2/HepB human IgG1 construct CDRL1 ($n = 87$). (B) Splenocytes from mice immunized 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

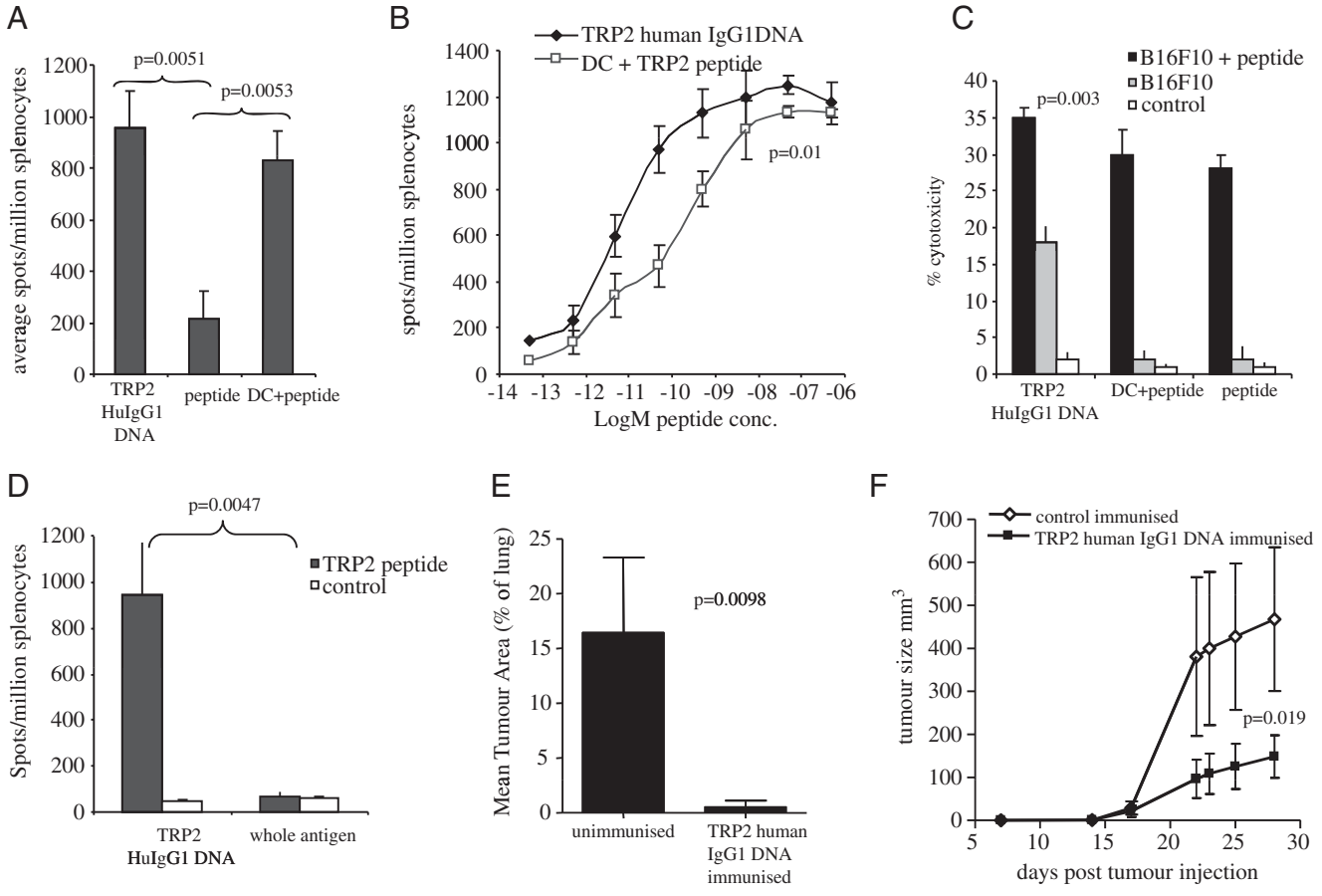

Figure 4. TRP2/HepB human IgG1 DNA immunisation is comparable to immunisation with peptide-pulsed DC. (A) C57BL/6 mice were vaccinated with human IgG1 DNA, peptide or peptide-pulsed DC. Splenocytes were assayed for frequency of TRP2-specific responses in IFN-γ ELISPOT assay against 0.5 μM peptide. (B) 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

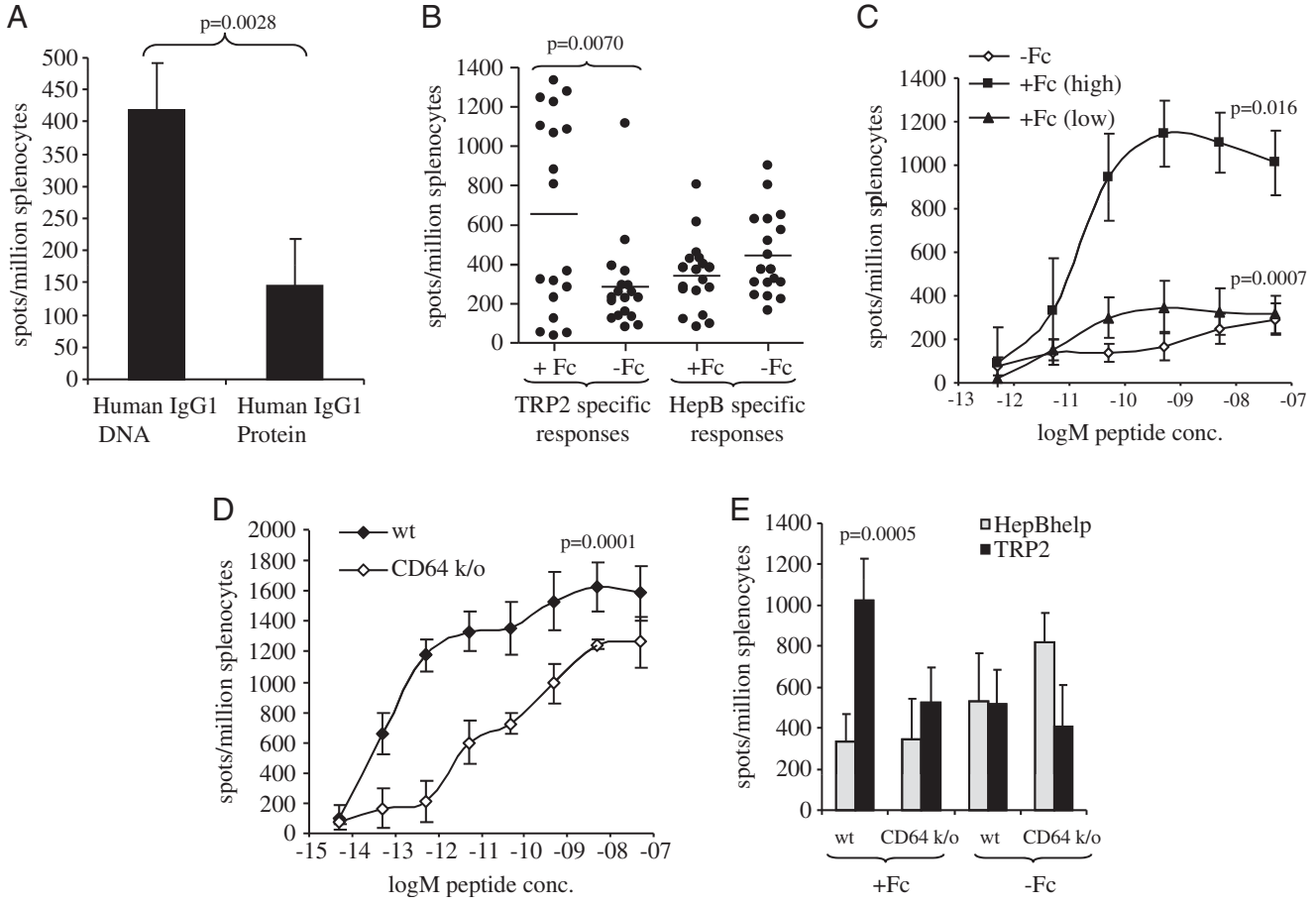

Figure 5. Fc region is important for the induction of CD8 responses. (A) Responses from C57BL/6 mice immunized with a human IgG1 DNA were compared to an identical protein construct in IFN-γ ELISPOT assay. (B) TRP2/HepB human IgG1 DNA was compared to a similar construct lacking the Fc region. (C) Avidity 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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