

Phase I/II study exploring ImMucin, a pan-major histocompatibility complex, anti-MUC1 signal peptide vaccine, in multiple myeloma patients

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Summary

ImMucin, a 21-mer cancer vaccine encoding the signal peptide domain of the MUC1 tumour-associated antigen, possesses a high density of T- and B-cell epitopes but preserves MUC1 specificity. This phase I/II study assessed the safety, immunity and clinical response to 6 or 12 bi-weekly intradermal ImMucin vaccines, co-administered with human granulocyte-macrophage colony-stimulating factor to 15 MUC1-positive multiple myeloma (MM) patients, with residual or biochemically progressive disease following autologous stem cell transplantation. Vaccination was well tolerated; all adverse events were temporal grade 1–2 and spontaneously resolved. ImMucin vaccination induced a robust increase in γ -interferon (IFN- γ -producing CD4+ and CD8+ T-cells (≤ 80 -fold), a pronounced population of ImMucin multimer CD8+ T-cells ($>2\%$), a 9.4-fold increase in peripheral blood mononuclear cells proliferation and 6.8-fold increase in anti-ImMucin antibodies, accompanied with T-cell and antibody-dependent cell-mediated cytotoxicity. A significant decrease in soluble MUC1 levels was observed in 9/10 patients. Stable disease or improvement, persisting for 17.5–41.3 months (ongoing) was achieved in 11/15 patients and appeared to be associated with low-intermediate PDL1 (CD274) bone marrow levels pre- and post-vaccination. In summary, ImMucin, a highly tolerable cancerous vaccine, induces robust, diversified T- and B-cell ImMucin-specific immunity in MM patients, across major histocompatibility complex-barrier, resulting in at least disease stabilization in most patients.

Keywords: ImMucin, signal peptide, MUC1, multiple myeloma, cancer vaccine.

Despite the remarkable improvement in multiple myeloma (MM) treatment outcomes (Attal *et al*, 2012; McCarthy *et al*, 2012), primarily attributed to the introduction of proteasome inhibitors and immunomodulatory agents, MM remains an incurable disease to which most patients succumb. Recently reported prolongation of progression-free survival (PFS), but a disputed improvement in overall survival (OS) (Attal *et al*, 2012, 2013; McCarthy *et al*, 2012), following the post-transplantation administration of immunomodulatory agents, support the proposed role of adoptive anti-MM immune responses under conditions of minimal residual disease (MRD). Cancer vaccines directed against tumour-associated antigens (TAAs) present a promising means of eliminating MRD, without inducing significant

toxicity and secondary malignancies (Gilboa, 2004; Morse & Whelan, 2010).

MUC1 (mucin 1, cell surface associated) is a glycoprotein that is highly expressed by carcinomas and haematological tumours, including MM (Kovjazin *et al*, 2014). Its broad tumour distribution, including on cancer stem cells (Engelmann *et al*, 2008), has established it as a promising target for active vaccination (Cheever *et al*, 2009). Most anti-MUC1 vaccines, targeting the entire molecule or the extracellular tandem repeat array (TRA) domain (Hareuveni *et al*, 1990), trigger inconsistent immunological responses and an inadequate long-term clinical impact, seemingly attributable to the presence of TRA-containing soluble MUC1 (sMUC1) in peripheral blood (PB), which decoys both endogenous and

vaccine-induced antibodies (Fan *et al*, 2010; Thie *et al*, 2011). Additionally, active suppression of T-cell function by sMUC1 may interfere with the desired response (van de Wiel-van Kemenade *et al*, 1993; Agrawal *et al*, 1998). Induction of a stronger and broader B- and T-cell response against MUC1 epitopes exclusively expressed on tumour cells (Kovjazin *et al*, 2011a, 2014), may lead to improved clinical outcomes.

ImMucin, a 21-mer synthetic long-peptide (LP) vaccine, containing the entire MUC1 signal peptide (SP) domain and free of sMUC1-related epitopes (Kovjazin *et al*, 2012), was predicted *in-silico* to strongly bind multiple MUC1-specific, major histocompatibility complex (MHC) class I, II (Carmon *et al*, 2000; Kovjazin *et al*, 2011a; Kovjazin & Carmon, 2014) and B-cell epitopes (Kovjazin *et al*, 2012, 2014; Kovjazin & Carmon, 2014), suggesting its capacity to promote robust and diversified MUC1-specific CD4⁺ and CD8⁺ T-cell and B-cell responses. Moreover, SP domains have a preferred transporter associated with antigen processing (TAP)-independent presentation, which may overcome immune escape and tumour resistance (Dorfel *et al*, 2005; Kovjazin *et al*, 2011b; Kovjazin & Carmon, 2014). Preclinical studies of ImMucin (Kovjazin *et al*, 2011a) and its internal epitopes in MM (Choi *et al*, 2005), suggested superior immunological and anti-tumour properties compared to other MUC1 TRA-derived epitopes (Kovjazin *et al*, 2011a). Here, we describe the first-in-human administration of ImMucin to MUC1-positive MM patients.

Materials and methods

Patients and design

The Phase I/II multi-centre trial explored the safety and toxicity (primary objective) of vaccination with ImMucin in MUC1-positive MM patients. Secondary objectives included (a) the induction of ImMucin-specific cellular and humoral immune responses and (b) the attainment of clinical response.

The study (NCT01232712) was approved by local Institutional Review Boards at the Hadassah and Rambam Medical Centres (HMC and RMC, respectively) and by the Israeli Ministry of Health.

Male or female MM patients, aged >18 years, previously treated with >1 anti-MM therapy including autograft, presenting biochemical evidence of either stable or progressive disease following autologous stem cell transplantation (ASCT), measurable disease, no calcium, renal insufficiency, anaemia, or bone lesions (CRAB) criteria (Durie *et al*, 2006), an Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2 , and adequate liver and kidney function were eligible to participate in this study. The expression of MUC1 SP by tumour plasma cells (PCs) was evaluated in bone marrow (BM) aspirates, whereas sMUC1 level was measured in serum. Patients exhibiting MUC1, either in serum

and/or BM PCs, were eligible for vaccination. Patients presenting a continued increase in monoclonal protein/free light chain level (without demonstrating organ impairment in blood tests and skeletal survey), underwent magnetic resonance imaging or computerized tomography scan, to confirm the lack of MM-related bone disease. Patients presenting active disease were excluded.

After informed, written consent, patients received six bi-weekly intradermal (i.d.) injections of 100 μ g ImMucin, divided over four injection sites near the armpit and in the upper thigh, close to the groyne. In order to increase antigen presentation, 250 μ g human granulocyte-macrophage colony-stimulating factor (hGM-CSF) (Leukine, Genzyme, Seattle, WA, USA), divided over four injection sites, was co-injected i.d. near the ImMucin vaccination sites. The vaccination schedule was determined based on preclinical data in mice, demonstrating that weekly subcutaneous administration of 100 μ g ImMucin over three consecutive weeks was well tolerated, and resulted in a robust induction of anti-tumour T-cell response (Kovjazin *et al*, 2011a). More recent experiments (R. Kovjazin and L. Carmon, unpublished data), showing that bi-weekly vaccination resulted in an even greater humoral immune response without attenuating T-cell response, promoted the adoption of a 100- μ g bi-weekly vaccination schedule. Patients undergoing vaccination without developing serious adverse events and/or progressive disease (PD) (Durie *et al*, 2006) were entitled to receive six additional bi-weekly immunizations with ImMucin plus hGM-CSF. Patients who attained at least stable disease (SD) at the end of the vaccination, were followed until PD.

No other anti-MM consolidative or maintenance therapies, including steroids, were permitted during the vaccination and follow-up periods.

Peptides

The 21-mer MUC1-SP-L (or VXL-100), 25-mer MUC1 TRA (MUC1-TRA-L or BP25) and 100-mer MUC1 TRA (MUC1-TRA-XL) peptides were synthesized by fully automated, solid-phase, peptide synthesis (EMC Tuebingen, Germany and Almac, Craigavon, UK).

Safety assessment

Adverse event (AEs) were graded for intensity according to the National Cancer Institute Common Terminology Criteria for AE, version 3.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcae3.pdf).

Clinical response assessment

Patients receiving at least four ImMucin doses were evaluable for disease responsiveness to treatment, assessed by serum tumour marker levels and the percentage of PCs by BM aspiration and biopsy. Disease response was assessed according

to the International Myeloma Working Group response criteria (Durie *et al*, 2006).

Statistical analysis

Continuous variables were compared using the 2-tailed Student's *t*-test. Pearson's correlation coefficients (*r* values) were calculated in Excel software (Microsoft, Redmond, WA, USA). PFS was considered to be the time from first vaccination to death or disease progression/relapse. OS was defined as the time from first vaccination to death or the last follow-up and survivals calculated using the Kaplan–Meier method (MedCalc Statistical Software version 13.0 (MedCalc Software bvba, Ostend, Belgium)). Significant observations were set at $P < 0.01$.

Immunomonitoring and tumour markers

Sera were maintained at -20°C until analysis. MM-related markers, excluding MUC1, were analysed at Shaarei-Zedek Medical Centre (Jerusalem, Israel), Maccabi Health Care Services (MHCS, Rehovot, Israel) and RMC (Haifa, Israel); MUC1 was analysed at Vaxil BioTherapeutics (Nes-Ziona, Israel).

Sera samples obtained pre-vaccination and at weeks 2, 4, 6, 8, 11, 13 and 26 were used to assess sMUC1 and anti-MUC1 antibody measurements. Peripheral blood mononuclear cells (PBMCs) samples, obtained pre-vaccination and at weeks 5, 8, 12 and 26 and separated with a Ficoll gradient (Histopaque, Sigma, Rehovot, Israel), were cryopreserved and then used to perform MUC1 SP HLA-2.1-specific multimer and T-cell proliferation analysis. PB samples, isolated at the same time points, were used to evaluate MUC1 SP-specific IFN- γ production, employing intracellular staining (ICS).

sMUC1 and BM MUC1 levels

Fluorescence-activated cell sorting analysis for MUC1 expression in fresh BM aspirates were performed as previously described (Kovjazin *et al*, 2014), using anti-MUC1 TRA monoclonal antibody H23 and fluorescein isothiocyanate (FITC)-conjugated anti-MUC1 SP polyclonal hyperimmune IgG R23IgG. Samples containing $\geq 5\%$ MUC1 SP+CD138+ cells were considered positive. Enzyme-linked immunosorbent assay (ELISA) for sMUC1 concentration was performed as previously described (Kovjazin *et al*, 2012). A standard curve of serially diluted MUC1 TRA 100-mer peptide; MUC-TRA-XL, was prepared for each assay. A sMUC1 concentration ≥ 600 pg/ml was considered as a positive result.

BM PDL1 (CD274) levels

Immunocytochemistry analysis for PDL1 expression was performed on methanol fixed, (5 min, room temperature) smears from fresh BM aspirates. Samples were stained with 1 $\mu\text{g}/\text{ml}$

of purified (B7-H1, PDL1) antibody (Biolegend, San Diego, CA, USA) for 1 h at room temperature. The PolyScan HRP/DAB detection system kit (Cell Marque, Rocklin, CA, USA) was then used according to the recommended protocol following by a standard giemsa stain. The amount of PDL1 expression on 50 PCs was a mean of ten different and distance slide area using the following score; (–), absence of positive cells; 1+, one to five positive cells; 2+, up to 20 positive cells per tissue section; 3+ >20 positive PCs.

Assessment of T-cell response

Intracellular staining. ICS for MUC1-SP-L specific IFN- γ producing CD4 and CD8 T-cells was performed according to the manufacturer's (BD, San Jose, CA, USA) protocol using MUC1-SP-L, MUC1-TRA-L, super antigen or phosphate-buffered saline (PBS) as stimulants. A reading of ≥ 2 -fold increase in MUC1-SP-L-specific IFN- γ -producing T-cells compared with prevaccination level, accounting for $\geq 0.5\%$ of gated T-cells, was considered as a positive and specific response.

MHC typing and multimer assay

MHC typing. High-resolution MHC typing was performed at HMC and MHCS, as previously described (Erlich *et al*, 2001).

Multimer assay. Multimer analysis for MUC1 SP specific CD8 T-cells was performed according to the manufacturer's (IMMUDEX, Copenhagen, Denmark) protocol, using MUC1-SP-S2 HLA-A2.1/LLLLTVLTV-APC-conjugated multimer (IMMUDEX). Any increase in the multimer binding level of ImMucin, compared with prevaccination level, was considered as a positive and specific response.

Proliferation assay. Proliferation analysis was performed as previously described (Kovjazin *et al*, 2011b, 2013). A stimulation index (SI) ≥ 2 and/or a two-fold increase from prevaccination level were considered as a positive and specific response.

Functional T-cell cytotoxicity assays. Viable prevaccination BM-derived cells were washed twice with PBS, and labelled with 185 kBq ^{35}S -methionine (Amersham, Little Chalfont, Buckinghamshire, UK) per 10^6 cells (18 h, 37°C , 5% CO_2). Cells were then washed four times with PBS and resuspended (5×10^5 cell/ml) in RPMI complete medium. Triplicate samples (100 μl each) were placed into 96-well plates (Greiner bio-one, Frickenhausen, Germany). Autologous PBMCs, collected at different time points before and during ImMucin vaccination, were thawed, washed twice with PBS, and resuspended in RPMI complete medium to a final concentration of 1.25×10^6 and 2.5×10^6 cells/ml. Serial dilutions (100 μl) were incubated with the target cells (5 h 37°C , 5%

CO₂). The reaction was terminated by centrifugation (280 g, 10 min, 4°C). Supernatants (50 µl) were mixed with 150 µl scintillation fluid (Microscint™ 40, PerkinElmer, USA) and measured in a β-counter. The percentage of specific lysis was calculated as: % lysis = (cpm in experimental well-cpm spontaneous release)/(cpm maximal release-cpm spontaneous release) × 100. Labelled cells (100 µl) incubated with 100 µl medium and labelled cells lysed in 100 µl 10% TritonX-100, served as a means of determining spontaneous and maximal release from target cells, respectively.

B-cell response

ELISA for antibodies against the MUC1 SP and MUC1 TRA epitopes was performed as previously described (Kovjazin *et al*, 2012). An anti-MUC1 SP antibody concentration of ≥300 µg/ml was considered as a positive and specific response.

Antibody-dependent cell-mediated cytotoxicity (ADCC). The ADCC assay was a modified T-cell cytotoxicity protocol, in which the target cells were pre-incubated (2 h 37°C, 5% CO₂) with 100 µl autologous patient serum, washed once with PBS and mixed with effector cells, as described above.

Results

Patient characteristics

Nineteen patients were screened and 15 were enrolled and vaccinated. Four patients were excluded during screening, due to lack of detectable MUC1 in both sera and BM ($n = 2$) or due to presentation of clinically significant progressive disease ($n = 2$).

The cohort included nine males and six females, with a median age of 57 (range: 49.8–70.3) years. Seven patients presented an ISS score of two and three patients with an ISS score of 3. FISH analysis defined 14 patients with standard risk disease and one patient with high-risk disease (Table I). Median lymphocyte count at trial entry was $1.5 \times 10^9/l$ (range 0.8–2.83) and most patients had immunoparesis (Table I). The number of prior therapies ranged between 1 and 3; last prior therapies were ASCT ($n = 9$), thalidomide ($n = 3$), bortezomib ($n = 2$), and a combination of thalidomide and bortezomib ($n = 1$) (Table I). Median time from diagnosis to first ImMucin vaccination was 25 months (range: 12–143 months) and median time from last therapy to vaccination was 15 months (range: 3–134 months). Nine patients were enrolled with stable residual disease and six with biochemical progression. Patients had a diversified MHC repertoire, where only 4/15 patients expressed the HLA-2.1 class I allele (Table I). Three out of the 15 patients had no MUC1+ CD138+ PCs in their BM aspirate and were enrolled into the study based on their abnormal sMUC1 sera levels.

Patient disposition and extent of exposure

A total of 167 ImMucin/hGM-CSF vaccinations were administered. Nine patients received all 12 vaccinations (Table II) and two patients, (01-014 and 02-004) received 6 and 10 vaccines only, due to recurrent grade 1, 2 non-ischemic chest pains (01-014), or withdrawal of consent (02-004) despite lack of side effects or evidence of PD (Table III). Four patients experienced PD after receiving 6 (01-001 and 01-012) or 9 (01-005 and 02-001) vaccine injections; their vaccination schedules were subsequently discontinued and they were excluded from the study and initiated a different therapy.

Vaccine-related toxicity

The vaccine was well tolerated (Table III), and no vaccine-related grade AEs ≥3 were reported. Grade 1 and 2 AEs included local inflammation (erythema and mild swelling) at the injection site, asthenia, bone pain and fatigue. All AEs self-resolved within 72 h.

T-cell response to vaccination

All vaccinated patients exhibited robust IFN-γ production by both CD4+ and CD8+ T-cells, with mean baseline and peak postvaccination IFN-γ levels generated by MUC1-SP-L (ImMucin's API)-specific T-cells of 0.21% vs. 4.07% ($P < 0.000014$, *t*-test) and 0.21% vs. 11.76%, ($P < 0.0001$, *t*-test), respectively (Fig 1A). In addition, a mean 35-fold increase in MUC1-SP-L-specific CD4+ T-cells (range: 4- to 80-fold) and a mean 43.4-fold increase in CD8+ (range: 18- to 80-fold) were observed post-vaccination (Fig 1A); Kinetics of the CD4+ and CD8+ IFN-γ producing T-cell responses are presented in Figure S1. The T-cell response was ascertained to be MUC-SP-L-specific, as demonstrated by the absence of IFN-γ production in response to treatment with the MUC1-TRA-L, MUC1 TRA 25-mer control peptide (Fig 1B). Moreover, stimulation with both PBS or with an irrelevant SP, failed to induce IFN-γ production (data not shown), further confirming an MUC1 SP-specific response.

Assessment of cytotoxicity of PBMCs, obtained post-vaccination from Patient 01-008, on autologous BM isolated at enrollment, showed a moderate (15%) yet specific tumour lysis reaction, which positively correlated ($R^2 = 0.8815$) with the increase in IFN-γ production by CD8+ T-cells (Fig 1D). Given that MUC1 SP CD138 double-positive cells were not sorted from the BM, it is reasonable to assume that the actual lysis of sorted MUC1-positive cells would have been higher. These results confirm the expression of MHC class I/MUC1 SP epitope complexes on BM-derived MM PCs and the efficacy of ImMucin in generating functional IFN-γ-producing CD8+ and, plausibly, also CD4+ T-cells with cytotoxic properties.

Multimer assessment of the induction of specific CD8+ T-cells to MUC1-SP-S2 (Kovjazin *et al*, 2011a), a 9-mer internal MUC1-SP-L epitope, in the four patients expressing HLA-A2.1 (Table I), demonstrated an increase in

Table 1. Patient characteristics.

Patient ID	Sex	Age (years)	MM subtype	ISS	Time from diagnosis (months)	Prior Tx (n)	Time from last Tx (months)	HLA Repertoire	Lymph ×10 ⁹ /l	IgA g/l	IgG g/l	FISH
01-001	F	59.4	IgG Kappa	2	113	3	1	A02-11,B35,DRB104-13	1	0.004	59.36	NA3
01-002	M	52.4	IgG Kappa	1	48	2	1	A3201,B3502-5001,CW0401-0602,DRB1 0701-1101	1.9	1.63	13.70	NA
01-003	M	70.3	IgG Kappa	3	12	1	5	A0301-2601,B3502-3801,CW0401-1203,DRB10402-1104	0.8	1.23	14.70	NA
01-005	M	50.9	IgG Lambda	2	17	1	11	A0201-2403,B1801-4006,CW0401-1502,DRB11104-1501	2.1	0.64	13.90	NA
01-007	F	52.7	IgG Kappa	3	24	2	1	A0101-6802,B1402-4403,CW0401-0802,DRB10102-0701	1.2	0.25	14.80	NA
01-008	M	51.2	IgG Kappa	1	78	3	12	A0101-3201,B0801-1801,CW0701-1203,DRB10301 1103	1.2	2.91	16.60	NA
01-010	M	58.5	IgG Kappa	3	27	1	15	A0301-3101,B3502-5801,CW0401-0602,DRB0403-1302	2.3	1.18	15.30	NA
01-012	M	64.9	IgG Kappa	2	21	1	15	A0101-3301,B1402-1517,c0701-0802,DRB1 0102-1001	1.5	0.55	10.00	NA
01-013	M	49.8	IgG Lambda	2	13	1	3	A0101-1101,B5201,C1202 DRB1 04021502	2.1	1.36	10.70	NA
01-014	F	65.4	IgG Kappa	2	27	2	9	A0101-0205,B4101-5201,C0701-1202,DRB1 1305-1502	1.5	0.64	6.81	NA
01-015	F	60.3	IgG Kappa	1	18	1	11	A0201-2403,B2702-5001,C0202-0602,DRB1 0701-1501	1	0.35	20.20	13q del
02-001	M	60.2	Lambda LC	1	110	1	98	A0201-2901,B4402-5601,C0102-0501,DRB1 0402-1104	1.35	0.58	21.80	13q del
02-003	F	52	IgG Kappa	1	153	1	134	A0201-0101,B0801-3801,C0701-1203,DRB1 0201-1301	1.48	0.28	36.30	NA
02-004	M	50.3	Lambda LC	2	68	1	71	A3001-3002,B3502-4101,C0401-1701,DRB10701-1104	1.91	2.04	17.20	+1q21
02-005	F	59.1	IgG Lambda	2	72	2	22	A0302-2402,B3801-4402,C1203-1604,DRB1 0402-1454	2.83	0.37	23.90	NA

ISS, International staging system; Tx, treatment; HLA, human leucocyte antigen; Lymph, lymphocytes; F, female; M, male; NA, no abnormalities by fluorescence *in situ* hybridization analysis; del, deletion.

Table II. Simplified study design.

	Screening			Vaccination (treatment) period										Follow up	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Visits															
Weeks	-4	0	2	4	6	8	10	11	12	14	16	18	20	22	26
ImMucin plus hGM-CSF Administration		X	X	X	X	X	X	Y/N*	X	X	X	X	X	X	
Adverse events assesment		X	X	X	X	X	X	X	X	X	X	X	X	X	X
BM aspiration and biopsy†	X														
sMUC1 and MM markers expression‡	X	X		X		X		X			X		X		X
Immunomonitoring§		X			X			X				X			X
Immunomonitoring¶		X		X		X		X			X		X		X

MM, multiple myeloma; PC, plasma cells; BM, bone marrow; hGM-CSF, human granulocyte-macrophage colony-stimulating factor.

*Yes/No for receiving a second cohort of vaccination.

†Sampling for evaluating disease status and MUC1 expression on MM PC in the bone marrow.

‡Sampling for evaluating sMUC1, MM markers including M-protein, free light chains, β 2-microglobulin and total immunoglobulins.

§Sampling for evaluating MUC1 SP-specific IFN- γ production by intracellular staining in CD4+ and CD8+ T-cells, multimer staining in CD8+ T-cells from HLA-A2.1-positive patients and specific proliferation.

¶Sampling for evaluating MUC1 SP-specific antibody concentrations.

Table III. Treatment-related adverse events

MedDRA system class*		Adverse Events		Study drug relationship		
		N/% of Events†	N/% of Patients‡	Possible	Probable	Grade
Blood and Lymphatic system disorders	Lymphadenopathy	1/1.14	1/6.6	1	–	2
Gastrointestinal disorders	Abdominal pain	2/2.27	2/13.3	2	–	1
	Diarrhoea	2/2.27	2/13.3	1	1	1
	Nausea	7/7.95	3/20	2	5	1
	Vomiting	2/2.27	2/13.3	–	2	2
	General disorders and administration site condition	Asthenia	10/11.36	6/40	6	4
	Axillary pain	4/4.54	2/13.3	4	–	1
	Chest pain	6/6.81	1/6.6	6	–	1
	Fatigue	6/6.81	5/33.3	3	3	1
	Influenza-like illness	4/4.54	2/13.3	3	1	1
	Inflammation at injection site	12/13.63	8/53.3	2	10	2
	Pyrexia	3/3.4	3/20	1	2	2
Immune system disorders	Allergy to vaccine	3/3.4	1/6.6	–	3	1
Musculoskeletal and connective tissue disorders	Back pain	1/1.14	1/6.6	1	–	1
	Bone pain	8/9.01	2/13.3	4	4	1
	Muscle weakness	1/1.14	1/6.6	1	–	1
	Musculoskeletal pain	4/4.54	3/20	3	1	1
	Neck pain	1/1.14	1/6.6	1	–	1
Nervous system disorders	Dizziness	2/2.27	1/6.6	–	2	1
	Headache	3/3.4	2/13.3	1	2	1
Respiratory, thoracic and mediastinal disorders	Cough	2/2.27	2/13.3	2	–	1
Skin and subcutaneous tissue disorders	Rash	4/4.54	2/13.3	1	3	1

*http://www.who.int/medical_devices/innovation/MedDRAintroguide_version14_0_March2011.pdf.

†Number and percentage of adverse events (AE) from the total treatment-related AE ($n = 88$).

‡Number and percentage of patients with AE from the number of treated patients (15 patients).

multimer-positive cells in response to vaccination, with mean pre- and post-vaccination peak levels of 0.33% vs. 2.11%, respectively (Fig 1C).

Ex vivo proliferation of PBMCs in response to MUC1-SP-L significantly increased in all patients, with mean baseline and peak post-vaccination levels of 3.24 and 15.92 SI values

respectively ($P < 0.024$), yielding a mean 9.4-fold amplification from baseline (range: 1.4–12.6) (Fig 2A left panel). In contrast, proliferation of PBMCs in response to MUC1-TRA-L was negative in all but three patients, with mean SI values of 1.49 pre-vaccination vs. 2.68 peak levels following vaccination (Fig 2A right panel).

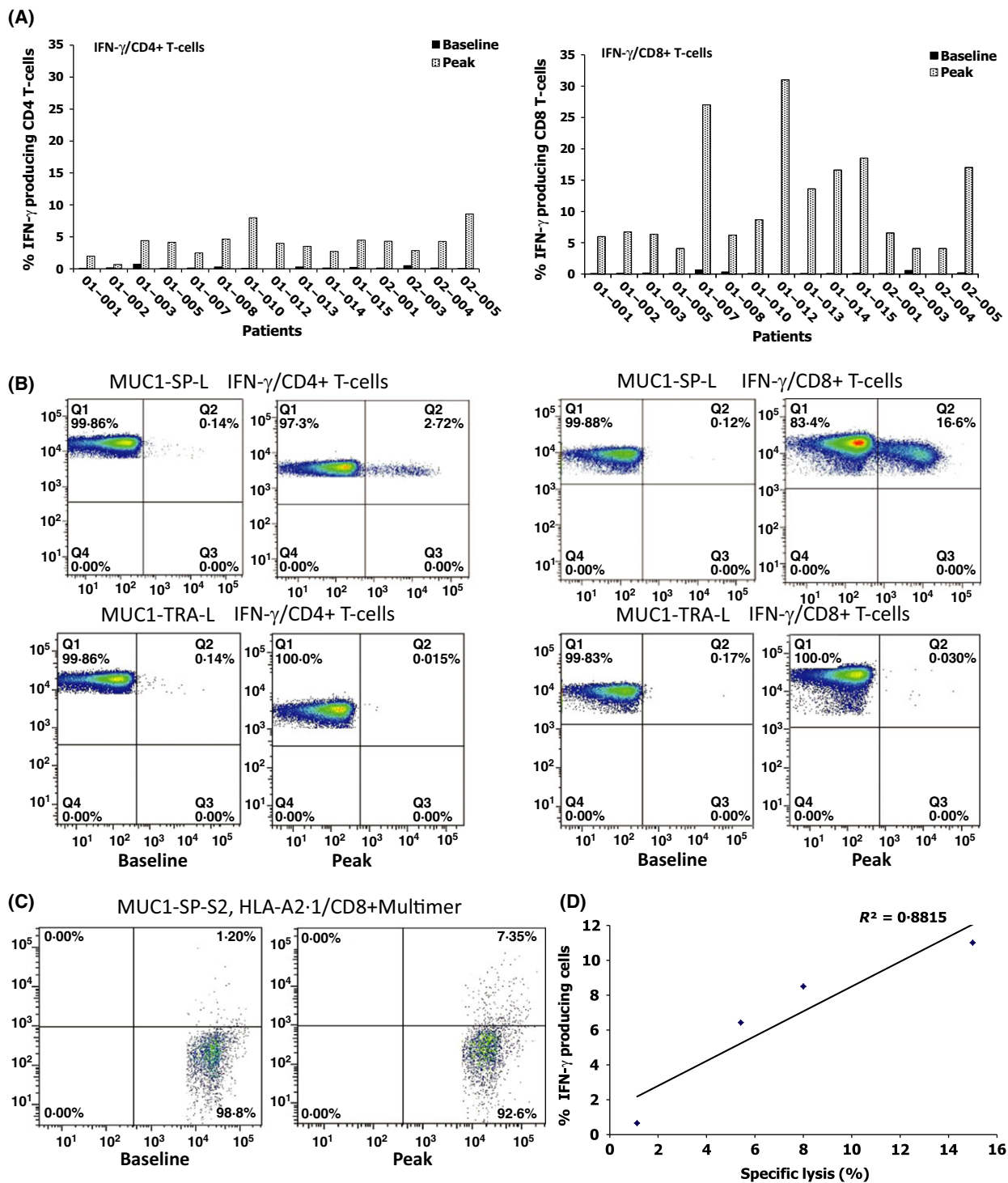


Fig 1. T-cell response as determined by cytotoxicity and production of IFN- γ . (A, B) Peripheral blood (PB) collected from all patients prevaccination and at visits 5, 8, 12 and 15, were incubated with MUC1-SP-L, MUC1-TRA-L, super antigen or phosphate-buffered saline, labelled with phycoerythrin-conjugated anti-Hu CD3, Fluorescein isothiocyanate-conjugated anti-Hu CD4, peridinin chlorophyll-cyanin5-5-conjugated Anti-Hu CD8 and allophycocyanin-conjugated anti-Hu IFN- γ antibodies, fixed in CellFIX (Becton Dickinson) and analysed by flow cytometry. Percentage of ImMucin-reactive CD4+ T-cells (A, B left panels), MUC1-TRA-L (B lower left panel) and ImMucin-reactive CD8+ T-cells (A, B, right panels) and MUC1-TRA-L (B lower right panel) measured pre- and post-vaccination (maximal levels) are demonstrated. (C) A representative % of positive MUC1-SP-S2-reactive multimer CD8+ T-cells measured pre- and postvaccination (maximal levels) in HLA-A2.1 patients. (D) Pearson correlation coefficient for ImMucin-specific IFN- γ production by CD8+ T-cells and T-cell cytotoxicity. For cytotoxicity evaluation, 35 S]-methionine labelled bone marrow cells isolated ($n = 1$, Patient 01-008) at screening (targets) were incubated with autologous PB, isolated at different time points before and during the ImMucin vaccination regimen.

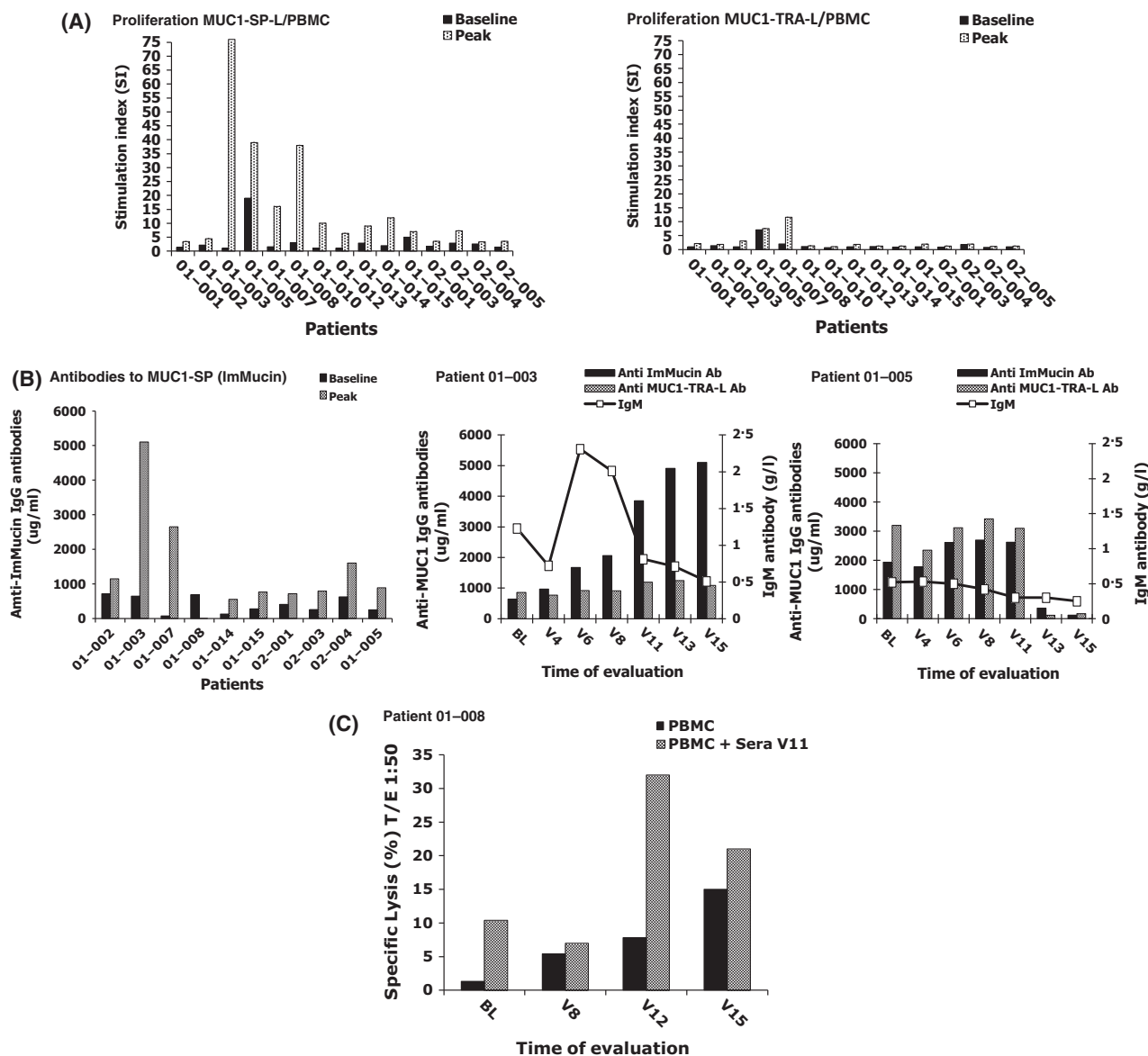


Fig 2. ImMucin-induced proliferation and antibody-dependent cell-mediated cytotoxicity (ADCC) responses. (A) peripheral blood mononuclear cells (PBMC) were collected from prevaccination and at visits 5, 8, 12 and 15 and incubated with 10 μ g/ml MUC1-SP-L or MUC1-TRA-L, for up to 6 d. Proliferation was measured by incorporation of 3 [H]-thymidine. T-cell proliferation in response to MUC1-SP-L (left panel) and MUC1-TRA-L (right panel), are demonstrated. Values are presented as mean SI; proliferation of stimulated/unstimulated cells. (B) For anti-MUC1 antibodies analysis, sera were collected prevaccination and at visits 2, 4, 6, 8, 11, 13 and 15, incubated (overnight, 4°C) with 5 μ g/ml peptides, followed by incubation (1 h, room temperature) with horseradish peroxidase-conjugated anti-human IgG. Anti-ImMucin IgG concentrations, measured pre and postvaccination (maximal levels) (left panel). Representative positive anti-ImMucin IgG concentrations, without antibody induction to MUC1-TRA-L ($n = 1$, Patient 01-003) (middle panel) and a negative anti-ImMucin IgG response ($n = 1$, Patient 01-005) (right panel) are shown, together with general non-MUC1-specific IgM concentrations. (C) For ADCC evaluation, 35 [S]-methionine-labelled bone marrow cells isolated at screening (targets) ($n = 1$, Patient 01-008), were incubated with autologous PBMC collected at visits 12 and 15, together with or in the absence of ImMucin hyperimmune sera obtained from the same patient at peak response (visit 11).

Humoral response to vaccination

A significant 6.86-fold (range: 1.4- to 40-fold) increase in anti-ImMucin IgG concentrations was observed at the response peak of 10/15 patients, after receiving 6 or 7 immunizations (Fig 2B, left panel), with mean baseline and peak post-vaccination levels of 410 μ g/ml vs. 1676 μ g/ml

($P < 0.01$). The anti-ImMucin IgG antibody response appeared 2–4 weeks after an increase in general IgM concentrations, suggesting the induction of a sustained humoral response (Fig 2B, middle panel, black bars). Notably, the response was ImMucin-specific, as shown by lack of a significant increase in the post-vaccination anti-MUC1-TRA IgG

antibody concentrations (Fig 2B, middle and right panels, grey bars).

The induced anti-ImMucin antibodies obtained from Patient 01-008 at peak response (visit 11) and mixed with autologous PBMCs collected at visits 12 and 15, triggered specific ADCC responses against autologous BM cells. A maximal specific lysis of 32% was induced by PBMCs from visit 12 (Fig 2C). As previously indicated, it is reasonable to assume that the actual lysis of MUC1-positive target cells would have been higher had the target population been an isolated group of MUC1-specific PCs.

Serum sMUC1 levels

sMUC1 levels, thought to reflect tumour mass, were shown to form complexes (Fan *et al*, 2010; Thie *et al*, 2011) with anti-MUC1 TRA antibodies, and may therefore be deceptively low following vaccination with anti-MUC1 vaccines containing a TRA domain (von Mensdorff-Pouilly *et al*, 2000). Given that ImMucin contains the MUC1 SP domain, which is not a part of sMUC1 and does not induce anti-MUC1 TRA antibody production, (Fig 2B) a reduction in sMUC1 concentrations following vaccination can be considered an indirect indication of tumour destruction. When considering the nine patients presenting abnormal sMUC1 levels (>600 pg/ml) at screening (mean 5129.44 pg/ml), a significant reduction in sMUC1 levels (2.6- to 21-fold) was observed following vaccination (Fig 3) [mean 792.333 pg/ml at maximal response ($P < 0.002$)]. Of note, Patient 01-12, the only patient who did not demonstrate a reduction in sMUC1 levels, experienced disease progression, while the other MUC1-overexpressing patients demonstrated at least SD. Normal sMUC1 levels were achieved in seven of these nine patients. In the other two patients (01-001 and 01-005), sMUC1 levels temporarily dropped after vaccination; disease progression was eventually observed and the patients were excluded from the study.

PDL1 expression in BM samples

Analysis of the PDL1 levels in BM aspirate obtained prior vaccination demonstrated increased levels in 3 out of the 10 evaluated patients. All three of the evaluated patients (01-001, 01-005 and 01-012) presenting with high BM, PC PDL1 levels pre- and post-vaccination (+++), experienced PD. Interestingly, the same three patients had no ($n = 1$) or transient ($n = 2$) reduction in sMUC1 levels. In contrast, patients in whom BM- PCs expressed low-intermediate PDL1 levels (+ or ++), attained at least disease stabilization and PDL1 levels remained negative ($n = 1$), low-intermediate ($n = 2$) or even decreased ($n = 4$) following vaccination.

Clinical response

Seven out of the nine patients who entered the study with stable residual biochemical disease experienced continuous

stabilization of their disease, lasting for ≥ 60 weeks in all except one. Of note, one patient experienced an improvement in depth of response; attaining a stringent complete response (CR) instead of CR. Six patients entered the study with a gradual biochemical progression. Two of these experienced continual biochemical progression whilst being vaccinated and the other four attained disease stabilization ($n = 2$) or deceleration in progression rate ($n = 2$), lasting for up to 26 months. One of these four responding patients, diagnosed with light chain MM, demonstrated a 30% decrease in light chain levels. At 17.5–41.3 months after study completion (measured for first and last patients, respectively), 12/15 patients were alive (Fig 4A). Median time from first vaccination was 24 months (range 5.5–41.3), at which time 10/15 patients had PD. Disease progressed during the vaccination period (up to week 26) in 4/15 patients, and during the follow up period in 6/15 patients (Fig 4B). Notably, 5/15 patients still maintained their CR ($n = 3$) or SD ($n = 2$). Median PFS of the entire cohort approached 17.5 ± 3.9 months (95% confidence interval for the median 7.5 to 20.0). Of note, response duration in those three patients (01-001, 01-005 and 01-012) who did not attain a durable decrease in sMUC1 levels was much shorter, approaching only 2.5, 4.5 and 6 months, respectively.

Discussion

Despite the significantly improved clinical response rates in MM (Attal *et al*, 2012; McCarthy *et al*, 2012), most patients experience disease progression, culminating in death. Administration of lenalidomide post-induction/ASCT resulted in significantly prolonged PFS, including in patients who attained 'complete remission', however, it was associated with reduced OS, increased risk for haematological toxicities and secondary malignancies (Attal *et al*, 2012, 2013; McCarthy *et al*, 2012).

An anti-MM vaccination approach has been proposed to provide a safer alternative for maintaining and plausibly inducing response in patients with low-tumour burden. However, while studies exploring the administration of anti-idiotypic vaccines in MM patients have generally resulted in enhanced anti-tumour immune responses, they have largely failed to demonstrate a significant improvement in patient outcome (Bogen *et al*, 2006; Rhee, 2007). These results probably reflect low immunogenicity of the administered antigen, negligible cell surface expression of IgG idiotype on myeloma cells (Rhee, 2007) and potentially, on progenitor cancer CS as well.

MUC1 presents a potent target for vaccination. All of the anti-MUC1 vaccines under clinical development target the extracellular TRA domain or its epitopes, and contain the sMUC1 sequence, which has been found to interfere with (Fan *et al*, 2010; Thie *et al*, 2011) or suppress (van de Wiele van Kemenade *et al*, 1993; Agrawal *et al*, 1998) vaccine-induced antibodies and T- cell responses. Moreover, these anti-MUC1 vaccines fail to induce a combined T- and B- cell adoptive immune response, which is required to achieve a

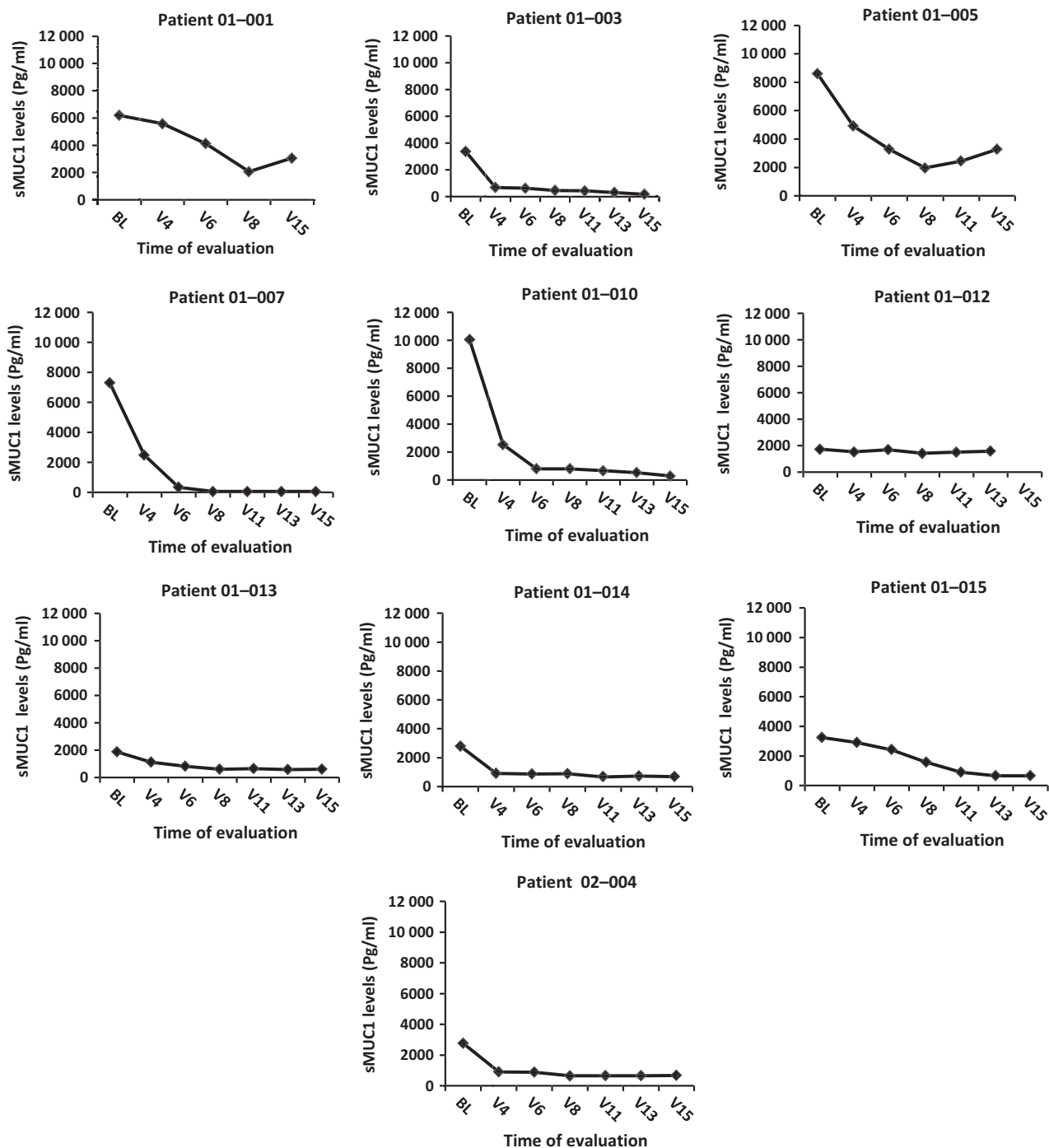


Fig 3. sMUC1 levels before and after vaccination. Sera were collected from all 15 patients prevaccination and at visits 2, 4, 6, 8, 11, 13 and 15, and analysed using a commercial anti-MUC1 TRA (clone M4H2) enzyme-linked immunosorbent assay kit (HyTest, Turku, Finland). BL, baseline; V, visit.

potent, long-lasting anti-tumour immune response (Morse & Whelan, 2010; Lakshminarayanan *et al*, 2012).

Long-peptide vaccines combining MHC class I and II TAA epitopes can efficiently potentiate broad T-cell effector function and long-term immunity (Melief & van der Burg, 2008; Perez *et al*, 2010). This therapeutic approach has been suggested to provide clinical responses in Human papilloma-

virus 16 (HPV16)-induced vulvar intraepithelial neoplasia (Kenter *et al*, 2009). However, currently, the few identified TAA-derived LPs bind a restricted repertoire of MHC alleles, resulting in limited antigen-specific activation of CD4+ and CD8+ T-cells in MHC-compatible subjects. Furthermore, most LPs do not contain B-cell epitopes and therefore fail to induce an antigen-specific ADCC response.

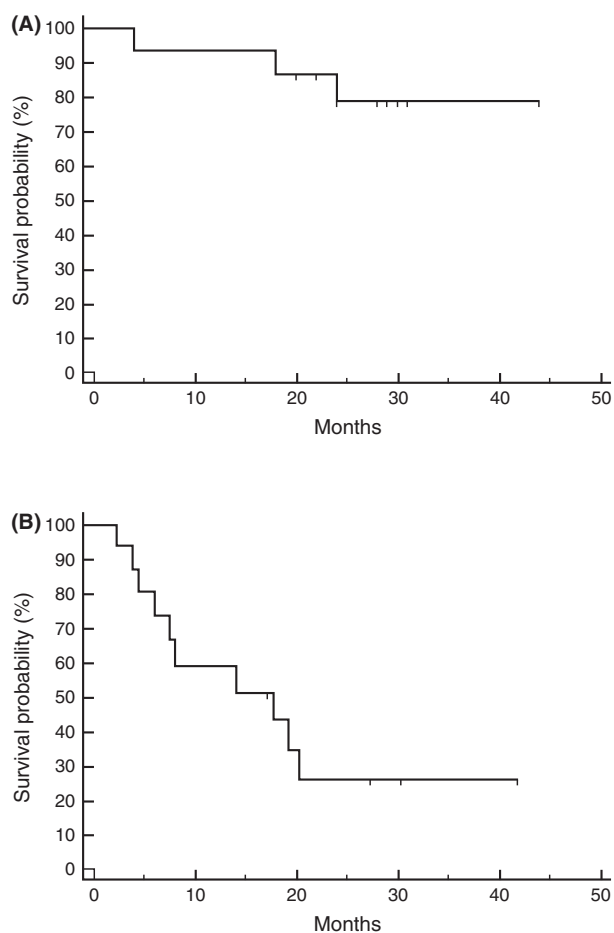


Fig 4. Overall survival and progression-free survival. Overall survival (A) and progression-free survival (B) for the entire group of vaccinated patients, starting from initial vaccination.

Sequential intradermal administration of ImMucin to MUC1-positive MM patients was well tolerated and associated with high quality of life. Side effects, all of low grade, were mainly localized and self-resolved, with no need for hospitalizations and no evidence of neuropathy or BM suppression.

ImMucin vaccination resulted in a significant increase in the percentage of both IFN- γ -producing CD4⁺ and CD8⁺ MUC1-SP-L-specific T-cells in all patients, irrespective of their MHC repertoire. The robust, yet specific T-cell immunity, demonstrated in both IFN- γ ICS, multimer and proliferation analyses, was MUC1 SP-specific, with negligible or no cross-reactivity with the control MUC1 TRA epitope and unrelated SP domains in the IFN- γ ICS and proliferation analysis.

This *in vivo* T-cell response corroborates with preclinical findings, where more robust and broader *in vitro* proliferation of a pool of cancer patient-derived PBMCs samples (Kovjazin *et al*, 2011a; Kovjazin & Carmon, 2014) across MHC barriers was induced by the MUC1-SP-L but not by MUC1-TRA-L and other MUC1 9-mer epitopes. In addition, strong and highly abundant CD4⁺ and CD8⁺ T-cell induction (Kovjazin *et al*, 2011a) toward MM cell lines was triggered *in vitro* by

human dendritic cells and *in vivo* in HLA-A2.1 transgenic mice (Carmon *et al*, 2000; Kovjazin, *et al* 2011a, Stepensky *et al*, 2006). Moreover, superior anti-tumour activity was observed with MUC1-SP-L when compared to that induced by MUC1-TRA-L, in the MUC1 BALB/c cancer model (Kovjazin *et al*, 2011a).

We can ascribe this strong and broad immune response to the lipophilic sequences within SP domains, such as MUC1-SP-L, which has been shown by us (Kovjazin *et al*, 2011a,b, 2013; Kovjazin & Carmon, 2014) and others (Wilkinson *et al*, 2012; Kerzerho *et al*, 2013) to be more immunogenic than other protein domains, and which, in the case of ImMucin, can generate a rapid response, using a low dose of naked LP administered in conjunction with hGM-CSF, without employing a dedicated 'carrier system' or specific adjuvants. In contrast, other anti-MUC1 vaccination strategies primarily induce humoral responses and/or selected CD8⁺ T-cell activation in a subset of patients (Roulois *et al*, 2013). In addition, MUC1-SP-L, as a LP, harbours many overlapping epitopes, with predicted binding to a wide range of MHC class I and II alleles, which is thought to enable broader and stronger activation of MUC1 SP specific CD4⁺ and CD8⁺ T-cell clones. Moreover, SP domains can induce preferred immunity via TAP-independent presentation (Martoglio & Dobberstein, 1998; Dorfel *et al*, 2005; Kovjazin *et al*, 2011b; Kovjazin & Carmon, 2014), suggesting its epitopes are potentially far more abundant on tumour cells (Aladin *et al*, 2007). Dorfel *et al* (2005) showed that TAP inhibition only affects the presentation of the MUC1 TRA epitope MUC1-TRA-S1, but not of MUC1-SP-L's internal epitope, MUC1-SP-S2.

In addition to the significant T-cell response, ImMucin administration resulted in a substantial increase in anti-MUC1 SP IgG titres, but not of anti-MUC1 TRA IgG. Importantly, the generated anti-ImMucin antibodies recognized autologous BM PCs, but failed to bind sMUC1, confirming previous observations regarding the presence of the MUC1 SP domain on MM cell lines and primary tumours, and the selective and prominent anti-tumour properties of the generated anti-MUC1 SP antibodies (Kovjazin *et al*, 2014).

The main challenge of immunotherapy lies in the induction of a potent anti-tumour response in tumour beds, with emphasis on defining the correlation between immune response evoked in the PB *versus* in the tumour itself. Our findings suggest that ImMucin can serve as a potent activator of adoptive immunity. These results also demonstrate that the MUC1 SP domain is presented on patient BM-derived MM PCs, both as independent epitopes (Kovjazin *et al*, 2014) targeted by antibodies via ADCC, and in association with MHC class I and II-complexes, targeted by T-cells. In parallel, the observed antibodies and IFN- γ -producing T-cells, suggest the induction of systemic anti-tumour responses, which may be associated with long-term survival in MM patients, as recently reported (Bryant *et al*, 2013).

Ninety percent of patients presenting abnormal baseline sMUC1 levels exhibited significantly reduced sMUC1 levels

following ImMucin vaccination, suggested to correlate with tumour cell destruction, as ImMucin fails to induce generation of antibody: sMUC1 complexes. Importantly, this is the first report of a correlation between reduction in sMUC1 levels and measured ImMucin-generated immunity in MM patients. In line with these findings, sMUC1 level did not decrease in one patient, who developed PD. Furthermore sMUC1 levels decreased temporarily in the two patients who experienced a temporary response. Interestingly, response duration in these three patients was much shorter than that obtained in the entire cohort. However, as sMUC1 is not a validated MM marker, larger studies are required to better determine the accuracy of this assay and its ability to predict clinical response.

Despite inclusion of a substantial number of heavily pre-treated patients, results were encouraging; as a minimum, disease stabilization and even deepening of response, accompanied with long-term PFS, was obtained in the majority of patients. Encouragingly, four of the six patients who entered the study with biochemically progressive disease experienced disease stabilization or a slower progression rate of their disease following vaccination, and this translated into a more extended time to next therapy. The inconsistency between a strong immune response and the poor clinical efficacy obtained in evaluable patients could be partly explained by the high PDL1 levels expressed by patients' tumour BM PCs. These results are in line with previous works emphasizing the importance of the PD1/PDL1 pathway on T-cell (Atanackovic *et al*, 2014) and Natural Killer cell (Benson *et al*, 2010) function. Moreover, the good clinical response to subsequent ImMucin therapy employed at clinical progression suggests this novel approach to be potentially valuable in the setting of early biochemical progression, and even a safe maintenance therapy, postponing the need for the administration of anti-myeloma agents.

In summary, the ImMucin vaccine presents an intuitive, yet unique immunotherapeutic approach, generating a com-

bined and diversified T- and B-cell immune response in a substantial number of MM subjects, irrespective of their MHC repertoire. In this manner, ImMucin overcomes the need for patient selection and treatment personalization. The induced immune response was highly specific and effective, resulting in *ex-vivo* killing of BM-derived MM PCs and in a remarkable decrease in sMUC1 levels. The observed clinical responses suggest that the immunological activity translated into relevant clinical activity. A larger randomized phase II study exploring efficacy of ImMucin in patients with residual myeloma is being planned to further strengthen the current encouraging findings.

Disclosure of potential conflicts of interest

LC is the founder and CEO, and RK is an employee at Vaxil BioTherapeutics Ltd. MYS and IA serve as consultants at Vaxil BioTherapeutics.

Author contributions

LC: designed the study, analysed the data and wrote the paper; IA: performed the research, analysed the data and wrote the paper; RK: performed the research and analysed the data; TZ: performed the research; LD: performed the research; MEG: performed the research; RO: performed the research; MYC: performed the research, analysed the data and wrote the paper.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Kinetic of MUC1-SP-L specific T-cell response as determined by production of INF- γ .

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