Wilms' Tumour 1 (WT1) peptide vaccination in patients with acute myeloid leukaemia induces short-lived WT1-specific immune responses

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Summary

Wilms' Tumour 1 (WT1) is a zinc finger transcription factor that is over-expressed in acute myeloid leukaemia (AML). Its restricted expression in normal tissues makes it a promising target for novel immunotherapies aiming to accentuate the cytotoxic T lymphocyte (CTL) response against AML. Here we report a phase I/II clinical trial of subcutaneous peptide vaccination with two separate HLA-A2-binding peptide epitopes derived from WT1, together with a pan-DR binding peptide epitope (PADRE), in Montanide adjuvant. Eight HLA-A2-positive patients with poor risk AML received five vaccination cycles at 3-weekly intervals. The three cohorts received 0·3, 0·6 and 1 mg of each peptide, respectively. In six patients, WT1-specific CTL responses were detected using enzyme-linked immunosorbent spot assays and pWT126/HLA-A*0201 tetramer staining, after *ex vivo* stimulation with the relevant WT1 peptides. However, re-stimulation of these WT1-specific T cells failed to elicit secondary expansion in all four patients tested, suggesting that the WT1-specific $CD8^+$ T cells generated following vaccination may be functionally impaired. No correlation was observed between peptide dose, cellular immune response, reduction in *WT1* mRNA expression and clinical response. Larger studies are indicated to confirm these findings.

Keywords: acute myeloid leukaemia, immunotherapy, tumour antigens, trials.

The potential of T lymphocytes to eliminate leukaemia is demonstrated by the graft-*versus*-leukaemia effect following haematopoietic stem cell transplantation (Odom *et al*, 1978; Goldman *et al*, 1988; Horowitz *et al*, 1990). The identification of target antigens on leukaemic cells has renewed interest in the use of peptide vaccines to stimulate leukaemia-specific T cell responses (Oka *et al*, 2004; Rezvani *et al*, 2008; Keilholz *et al*, 2009; Maslak *et al*, 2010; Kuball *et al*, 2011). A target antigen of particular interest is the zinc finger transcription factor Wilms' Tumour 1 (WT1), which is over expressed in several solid tumours as well as acute myeloid leukaemia (AML) and myelodysplasia (Inoue *et al*, 1997; Miyoshi *et al*, 2002; Oji *et al*, 2002). WT1 has several characteristics that render it a highly attractive target for immunotherapies: its expression in normal tissues is limited (Call *et al*, 1990); it is expressed by leukaemic stem cells in addition to more mature myeloid cells (Saito *et al*, 2010); and its expression increases as myeloid disease progresses (Cilloni *et al*, 2003). WT1 is required for proliferation of leukaemia cells (Algar *et al*, 1996), reducing the likelihood of tumour escape by down-modulation of the antigen. We have previously shown that WT1-specific cytotoxic T lymphocytes (CTLs) kill leukaemia cells and leukaemia stem cells without affecting normal stem cell function (Gao *et al*, 2000, 2003).

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Although WT1 is expressed in normal tissues during embryogenesis, immunological tolerance to WT1 is not complete: WT1-specific CTLs have been detected and expanded following exposure to the peptide both in healthy donors (Rezvani *et al*, 2003) and in patients with AML (Scheibenbogen *et al*, 2002). Recent studies have shown immune and clinical responses with peptide vaccinations against single epitopes of WT1 (Oka *et al*, 2004; Keilholz *et al*, 2009); however, naturally occurring $CD8^+$ T cell responses against myeloid leukaemias target multiple epitopes, potentially enhancing the strength and diversity of these responses (Gannagé *et al*, 2005; Rezvani *et al*, 2005). We have identified two distinct peptide epitopes of WT1 that are presented by HLA-A0201 (A2) and function as targets for leukaemia-reactive $CD8^+$ CTL: pWT126 and pWT235 (Bellantuono *et al*, 2002). We hypothesized that the use of peptide vaccination with both epitopes would result in a robust immunological response with reduced risk of tumour escape. Here we report a phase I/II clinical trial of peptide vaccination in HLA-A2-positive patients with poor risk AML, using pWT126, pWT235 and the pan HLA-DR T helper cell epitope (PADRE) together with Montanide adjuvant.

Methods

Study design and patient population

This was a phase I/II multi-centre dose escalation trial in HLA-A*0201-positive patients with poor risk AML. The primary objectives of the study were to determine the safety and toxicity profile of peptide vaccination with a combination of WT1 peptides and PADRE in Montanide adjuvant, and to evaluate the induction of autologous WT1-specific CTL responses; the secondary objective was to document any disease response. Details of the clinical trial inclusion criteria are given in Table I.

The trial was approved by the UK National Research Ethics Service and all participants gave written informed consent.

Vaccine preparation and administration

pWT126 (WT1 126–134: RMFPNAPYL), pWT235 (WT1 235–243: CMTWNQMNL) and PADRE [H-D-Ala-Cha -Val-Ala-Ala-Trp-Thr-Leu-Lys-Ala-Ala-D-Ala-εAhx-Cys-OH (Alexander *et al*, 1994)] peptides were synthesized to GMP grade by Bachem UK Ltd. (St Helens, Merseyside, UK). Peptides were reconstituted to 3 mg/ml in water with a maximum dimethyl sulfoxide (DMSO) concentration of 25% v/v and stored at −20°C. On the day of injection, peptides were thawed and a water-in-oil emulsion vaccine was prepared by mixing the peptides in 1:1 ratio (aqueous phase) with an equal volume of the adjuvant Montanide ISA-51 (oil phase; Seppic, Puteaux, France). Vaccines containing pWT126 with PADRE, and pWT235 with PADRE, were administered subcutaneously within 4 h of preparation at two separate sites, to avoid competitive binding of HLA-A*0201 by the WT1 peptides.

Dose escalation schedule

Three dose levels were used, comprising 0·3, 0·6 or 1·0 mg peptide. One vaccination cycle comprised separate subcutaneous injections of 0·3/0·6/1·0 mg pWT126 + 0·3/0·6/1·0 mg PADRE peptide, and of 0·3/0·6/1·0 mg pWT235 + 0·3/0·6/1·0 mg PADRE peptide, each admixed with Montanide ISA-51. Vaccination cycles were repeated every 3 weeks to a maximum of five cycles. After three of three patients had completed the initial 0·3 mg dose level with no evidence of grade 3–4 toxicity attributable to vaccination, a further three patients were treated at the 0·6 mg dose level. In the absence of grade 3–4 toxicity at this level, further patients received treatment at the 1·0 mg dose level.

Assessment of toxicity

At each outpatient visit, patients were evaluated for toxicities according to the National Cancer Institute, COMMON TOXICITY

Table I. Inclusion criteria.

HLA-A*0201-positive
18–75 years
No fludarabine in previous 3 months
World Health Organization performance status 0–2
Life expectancy 6 months or greater
Haemoglobin ≥70 g/l; neutrophil count ≥0·2 × 10^9/l; lymphocyte count >0·5 × 10^9/l; platelet count ≥40 × 10^9/l
Serum bilirubin, alanine aminotransferase and/or aspartate aminotransferase <3 times upper limit of normal reference range
Creatinine clearance ≥30 ml/min
Patients NOT eligible for haematopoietic stem cell transplantation, with:
AML in CR2 or greater
Good and standard risk AML in CR1 or stable PR (<20% blasts) in patients >60 years
Poor risk AML in CR1 or PR (slow remitters and/or adverse cytogenetics)
AML at first relapse post-HCST in CR or PR following re-induction and consolidation

AML, acute myeloid leukaemia; CR, complete remission; CR1, first CR; CR2, second CR; PR, partial remission; HSCT, haematopoietic stem cell transplantation.

CRITERIA v2.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcmanual_v4_10-4-99.pdf). Dose limiting toxicity was defined as grade 3–4 non-haematological toxicity, or grade 4 haematological toxicity, that had been determined by defined criteria to be certainly or probably related to the vaccine.

Detection of antigen-specific CD8$^+$ T cells

WT1-specific T cells were detected by staining with phycoerythrin-conjugated HLA-A*0201-pWT126 tetramer (Beckman Coulter, High Wycombe, Buckinghamshire, UK) or dextramer (Immudex, Copenhagen, Denmark), allophycocyanin-conjugated antibody to CD3 and fluorescein isothyocyanate-conjugated antibody to CD8 (BD Biosciences, Oxford, UK), as described previously (King *et al*, 2009). The multimers have mutations in the alpha-3 domain of the major histocompatibilty complex (MHC) molecule that reduces binding to CD8 molecules. The frequency of WT1-specific T cells was expressed as the percentage of gated CD3$^+$ CD8$^+$ T cells that were HLA-A*0201-pWT126-positive. The detection limit of this tetramer had previously been determined as 0·05% of viable CD3$^+$ CD8$^+$ T cells in patients with breast cancer and prostate cancer (Gillmore *et al*, 2006; King *et al*, 2009). Control cytomegalovirus (CMV)-specific T cells were detected by substituting the HLA-A*0201-pWT126 tetramer with HLA-A*0201-NLV tetramer (Beckman Coulter). Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences) and analysed using FacsDiva software (BD Biosciences).

Heparinized peripheral blood samples were obtained prior to the first vaccination cycle, at weeks 3, 6, 9 and 12, and after the last vaccination at weeks 15 and 18–20. Serial cryopreserved mononuclear cell samples from each patient were analysed simultaneously. As primary assay, WT1-specific T cells were detected by tetramer staining following 8 d in culture with pWT126 peptide in the presence of interleukin (IL) 2 and IL7 at 20 units/ml and 5 ng/ml respectively (Roche, Welwyn Garden City, Herts, UK and R&D Systems, Abingdon, Oxfordshire, UK), as described previously (King *et al*, 2009). Induction of a T-cell response to WT1 vaccination was defined as an increase in frequency of tetramer-positive CD8$^+$ T cells of >1·5 times that at baseline, as in previous studies (Oka *et al*, 2004).

Analysis of antigen-specific CD8$^+$ T cell function in vitro

Functional analysis of WT1-specific T cell responses was carried out by detection of γ-interferon (IFN-γ) secretion using an enzyme-linked immunosorbent spot (ELISPOT) assay (BD Biosciences), as described previously (King *et al*, 2009). Autologous peripheral blood mononuclear cells (PBMC) were used to present peptides to the expanded T cells. ELISPOT assays were performed in triplicate, with each well containing 2×10^5 autologous PBMC, 5 μmol/l pWT126 or pWT235 peptide and 2×10^5 responder T cells. After incubation for 20 h at 37°C, the cells were discarded and plates were washed and developed with a second, biotinylated, antibody to human IFN-γ and streptavidin-alkaline phosphatase, according to the manufacturer's instructions. ELISPOT wells containing responder T cells and autologous PBMC with irrelevant peptides served as negative controls. Stimulation of 5×10^4 responder T cells with 200 ng/ml of staphylococcal enterotoxin B was used as a positive control. ELISPOT plates were read using an AxioCamMRc plate reader and KS Elispot software (Zeiss, Cambridge, UK). Results were expressed as the mean number of spot-forming units per 10^6 cells from triplicate wells, and a cytokine response was considered positive if the mean number of spot-forming units per 10^6 cells was greater than two standard deviations above that in the absence of cognate peptide.

The capacity of vaccination-induced WT1-specific CD8$^+$ T cells to undergo antigen-specific expansion *ex vivo* was measured by HLA-A*0201-pWT126 dextramer staining and flow cytometric analysis following restimulation over 14 d: 3×10^6 responder T cells were incubated with 2×10^6 autologous PBMC and 5 μmol/l peptide in culture medium supplemented with 20 units/ml IL2 (Roche), 5 ng/ml IL7 (R&D Systems), together with additional 10 ng/ml IL15 (R&D Systems) and 30 ng/ml IL21 (Peprotech, London, UK), as previously shown (King *et al*, 2009) to maximize antigen-specific *in vitro* expansion. pWT126 peptide was used as test peptide, with CMV pp65 NLVPMVATV (NLV) peptide (ProImmune, Oxford, UK) as positive control in CMV-responsive patients.

Measurement of WT1 *mRNA levels by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)*

All samples for qRT-PCR were blinded. Total RNA was extracted from PBMC and reverse transcribed using the standardized protocol developed within the Europe Against Cancer (EAC) programme (Gabert *et al*, 2003). qRT-PCR assays were performed on an Applied Biosystems ABI7900 instrument (Applied Biosystems, Foster City, CA, USA) according to EAC conditions, with standardized *WT1*-specific primers (Van Dijk *et al*, 2002; Cilloni *et al*, 2009) and probes at final concentrations of 300 and 200 nmol/l respectively (Gabert *et al*, 2003). In patients with *NPM1* mutations, the mutant transcript was monitored by qRT-PCR in parallel with *WT1*, according to the method of Gorello *et al* (2006). Reactions were performed in triplicate wells with appropriate water controls. Expression of the housekeeping gene *ABL1* was used as the endogenous cDNA quantity control for all samples (Cilloni *et al*, 2009).

Statistics

The pre-specified primary immunological trial objective was to evaluate induction of autologous WT1-specific T cell responses. Baseline and maximum post-vaccination responses were compared using the paired two-tailed *t*-test. Spearman

ρ was used to correlate antigen-specific responses to pWT126 and pWT235 peptides. GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. *P* values of 0·05 or less were considered statistically significant.

Results

Patient characteristics

Demographics of the patients are shown in Table II. The eight patients comprised three males and five females with AML. The median age was 65 years (range 56–75 years). All had received previous chemotherapy. At the start of vaccination, four were in morphological first complete remission (CR1) (one with persistent cytogenetic abnormalities), one in CR2 post-autograft, two in partial remission with 15% blasts and one with slowly progressive disease.

Protocol administration and toxicity

Three patients received vaccination at the 0·3 mg dose level, three at the 0·6 mg dose level and two at the 1·0 mg dose level. Five patients completed all five vaccination cycles; two received four cycles and one received two cycles, owing to disease progression. No toxicity greater than grade 3 was observed in any cohort; of note, no renal toxicity, no haematological toxicity and no autoimmune phenomena were attributable to vaccination. All patients developed local reactions comprising erythema, swelling and induration of up to grade 3 at vaccination sites, in one case requiring subsequent vaccination to be deferred by 3 weeks. These results indicate that vaccination with a combination of WT1 peptides and PADRE in Montanide adjuvant is well tolerated.

Vaccination induces WT1-specific immune responses

Seven of eight patients were evaluable for assessment of T cell response. PBMC samples were stained with HLA-A*0201-pWT126 tetramer and analysed for circulating CD8+ T cells specific for WT1 using flow cytometry. Overall, the frequency of WT1 tetramer-positive CD8+ T cells increased from a median of 0·1% at baseline to a median of 0·45% at maximum post-vaccination response (Fig 1A and B). Six of seven patients showed an increase in frequency of WT1 tetramer-positive CD8+ T cells >1·5 times that at baseline, a criterion for response used in previous studies (Oka et al, 2004). However, the increase in WT1 tetramer-positive T cells was not significant when the pre- and post-vaccination data were analysed using a paired two-tailed t-test.

We next analysed functional CD8+ T cell responses to pWT126, pWT235 and PADRE peptides by IFN-γ ELISPOT. Six of seven patients showed antigen-specific IFN-γ responses to all three peptides following vaccination (Fig 1C and Table III), the earliest response being detected after a single vaccination. Four of five patients in whom pWT126 and pWT235-specific responses were detected continued to demonstrate these responses at follow-up in weeks 15–18;

Table II. Patient characteristics.

Patient	Sex	Age (years)	Diagnosis (morphological subtype)	Cytogenetics at diagnosis	Previous treatment	Disease status pre-trial
RFH 001	Male	61	AML (M0)	46, XY, t(1;2) (p32;p13)	ADE 3 + 10 (no response); FlAG-Ida (to CR); FlAG; MACE	Morphological CR1; MRD positive
RFH 002	Female	68	AML (M1)	Failed	DA + GO (to CR); DA	CR1; low platelet count
RFH 003	Female	56	Secondary AML (M5)	Normal	MDS RAEB-1 treated with FlAG (to CR1); DA; MACE; MidAC Relapsed with AML after 3 years: treated with FlAG; FlAG-Ida (to CR2); Bu/Cy ASCT.	CR2
UCH 001	Female	64	AML (M1)	Normal	DA × 1; LD ara-C × 3	Stable PR; 15% blasts
UCH 002	Female	65	AML (M1)	Normal	DA × 1; LD ara-C × 8	PR; 15% blasts
UCH 003	Female	75	AML (M4)	Normal	LD ara-C + GO × 2 (to CR); LD ara-C × 2	CR1
BHM 001	Male	66	AML	46, XY, der(7) t(7;11) (q22;q13)	DA × 1; MidAC × 2	PR; slowly progressive
BHM 002	Male	68	AML (M0)	Normal	DA × 2; MidAC × 1	CR1

RFH, Royal Free Hospital, London; UCH, University College Hospital, London; BHM, Queen Elizabeth Hospital, Birmingham; AML, acute myeloid leukaemia; CR, complete remission; CR1, first CR; CR2, second CR; PR, partial remission; MRD, minimal residual disease; MDS RAEB-1, myelodysplastic syndrome refractory anaemia with excess blasts-1; ADE, daunorubicin, cytarabine and etoposide; Bu/Cy ASCT, busulfan and cyclophosphamide-conditioned autologous stem cell transplantation; DA, daunorubicin and cytarabine; FlAG, fludarabine, cytarabine and granulocyte colony-stimulating factor; GO, gemtuzumabozogamicin; Ida, idarubicin; LD ara-C, low-dose cytarabine; MACE, amsacrine, cytarabine and etoposide; MidAC, mitoxantrone and cytarabine.

however, one patient had lost pWT126- and pWT235-specific responses. Responses to pWT126 and pWT235 were highly correlated (P = 0·0008).

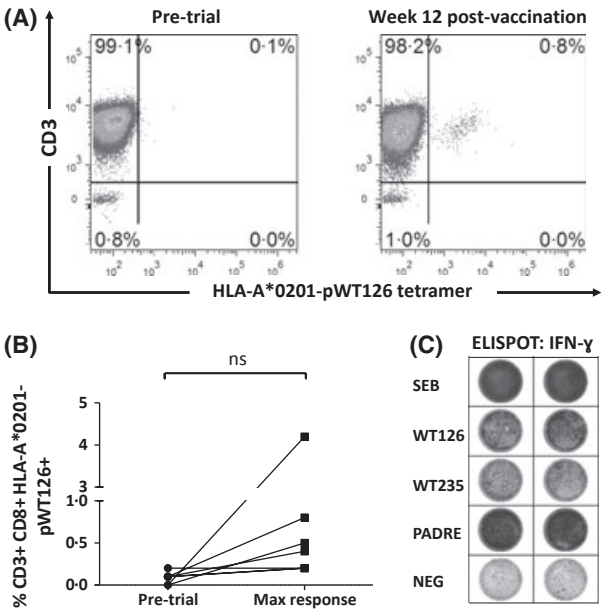

Fig 1. WT1-specific CD8$^+$ T cell response to vaccination. Tetramer analysis of peripheral blood mononuclear cells was performed by flow cytometry. Plots shown are gated on viable CD8$^+$ T cells. Representative data from Patient RFH003 (A) and maximal responses for all patients (B) are shown. (C) Functional responses were assessed by ELISPOT for γ-interferon (IFN-γ). Representative responses from Patient RFH003 are shown.

Vaccination-induced WT1-specific T cells do not expand in vitro

We then examined the potential for WT1-specific CD8$^+$ T cells isolated from patients after vaccination to expand following re-challenge with the WT1 pWT126 peptide *in vitro*. PBMC samples from four patients with at least four-fold WT1 tetramer response were stimulated over 2 weeks *in vitro* with WT1 pWT126 peptide or CMV pp65 NLV peptide. Two of these patients were CMV IgG seropositive. Following stimulation, both of these patients showed a two-fold or greater increase in the frequency of CMV pp65-specific CD8$^+$ T cells (Fig 2). In contrast, WT1-specific CD8$^+$ T cells did not respond to stimulation (Fig 2 and data not shown), indicating that the vaccine-induced WT1-specific CD8$^+$ T cells are unable to proliferate and expand upon re-stimulation with peptide antigen *in vitro*.

Disease activity after vaccination

To assess the potential effect of vaccination on leukaemia, *WT1* mRNA expression was used as a surrogate marker of minimal residual disease. In two patients, *NPM1* mutations were present, allowing disease progression to be assessed independently of the targeted antigen.

In two of five evaluable patients (RFH001, RFH003), the emergence of functional WT1-specific T cells after vaccination at weeks 9 and 12 respectively, was followed by a decrease in WT1 mRNA levels to below the upper threshold limit observed in normal peripheral blood samples (Fig 3). Patients in whom there was no increase in

Table III. Immunological and clinical response to vaccination.

Patient	Vaccine dose (mg)	Cycles completed	Pre-trial WT1 tetramer (% of CD8$^+$)	Maximum post-vaccination tetramer (% of CD8$^+$) and time point	Tetramer response >1·5 × pre-trial?	IFN-γ response by ELISPOT			Time from start of study to disease progression
						pWT126	pWT235	PADRE	
RFH 001	0·3	5	0·1	0·2 maximum week 9	+	+	+	+	9 months
RFH 002	1·0	4	0·1	0·2 maximum week 9	+	+	+	+	39 months
RFH 003	1·0	5	0·1	0·8 maximum week 12	+	+	+	+	>41 months
UCH 001	0·3	5	N/A	1·4 N/A	N/A	N/A	N/A	N/A	18 months
UCH 002	0·3	4	0·2	0·2 N/A	−	+	+	+	9 weeks
UCH 003	0·6	5	0·0	0·5 maximum week 15	+	+	+	+	7 months
BHM 001	0·6	2	0·1	0·4 maximum week 6	+	−	−	−	6 weeks
BHM 002	0·6	5	0·0	4·2 maximum week 6	+	+	+	+	10 months

RFH, Royal Free Hospital, London; UCH, University College Hospital, London; BHM, Queen Elizabeth Hospital, Birmingham; ELISPOT, enzyme-linked immunosorbent spot; N/A, not applicable.

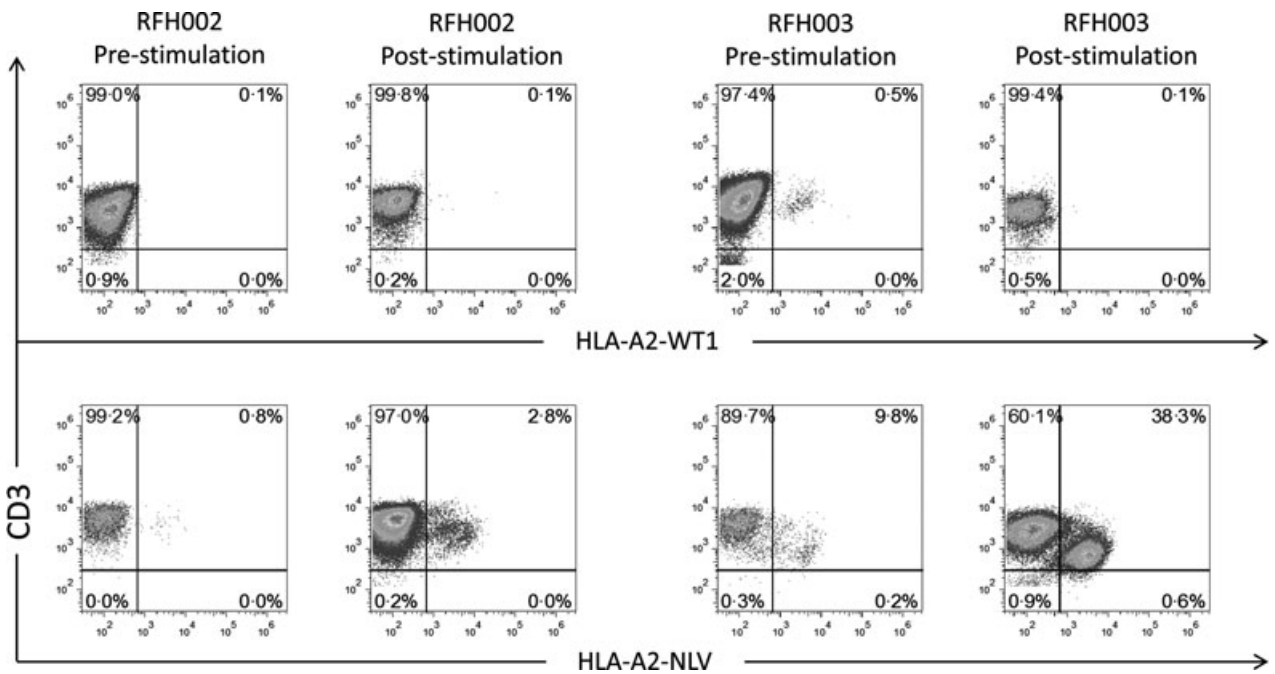

Fig 2. Vaccination-induced WT1-specific immune responses are short-lived. Peripheral blood mononuclear cells were isolated from two pWT126/pWT235/PADRE-vaccinated, cytomegalovirus (CMV) IgG seropositive patients and re-stimulated with WT126 (WT1) or NLV (CMV) peptides for 2 weeks. Frequencies of WT1- and CMV-specific $CD8^+$ T cells were measured by dextramer staining and flow cytometric analysis. Plots shown are gated on viable $CD8^+$ T cells.

WT1-specific T cells, or in whom functional IFN-γ responses could not be detected, showed stable or increasing WT1 mRNA levels. However, there was no correlation between vaccine dose, emergence of WT1-specific T cells or disease response.

Discussion

Vaccination with a combination of WT1 and PADRE peptides in Montanide adjuvant was well tolerated and safe, with no evidence of renal toxicity nor the haematological toxicity that was previously observed in myelodysplastic syndrome patients in one study (Oka *et al*, 2003). The primary immunological objective of this trial was the assessment of WT1-specific $CD8^+$ T cell responses following vaccination. In accordance with the recommendations of the Society for Biological Therapy (Keilholz *et al*, 2002), tetramer-based quantitative assays and ELISPOT assays of function were used to determine T cell responses. Both assays showed responses to vaccination in six/seven patients (86%), a response rate that compares favourably with that achieved in other trials of WT1 peptide vaccination in AML (Oka *et al*, 2004; Rezvani *et al*, 2008; Keilholz *et al*, 2009; Maslak *et al*, 2010; Kuball *et al*, 2011). Using WT1 peptides with Montanide adjuvant, several groups have reported immunological responses in 62·5–87·5% of patients with AML in CR (Oka *et al*, 2004; Rezvani *et al*, 2008; Maslak *et al*, 2010). In patients with residual disease, Keilholz *et al* (2009) used keyhole-limpet-haemocyanin (KLH) adjuvant with granulocyte-macrophage colony-stimulating factor to enhance immunogenicity, achieving WT1-specific tetramer responses in 44%: it was suggested that this lower response rate might be due either to an immunosuppressive effect of leukaemic blasts or to responses falling below the limits of detection of the assays used (Keilholz *et al*, 2009). In our study, immunological responses were seen within 2 weeks of a single vaccination: similarly rapid responses have been observed previously, suggesting that the vaccination-induced expansion of self-restricted WT1-specific cells originates from pre-existing memory $CD8^+$ T cells (Oka *et al*, 2003; Rezvani *et al*, 2008). However, we did not find these early immunological responses to correlate with clinical outcome, possibly owing to the small sample size; only one group has previously been able to show a correlation between clinical and immunological responses (Oka *et al*, 2004).

Importantly, we were unable to elicit secondary expansion of WT1-specific $CD8^+$ cells on re-stimulation with the cognate pWT126 antigen *in vitro*. By contrast, CMV-specific $CD8^+$ T cells from the same patients exhibited robust potential for expansion, suggesting that the WT1-specific $CD8^+$ T cells generated following vaccination were functionally impaired, or were of insufficient functional avidity. Such antigen-specific T cell expansion is a key feature of immune memory, and predicts tumour regression following cancer vaccination in mice (Janssen *et al*, 2005; Rosato *et al*, 2006). The failure of vaccination to establish functional immune

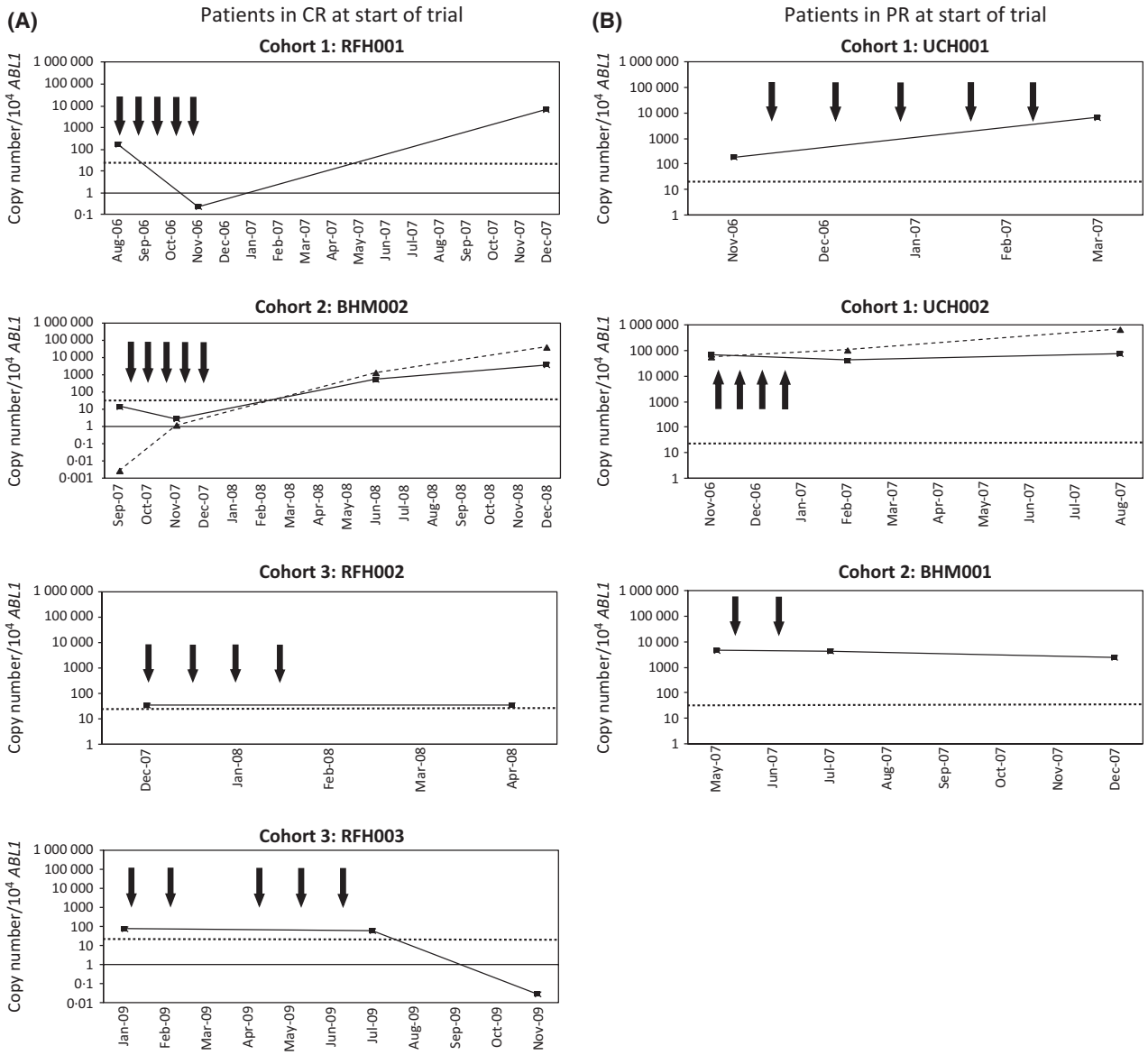

Fig 3. Disease response to vaccination with pWT126, pWT235 and PADRE peptides as measured by *WT1*/*ABL1* mRNA quantification. Results are shown for individual patients in complete remission (CR) (A) or partial remission (PR) (B) at the start of the trial. Expression of *WT1* mRNA (solid, black squares) and, in patients with mutations in *NPM1*, *NPM1* mutant mRNA (dashed, triangles) are shown as a ratio of the gene of interest to *ABL1*. Months from the start of trial are shown on the *X* axis. Bold arrows denote times of vaccination and the time at which maximal tetramer responses were detected are given in the results section. The horizontal dotted line corresponds to the upper limit of *WT1* expression in normal peripheral blood, as previously defined (Cilloni *et al*, 2009). RFH, Royal Free Hospital, London; UCH, University College Hospital, London; BHM, Queen Elizabeth Hospital, Birmingham.

memory may explain the transient nature of the immune responses typically observed in cancer vaccination trials. Rezvani *et al* (2011) recently found that initial WT1- and PR1-specific T cell responses were lost in all patients following repeated vaccination over 12 weeks; and although long-term follow-up of three patients from the first trial of WT1 peptide vaccination has demonstrated persistence of WT1-specific $CD8^+$ T cells for over 8 years, in all cases these cells were detectable before vaccination, raising the possibility that their initial priming and expansion was a physiological response to tumour rather than to vaccination (Tsuboi *et al*, 2011).

Different approaches have been used to provide $CD4^+$ T cell help following vaccination. In our study, PADRE peptide was used to stimulate $CD4^+$ T cells through MHC class II. Both PADRE and WT1 peptides elicited an IFN-γ response following vaccination, suggesting that concurrent $CD4^+$ and $CD8^+$ T cell responses were achieved, but this did not correlate with establishment of a memory response. Similarly, Kuball *et al* (2011) were unable to demonstrate antigen-specific

T cell responses in any of four patients with AML who were vaccinated with WT1, PR3 and PADRE peptides in Montanide and CpG adjuvant; PADRE-specific $CD4^+$ T cells in their study were shown to lack the capacity to produce IL2, a cytokine that is required for potent $CD8^+$ T cell responses to a vaccinated antigen.

The choice of adjuvant may also be important in determining the longevity of $CD8^+$ T cell responses to vaccination. Following early murine experiments showing that oil-in-water adjuvants, such as Incomplete Freund's Adjuvant (IFA) and Montanide adjuvant, could enhance immune responses to peptide vaccination (Kast *et al*, 1993), Montanide adjuvant has been used by our group and others in clinical trials (Tsuboi *et al*, 2004; Rezvani *et al*, 2008; Maslak *et al*, 2010; Kuball *et al*, 2011). However, sustained systemic presentation of peptides from the oil-in-water depot in the absence of a continuous helper/danger signal may contribute to induction of $CD8^+$ T cell tolerance, a hypothesis formed by Bijker *et al* (2007) and reinforced by their finding that substitution of IFA with phosphate-buffered saline shortened the duration of antigen presentation and resulted in durable $CD8^+$ T cell responses to a minimal $CD8^+$ epitope.

Although we found that, in two patients, the emergence of functional WT1-specific T cells after vaccination was followed by a decrease in *WT1* mRNA levels to below the upper threshold limit observed in normal control samples, there was no significant correlation between the immunological and disease responses to vaccination. This may relate to the observation that secondary expansion of WT1-specific T cells *in vitro* failed despite IL2, IL7, IL15 and IL21 cytokine cocktail and good CMV-specific T cell expansion. It is well recognized that effective anti-tumour activity of cytotoxic T cells correlates with high avidity for tumour-associated antigens (Zeh *et al*, 1999; Dutoit *et al*, 2001; Yang *et al*, 2002); the lack of consistent clinical response in our study may reflect a failure of vaccination to result in sustained expansion of high-avidity WT1-specific T cell clones. Functional avidity using peptide titration experiments was not tested in our study. In common with other tumour-associated antigens, WT1 is expressed at low levels in some normal tissues (Mundlos *et al*, 1993; Ramani & Cowell, 1996; Baird & Simmons, 1997; Menssen *et al*, 1997), and high-avidity cytotoxic T cell clones may have been deleted or rendered unresponsive during the establishment of tolerance (Theobald *et al*, 1997; Nugent *et al*, 2000). In mice, repeated immunization with foreign antigen generates memory $CD8^+$ T cells that have increased avidity for that antigen; in contrast, such avidity maturation is limited for self antigen, such as WT1 (Turner *et al*, 2008). Similarly, antigen-specific memory $CD8^+$ T cells in patients vaccinated with a melanoma peptide failed to acquire the enhanced functional avidity usually associated with competent memory T cell maturation (Walker *et al*, 2008), while Rezvani *et al* (2011) demonstrated selective deletion of high-avidity leukaemia-specific $CD8^+$ T cells following vaccination with WT1 and PR1 peptides.

Although early trials of peptide vaccination in cancer patients showed promising immunological responses, more recent reports have been predominantly negative (Kuball *et al*, 2011; Rezvani *et al*, 2011). Our results illustrate further the difficulties of expansion of high-avidity leukaemia-specific effector and memory $CD8^+$ T cell clones through vaccination with short self-peptides, demonstrating that translational studies are still required to define the site, time period, dose and adjuvants that lead to optimal activation of both $CD8^+$ and $CD4^+$ T cell responses following delivery of peptide. The low toxicity and relative simplicity of administration of peptide vaccines remain attractive, with almost 100 open studies of peptide vaccination currently listed on clinicaltrials.gov; however, our data lend impetus to alternative strategies, such as adoptive cellular immunotherapy with T cell receptor gene-modified T cells, that have the potential to generate potent antigen-specific cytotoxic responses against cancer while circumventing the difficulties of peptide vaccination.

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Author contributions

BU analysed the data and wrote the paper; IM-D performed the research and analysed the data; AI performed the research; CC performed the research; FC performed the research and analysed the data; AV performed the research; PK performed the research; DG performed the research, provided reagents and analysed the data; AK performed the research; HS designed the study and analysed the data; EM designed the study, performed the research, analysed the data and wrote the paper.

References

Alexander, J., Sidney, J., Southwood, S., Ruppert, J., Oseroff, C., Maewal, A., Snoke, K., Serra, H.M., Kubo, R.T. & Sette, A. (1994) Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. *Immunity*, **1**, 751–761.

Algar, E.M., Khromykh, T., Smith, S.I., Blackburn, D.M., Bryson, G.J. & Smith, P.J. (1996) A WT1 antisense oligonucleotide inhibits proliferation and induces apoptosis in myeloid leukaemia cell lines. *Oncogene*, **12**, 1005–1014.

Baird, P.N. & Simmons, P.J. (1997) Expression of the Wilms' tumor gene (WT1) in normal hemopoiesis. *Experimental Hematology*, **25**, 312–320.

Bellantuono, I., Gao, L., Parry, S., Marley, S., Dazzi, F., Apperley, J., Goldman, J.M. & Stauss, H.J. (2002) Two distinct HLA-A0201-presented epitopes of the Wilms tumor antigen 1 can function as targets for leukemia-reactive CTL. *Blood*, **100**, 3835–3837.

Bijker, M.S., van den Eeden, S.J.F., Franken, K.L., Melief, C.J.M., Offringa, R. & van der Bur, S.H. (2007) CD8+ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. *Journal of Immunology* (Baltimore, Md.: 1950), **179**, 5033–5040.

Call, K.M., Glaser, T., Ito, C.Y., Buckler, A.J., Pelletier, J., Haber, D.A., Rose, E.A., Kral, A., Yeger, H. & Lewis, W.H. (1990) Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell*, **60**, 509–520.

Cilloni, D., Gottardi, E., Messa, F., Fava, M., Scaravaglio, P., Bertini, M., Girotto, M., Marinone, C., Ferrero, D., Gallamini, A., Levis, A. & Saglio, G. (2003) Significant correlation between the degree of WT1 expression and the International Prognostic Scoring System Score in patients with myelodysplastic syndromes. *Journal of Clinical Oncology*, **21**, 1988–1995.

Cilloni, D., Renneville, A., Hermitte, F., Hills, R.K., Daly, S., Jovanovic, J.V., Gottardi, E., Fava, M., Schnittger, S., Weiss, T., Izzo, B., Nomdedeu, J., van der Heijden, A., van der Reijden, B.A., Jansen, J.H., van der Velden, V.H.J., Ommen, H., Preudhomme, C., Saglio, G. & Grimwade, D. (2009) Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute myeloid leukemia: a European Leukemia Net study. *Journal of Clinical Oncology*, **27**, 5195–5201.

Dutoit, V., Rubio-Godoy, V., Dietrich, P.Y., Quiqueres, A.L., Schnuriger, V., Rimoldi, D., Liénard, D., Speiser, D., Guillaume, P., Batard, P., Cerottini, J.C., Romero, P. & Valmori, D. (2001) Heterogeneous T-cell response to MAGE-A10 (254-262): high avidity-specific cytolytic T lymphocytes show superior antitumor activity. *Cancer Research*, **61**, 5850–5856.

Gabert, J., Beillard, E., van der Velden, V.H.J., Bi, W., Grimwade, D., Pallisgaard, N., Barbany, G., Cazzaniga, G., Cayuela, J.M., Cavé, H., Pane, F., Aerts, J.L.E., De Micheli, D., Thirion, X., Pradel, V., González, M., Viehmann, S., Malec, M., Saglio, G. & van Dongen, J.J.M. (2003) Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. *Leukemia*, **17**, 2318–2357.

Gannagé, M., Abel, M., Michallet, A.-S., Delluc, S., Lambert, M., Giraudier, S., Kratzer, R., Niedermann, G., Saveanu, L., Guilhot, F., Camoin, L., Varet, B., Buzyn, A. & Caillat-Zucman, S. (2005) Ex vivo characterization of multiepitopic tumor-specific CD8 T cells in patients with chronic myeloid leukemia: implications for vaccine development and adoptive cellular immunotherapy. *Journal of Immunology* (Baltimore, Md.: 1950), **174**, 8210–8218.

Gao, L., Bellantuono, I., Elsasser, A., Marley, S.B., Gordon, M.Y., Goldman, J.M. & Stauss, H.J. (2000) Selective elimination of leukemic CD34+ progenitor cells by cytotoxic T lymphocytes specific for WT1. *Blood*, **95**, 2198–2203.

Gao, L., Xue, S.-A., Hasserjian, R., Cotter, F., Kaeda, J., Goldman, J.M., Dazzi, F. & Stauss, H.J. (2003) Human cytotoxic T lymphocytes specific for Wilms' tumor antigen-1 inhibit engraftment of leukemia-initiating stem cells in non-obese diabetic-severe combined immunodeficient recipients. *Transplantation*, **75**, 1429–1436.

Gillmore, R., Xue, S.-A., Holler, A., Kaeda, J., Hadjiminas, D., Healy, V., Dina, R., Parry, S.C., Bellantuono, I., Ghani, Y., Coombes, R.C., Waxman, J. & Stauss, H.J. (2006) Detection of Wilms' tumor antigen–specific CTL in tumor-draining lymph nodes of patients with early breast cancer. *Clinical Cancer Research*, **12**, 34–42.

Goldman, J.M., Gale, R.P., Horowitz, M.M., Biggs, J.C., Champlin, R.E., Gluckman, E., Hoffmann, R.G., Jacobsen, S.J., Marmont, A.M. & McGlave, P.B. (1988) Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion. *Annals of Internal Medicine*, **108**, 806–814.

Gorello, P., Cazzaniga, G., Alberti, F., Dell'Oro, M.G., Gottardi, E., Specchia, G., Roti, G., Rosati, R., Martelli, M.F., Diverio, D., Lo Coco, F., Biondi, A., Saglio, G., Mecucci, C. & Falini, B. (2006) Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. *Leukemia*, **20**, 1103–1108.

Horowitz, M.M., Gale, R.P., Sondel, P.M., Goldman, J.M., Kersey, J., Kolb, H.J., Rimm, A.A., Ringdén, O., Rozman, C. & Speck, B. (1990) Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*, **75**, 555–562.

Inoue, K., Ogawa, H., Sonoda, Y., Kimura, T., Sakabe, H., Oka, Y., Miyake, S., Tamaki, H., Oji, Y., Yamagami, T., Tatekawa, T., Soma, T., Kishimoto, T. & Sugiyama, H. (1997) Aberrant over expression of the Wilms Tumor gene (WT1) in human leukemia. *Blood*, **89**, 1405–1412.

Janssen, E.M., Droin, N.M., Lemmens, E.E., Pinkoski, M.J., Bensinger, S.J., Ehst, B.D., Griffith, T.S., Green, D.R. & Schoenberger, S.P. (2005) CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*, **434**, 88–93.

Kast, W.M., Brandt, R.M. & Melief, C.J. (1993) Strict peptide length is not required for the induction of cytotoxic T lymphocyte-mediated antiviral protection by peptide vaccination. *European Journal of Immunology*, **23**, 1189–1192.

Keilholz, U., Weber, J., Finke, J.H., Gabrilovich, D.I., Kast, W.M., Disis, M.L., Kirkwood, J.M., Scheibenbogen, C., Schlom, J., Maino, V.C., Lyerly, H.K., Lee, P.P., Storkus, W., Marincola, F., Worobec, A. & Atkins, M.B. (2002) Immunologic monitoring of cancer vaccine therapy: results of a workshop sponsored by the Society for Biological Therapy. *Journal of Immunotherapy* (Hagerstown, Md.: 1997), **25**, 97–138.

Keilholz, U., Letsch, A., Busse, A., Asemissen, A.M., Bauer, S., Blau, I.W., Hofmann, W.-K., Uharek, L., Thiel, E. & Scheibenbogen, C. (2009) A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. *Blood*, **113**, 6541–6548.

King, J.W., Thomas, S., Corsi, F., Gao, L., Dina, R., Gillmore, R., Pigott, K., Kaisary, A., Stauss, H.J. & Waxman, J. (2009) IL15 can reverse the unresponsiveness of Wilms' tumor antigen-specific CTL in patients with prostate cancer. *Clinical Cancer Research*, **15**, 1145–1154.

Kuball, J., de Boer, K., Wagner, E., Wattad, M., Antunes, E., Weeratna, R.D., Vicari, A.P., Lotz, C., van Dorp, S., Hol, S., Greenberg, P.D., Heit, W., Davis, H.L. & Theobald, M. (2011) Pitfalls of vaccinations with WT1-, Proteinase3- and MUC1-derived peptides in combination with Montanide ISA51 and CpG7909. *Cancer Immunology, Immunotherapy: CII*, **60**, 161–171.

Maslak, P.G., Dao, T., Krug, L.M., Chanel, S., Korontsvit, T., Zakhaleva, V., Zhang, R., Wolchok, J.D., Yuan, J., Pinilla-Ibarz, J., Berman, E., Weiss, M., Jurcic, J., Frattini, M.G. & Scheinberg, D.A. (2010) Vaccination with synthetic analog peptides derived from WT1 oncoprotein induces T-cell responses in patients with complete remission from acute myeloid leukemia. *Blood*, **116**, 171–179.

Menssen, H.D., Renkl, H.J., Entezami, M. & Thiel, E. (1997) Wilms' tumor gene expression in human CD34+ hematopoietic progenitors during fetal development and early clonogenic growth. *Blood*, **89**, 3486–3487.

Miyoshi, Y., Ando, A. & Egawa, C. (2002) High expression of Wilms 'Tumor suppressor gene predicts poor prognosis in breast cancer patients. *Clinical Cancer Research*, **8**, 1167–1171.

Mundlos, S., Pelletier, J., Darveau, A., Bachmann, M., Winterpacht, A. & Zabel, B. (1993) Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. *Development (Cambridge, England)*, **119**, 1329–1341.

Nugent, C.T., Morgan, D.J., Biggs, J.A., Ko, A., Pilip, I.M., Pamer, E.G. & Sherman, L.A. (2000) Characterization of CD8+ T lymphocytes that persist after peripheral tolerance to a self antigen expressed in the pancreas. *Journal of Immunology* (Baltimore, Md.: 1950), **164**, 191–200.

Odom, L., Githens, J., Morse, H., Sharma, B., August, C., Humbert, J., Peakman, D., Rusnak, S. & Johnson, F. (1978) Remission of relapsed leukæmia during a graft-versus-host reaction a "graft-versus-leukæmia reaction" in man? *The Lancet*, **312**, 537–540.

Oji, Y., Miyoshi, S., Maeda, H., Hayashi, S., Tamaki, H., Nakatsuka, S.-I., Yao, M., Takahashi, E., Nakano, Y., Hirabayashi, H., Shintani, Y., Oka, Y., Tsuboi, A., Hosen, N., Asada, M., Fujioka, T., Murakami, M., Kanato, K., Motomura, M., Kim, E.H., Kawakami, M., Ikegame, K., Ogawa, H., Aozasa, K., Kawase, I. & Sugiyama, H. (2002) Over expression of the Wilms' tumor gene WT1 in de novo lung cancers. *International Journal of Cancer*, **100**, 297–303.

Oka, Y., Tsuboi, A., Murakami, M., Hirai, M., Tominaga, N., Nakajima, H., Elisseeva, O.A., Masuda, T., Nakano, A., Kawakami, M., Oji, Y., Ikegame, K., Hosen, N., Udaka, K., Yasukawa, M., Ogawa, H., Kawase, I. & Sugiyama, H. (2003) Wilms tumor gene peptide-based immunotherapy for patients with overt leukemia from myelodysplastic syndrome (MDS) or MDS with myelofibrosis. *International Journal of Hematology*, **78**, 56–61.

Oka, Y., Tsuboi, A., Taguchi, T., Osaki, T., Kyo, T., Nakajima, H., Elisseeva, O.A., Oji, Y., Kawakami, M., Ikegame, K., Hosen, N., Yoshihara, S., Wu, F., Fujiki, F., Murakami, M., Masuda, T., Nishida, S., Shirakata, T., Nakatsuka, S.-I., Sasaki, A., Udaka, K., Dohy, H., Aozasa, K., Noguchi, S., Kawase, I. & Sugiyama, H. (2004) Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 13885–13890.

Ramani, P. & Cowell, J.K. (1996) The expression pattern of Wilms' tumour gene (WT1) product in normal tissues and paediatric renal tumours. *The Journal of Pathology*, **179**, 162–168.

Rezvani, K., Grube, M., Brenchley, J.M., Sconocchia, G., Fujiwara, H., Price, D.A., Gostick, E., Yamada, K., Melenhorst, J., Childs, R., Hensel, N., Douek, D.C. & Barrett, A.J. (2003) Functional leukemia-associated antigen-specific memory CD8+ T cells exist in healthy individuals and in patients with chronic myelogenous leukemia before and after stem cell transplantation. *Blood*, **102**, 2892–2900.

Rezvani, K., Brenchley, J.M., Price, D.A., Kilical, Y., Gostick, E., Sewell, A.K., Li, J., Mielke, S., Douek, D.C. & Barrett, A.J. (2005) T-cell responses directed against multiple HLA-A*0201-restricted epitopes derived from Wilms' tumor 1 protein in patients with leukemia and healthy donors: identification, quantification, and characterization. *Clinical Cancer Research*, **11**, 8799–8807.

Rezvani, K., Yong, A.S.M., Mielke, S., Savani, B.N., Musse, L., Superata, J., Jafarpour, B., Boss, C. & Barrett, A.J. (2008) Leukemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. *Blood*, **111**, 236–242.

Rezvani, K., Yong, A.S.M., Mielke, S., Jafarpour, B., Savani, B.N., Le, R.Q., Eniafe, R., Musse, L., Boss, C., Kurlander, R. & Barrett, A.J. (2011) Repeated PR1 and WT1 peptide vaccination in Montanide-adjuvant fails to induce sustained high-avidity, epitope-specific CD8+ T cells in myeloid malignancies. *Haematologica*, **96**, 432–440.

Rosato, A., Zoso, A., Santa, S.D., Milan, G., Del Bianco, P., De Salvo, G.L. & Zanovello, P. (2006) Predicting tumor outcome following cancer vaccination by monitoring quantitative and qualitative CD8+ T cell parameters. *Journal of Immunology*, **176**, 1999–2006.

Saito, Y., Kitamura, H., Hijikata, A., Tomizawa-Murasawa, M., Tanaka, S., Takagi, S., Uchida, N., Suzuki, N., Sone, A., Najima, Y., Ozawa, H., Wake, A., Taniguchi, S., Shultz, L.D., Ohara, O. & Ishikawa, F. (2010) Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. *Science Translational Medicine*, **2**, 17ra9.

Scheibenbogen, C., Letsch, A., Thiel, E., Schmittel, A., Mailaender, V., Baerwolf, S., Nagorsen, D. & Keilholz, U. (2002) CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. *Blood*, **100**, 2132–2137.

Theobald, M., Biggs, J., Hernández, J., Lustgarten, J., Labadie, C. & Sherman, L.A. (1997) Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. *The Journal of Experimental Medicine*, **185**, 833–841.

Tsuboi, A., Oka, Y., Osaki, T., Kumagai, T., Tachibana, I., Hayashi, S., Murakami, M., Nakajima, H., Elisseeva, O.A., Fei, W., Masuda, T., Yasukawa, M., Oji, Y., Kawakami, M., Hosen, N., Ikegame, K., Yoshihara, S., Udaka, K., Nakatsuka, S.-I., Aozasa, K., Kawase, I. & Sugiyama, H. (2004) WT1 peptide-based immunotherapy for patients with lung cancer: report of two cases. *Microbiology and Immunology*, **48**, 175–184.

Tsuboi, A., Oka, Y., Kyo, T., Katayama, Y., Elisseeva, O.A., Kawakami, M., Nishida, S., Morimoto, S., Murao, A., Nakajima, H., Hosen, N., Oji, Y. & Sugiyama, H. (2011) Long-term WT1 peptide vaccination for patients with acute myeloid leukemia with minimal residual disease. *Leukemia*, **26**, 1410–1413.

Turner, M.J., Jellison, E.R., Lingenheld, E.G., Puddington, L. & Lefrançois, L. (2008) Avidity maturation of memory CD8 T cells is limited by self-antigen expression. *The Journal of Experimental Medicine*, **205**, 1859–1868.

Van Dijk, J.P., Knops, G.H.J.N., Van De Locht, L.T.F., Menke, A.L., Jansen, J.H., Mensink, E.J.B.M., Raymakers, R.A.P. & De Witte, T. (2002) Abnormal WT1 expression in the CD34-negative compartment in myelodysplastic bone marrow. *British Journal of Haematology*, **118**, 1027–1033.

Walker, E.B., Haley, D., Petrausch, U., Floyd, K., Miller, W., Sanjuan, N., Alvord, G., Fox, B.A. & Urba, W.J. (2008) Phenotype and functional characterization of long-term gp100-specific memory CD8+ T cells in disease-free melanoma patients before and after boosting immunization. *Clinical Cancer Research*, **14**, 5270–5283.

Yang, S., Linette, G.P., Longerich, S. & Haluska, F.G. (2002) Antimelanoma activity of CTL generated from peripheral blood mononuclear cells after stimulation with autologous dendritic cells pulsed with melanoma gp100 peptide G209-2M is correlated to TCR avidity. *Journal of Immunology* (Baltimore, Md.: 1950), **169**, 531–539.

Zeh, H.J., Perry-Lalley, D., Dudley, M.E., Rosenberg, S.A. & Yang, J.C. (1999) High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *Journal of Immunology* (Baltimore, Md.: 1950), **162**, 989–994.