RESEARCH PAPER

Human Vaccines & Immunotherapeutics 10:11, 3394–3408; November 2014; © 2014 Taylor & Francis Group, LLC

Mucin 1-specific active cancer immunotherapy with tecemotide (L-BLP25) in patients with multiple myeloma: An exploratory study

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Keywords: immunotherapy, L-BLP25, multiple myeloma, mucin 1, MUC1, tecemotide

Abbreviations: ASCI, antigen-specific cancer immunotherapy; AUC, area under the curve; Cy, cyclophosphamide; ELISpot, enzyme-linked immunosorbent spot; GM-CSF, granulocyte-macrophage colony-stimulating factor; HR, hazard ratio; IDA, Immunologic Diagnostic Analysis; IFN-g, interferon-g; IL-17, interleukin-17; IQR, interquartile range; MM, multiple myeloma; MUC1, mucin 1; NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear cell; TNF-α, tumor necrosis factor-α; Treg, regulatory T cell; URR, upper reference range

Patients (n = 34) with previously untreated, slowly progressive asymptomatic stage I/II multiple myeloma or with stage II/III multiple myeloma in stable response/plateau phase following conventional anti-tumor therapy were immunized repeatedly with the antigen-specific cancer immunotherapeutic agent tecemotide (L-BLP25). Additionally, patients were randomly allocated to either single or multiple low doses of cyclophosphamide to inhibit regulatory T cells (Treg). Immunization with tecemotide resulted in the induction/augmentation of a mucin 1-specific immune response in 47% of patients. The immune responses appeared to involve a Th1-like cellular immune response involving CD4 and CD8 T cells. The rate of immune responses was similar with single versus multiple dosing of cyclophosphamide and in patients with vs. without pre-existing mucin 1 immunity. On-treatment reductions in the slope of M-protein concentration over time (but not fulfilling clinical criteria for responses with conventional anti-tumor agents) were observed in 45% of evaluable patients, predominantly in those without versus with pre-existing mucin 1 immunity and in patients with early stage disease. No differences were seen in patients receiving single or multiple cyclophosphamide dosing. Treatment with tecemotide was generally well tolerated. Repeated vs. single dosing of cyclophosphamide had no impact on Treg numbers and was stopped after a case of fatal encephalitis that was assessed as possibly study-related. Tecemotide immunotherapy induces mucin 1-specific cellular immune responses in a substantial proportion of patients, with preliminary evidence of changes in the M-protein concentration time curve in a subset of patients.

Introduction

Despite improvements in recent decades, management of multiple myeloma (MM) remains suboptimal. There is no established therapy for asymptomatic MM and the standard of care for most patients following an initial response to primary therapy is 'watchful waiting'.[1] Studies of conventional chemotherapy as maintenance therapy proved disappointing.[2,3] Newer agents including thalidomide,[4] lenalidomide[5,6] or bortezomib[7] can improve progression-free – and sometimes overall – survival but can also be associated with toxicity, and are not approved in most countries for maintenance therapy.[1]

A potential new strategy in the early disease phase is the use of immunotherapy to direct an immune response against malignant cells. Tecemotide is a liposomal antigen-specific cancer immunotherapeutic agent targeting mucin 1 (MUC1). It incorporates a synthetic, 25 amino acid, non-glycosylated MUC1 lipopeptide (BLP25) and monophosphoryl lipid A immunoadjuvant in a liposomal (L) delivery system. Results from a global, randomized, placebo-controlled trial of tecemotide in stage III non-small cell lung cancer (NSCLC) have been reported recently.[8] While prolongation of overall survival in the primary analysis population failed to achieve statistical significance, there was a trend toward prolonged survival and time-to-tumor progression with tecemotide. Also, tecemotide maintenance therapy resulted in a notable improvement in survival in the predefined subgroup of patients who had previously received concurrent chemoradiotherapy, but not when administered after sequential chemoradiotherapy.

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Submitted: 05/22/2014; Revised: 07/01/2014; Accepted: 07/12/2014
http://dx.doi.org/10.4161/hv.29918

The potential utility of tecemotide in treating MM is supported by studies reporting MUC1 expression on myeloma cells,[9,10] recognition of MUC1-positive MM cell lines by cytotoxic T lymphocytes from MM patients[11] and elevated levels of MUC1-specific CD8+ T-cells in peripheral blood and bone marrow from patients with MM.[12] MUC1 normally carries extensive O-linked glycans, but tumor-associated MUC1 is frequently hypoglycosylated. Furthermore, antigen processing by dendritic cells is more efficient when MUC1 is less extensively glycosylated, leading to stronger T cell responses.[13-15] Therefore, it seems reasonable to anticipate that immunization with non-glycosylated MUC1 peptides may induce a specific immune response to tumor-associated MUC1.

The primary objective of this exploratory study was to investigate the MUC1-specific immune response to tecemotide in patients with previously untreated, asymptomatic stage I/II MM or with stage II/III disease in stable response/plateau phase after primary anti-tumor therapy. Secondary objectives were to clarify the nature of the immune response and gain a preliminary assessment of the safety and clinical efficacy of tecemotide combined with single or repeated administration of low-dose cyclophosphamide (Cy).

Multiple myeloma is associated with alterations in immune status, including increased regulatory T (Treg) cells that could suppress anti-tumor immune responses.[16] Low-dose Cy might reduce Treg numbers[17] and was administered as a single dose before the first immunization in all clinical trials with tecemotide.[8,18,19] Identical single dosing of Cy in a recent phase II study in renal cell carcinoma of immunization with multiple tumor-associated peptides led to a 20% reduction in Tregs 3 d after Cy administration that persisted for at least 3 weeks. Treg levels were not reduced in the absence of Cy.[20] Animal studies suggest that a single administration of Cy induces a durable Treg depletion that may persist for only a few weeks[21] and repeated administration of low-doses of Cy improved survival versus single dosing in a mouse model of MM.[22] Therefore, in this study we explored the effects of single or repeated low doses of Cy to gain preliminary insights on whether repeated Cy dosing could affect Treg levels and possibly enhance the response to tecemotide.

Results

Patient characteristics

Thirty-four patients were enrolled and randomly allocated to receive tecemotide with either single or repeated Cy dosing (17 per group). Two patients in the repeated Cy group were missing immunologic samples up to week 9 and were excluded from the Immunologic Diagnostic Analysis (IDA) Set (**Fig. 1**). Patient demographics and baseline characteristics are presented in **Table 1**. The 2 groups were comparable in terms of gender, age, performance status and MM duration. However, the proportion of previously treated stage II/III patients was higher, and of chemotherapy-naïve stage I/II patients was lower, in the group with single compared with repeated Cy dosing.

Treatment duration and dosing

Median treatment duration was 54 weeks (interquartile range [IQR] = 50–93) and 87 weeks (39-116) and median number of tecemotide administrations was 15 (IQR = 12–19) and 21 (13-23) for Groups A and B respectively. All patients in Group A received a single Cy infusion per protocol and the median number of infusions in Group B was 11 (IQR = 3–13). The study protocol allowed the investigator to reduce the Cy dose at their discretion. While this occurred frequently in Group B, most deviations were minor and at each administration only 0–2 patients received <50% of the target dose. The median number of Cy infusions in Group B was less than expected from the treatment duration, partly reflecting suspension of Cy dosing after the clinical hold. All patients had completed the weekly tecemotide treatment before the hold. The 6-weekly treatment phase was interrupted in 25 of 34 patients (74%), 16 of whom restarted treatment after the lift of the clinical hold after a median of 175 d (range 154–215).

Frequency of MUC1-specific immune responses

Seventeen of 32 (53%) patients showed a spontaneous MUC1-specific immune response at study entry prior to starting tecemotide treatment. Rates of pre-existing MUC1 immune responses were somewhat higher in Group A (10 of 17; 59%) than Group B (7 of 15; 47%) and in patients with previously treated stage II/III disease (11 of 19, 58%) compared with chemotherapy-naïve stage I/II MM (6 of 13, 46%).

Specific on-treatment induction or augmentation of MUC1 overall induced immune responses occurred in 15 of 32 (47%) patients. On-treatment induction of an overall immune response was seen in 8 of 17 (47%) patients with, and 7 of 15 patients (47%) without, a baseline MUC1-specific immune response. The rate of on-treatment induction/augmentation was somewhat higher among patients with chemotherapy naïve stage I/II MM than those with previously treated stage II/III disease (**Table 2**).

Figure 2 shows the frequency of MUC1-specific immune responses in each assay at baseline (red shading) and on-treatment (blue shading) for each patient in group A (**Fig. 2A**) and group B (**Fig. 2B**). For the display of responses by assessment week, the number of antigens (parameters) for which the criteria for an immune response were met is indicated by the numbers in the blue boxes. Induction or augmentation of MUC1-specific immune responses occurred early in the course of treatment, typically within the first 9 weeks. The proportion of patients with an early induced MUC1-specific immune response (i.e. in any assay at $\geq$1 time-point within the first 9 weeks) was 47% in both groups, similar to the proportion of patients with an overall tecemotide-induced immune response (in any of the individual assays at 2 time-points up to week 50). Appendix S5 shows the time course of changes in the ELISpot assay for a representative patient with an overall immune response.

Characterization of the nature of the immune response

There were no apparent differences in the rates of overall induced MUC1-specific immune responses according to HLA subtype (Appendix S6). The absolute count of CD8+ effector T

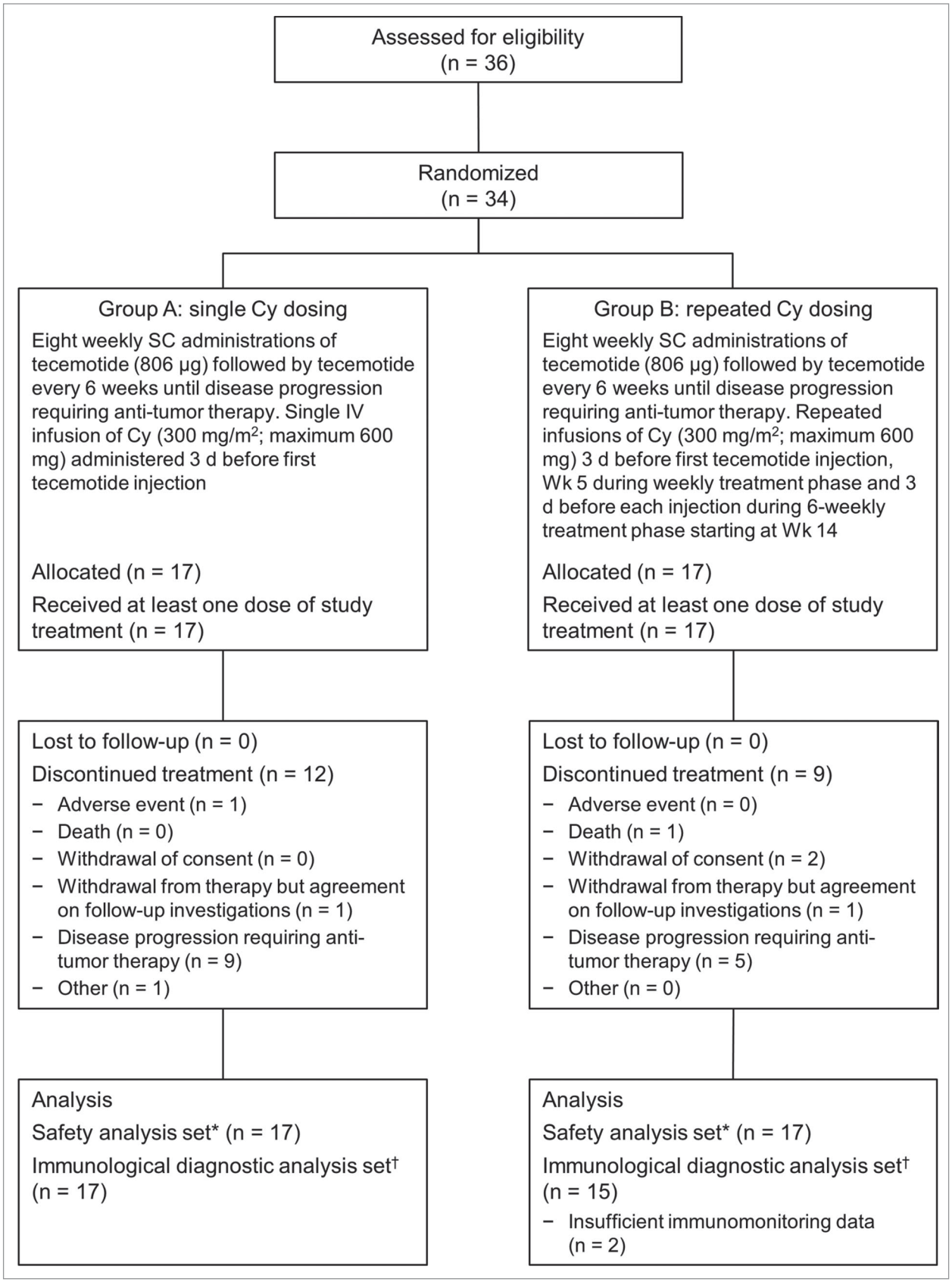


Figure 1. CONSORT flow diagram for enrolment and treatment of patients. Cy: cyclophosphamide; d: day; IV: intravenous; SC: subcutaneous; Wk: week. *Patients who received at least one dose of study treatment; †Patients with at least one complete set of baseline, week 5 and week 9 data of either ELISpot, proliferation or cytokine assay.

Table 1. Patient demographics and baseline disease characteristics (Safety Analysis Set)

		Group A Single Cy dosing (n = 17)	Group B Repeated Cy dosing (n = 17)
Sex, n (%)	Male	8 (47)	7 (41)
	Female	9 (53)	10 (59)
Race, n (%)	White	16 (94)	17 (100)
	Other	1 (6)	0 (0)
Median age, yr (range)		64 (45–72)	64 (46–79)
ECOG performance status, n (%)	0	14 (82)	12 (71)
	1	3 (18)	5 (29)
Median duration of MM, months (IQR)		34 (22–60)	37 (23–74)
MM stage at study entry, n (%)	Untreated asymptomatic stage I/II	5 (29)	8 (47)
	Previously treated stage II/III	12 (71)	9 (53)
Previous high-dose chemotherapy with ASCT, n (%)	All patients	10/17 (59)	9/17 (53)
	Previously treated patients	10/12 (83)	9/9 (100)

ASCT: autologous stem cell transplantation; ECOG: Eastern Cooperative Oncology Group; IQR: interquartile range; MM: multiple myeloma.

cells in peripheral blood tended to increase over time in Group B but decrease in Group A (median normalized AUC = 0.246 and −0.243, respectively; p = 0.08). There were no other significant differences in the normalized AUC values of effector and memory T cells between treatment groups (data not shown).

There were no clear differences between the treatment groups in Treg frequencies. Although the normalized AUC of several Treg subpopulations tended to be higher in Group B, none of the p-values from the Mann-Whitney U-test of between-group differences was <0.05 (**Fig. 3A**). Treg levels decreased modestly in the days immediately following repeat Cy administrations in some patients, but tended to increase in the long term (data not shown). Some differences in effector and memory T cells in blood were seen between those patients with, compared to those patients without, an overall induced MUC1 immune response (**Fig. 3B**).

Cytokine production following *in vitro* stimulation of PBMC with MUC1-derived peptides appeared to involve a Th1/T-cytotoxic 1-like response (**Fig. 4**). The majority of patients (26 of 32; 81%) across both groups showed an on-treatment induction (≥50% increase, blue shading) of TNF-α production and in Group A IFN-γ production was induced for 6 of 10 (60%) patients with evaluable samples.

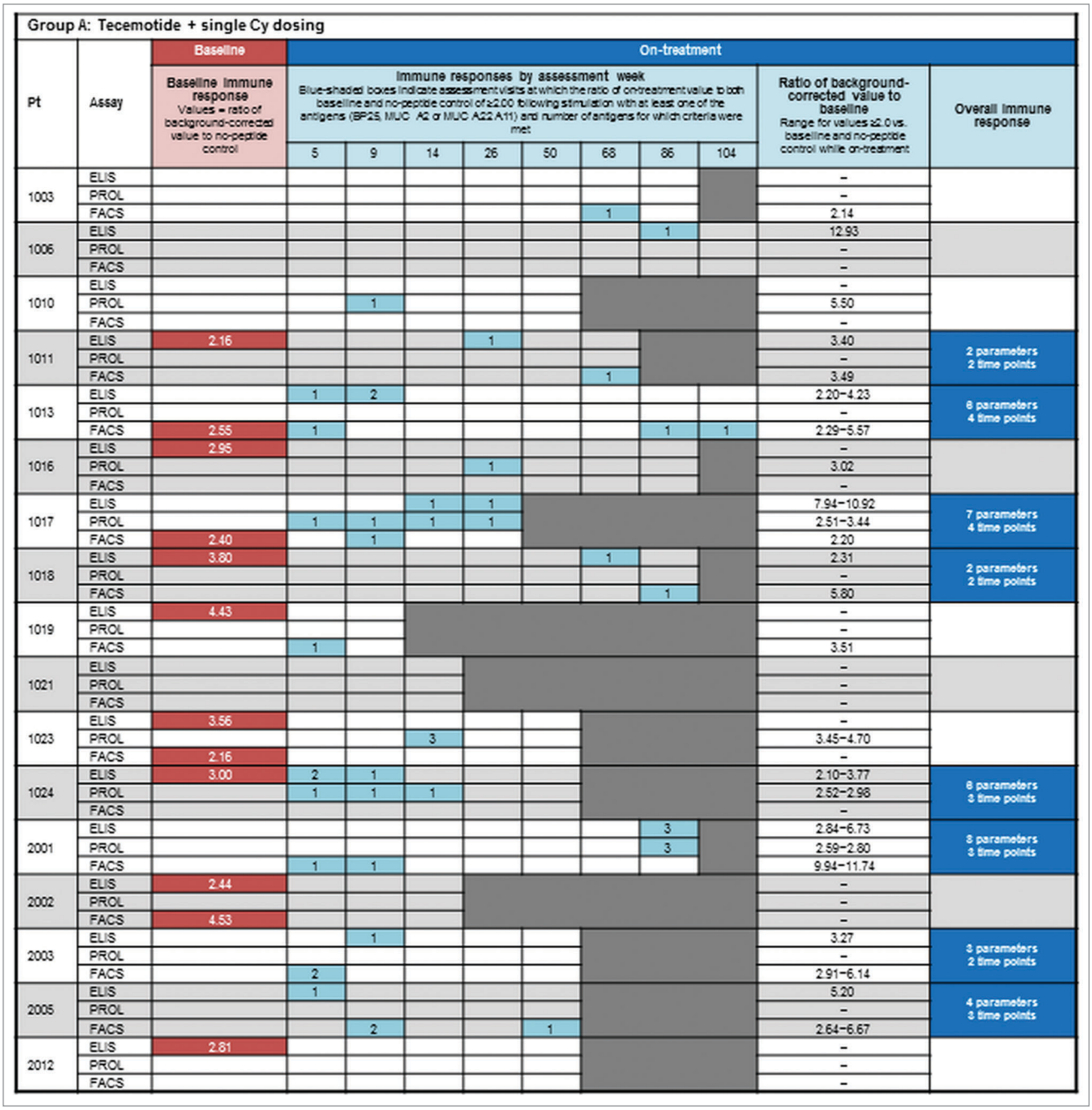
Safety and tolerability

All patients reported at least one treatment-emergent AE; **Table 3** summarizes the most frequently reported. Adverse events related to Cy (nausea, constipation, fatigue) occurred more frequently with repeated than single Cy dosing. The most common AEs reported as related to tecemotide were injection-site reactions (ISRs) (Group A: 8/17 [47%]; Group B: 10/17 [59%]) and flu-like symptoms (both Groups: 2/17 [12%]). The majority of ISRs involved nodulation or erythema. All ISRs were mild-to-moderate in severity. One patient in each group had an ISR that lasted >72 d and one patient (Group B) had an ISR lasting 56–72 d. All other ISRs lasted <56 d. At least one injection site nodule was reported in 23.5% of patients in both groups. When present, the median nodule size was 20 mm in Group A and 16 mm in Group B.

Two of the 34 patients permanently discontinued study treatment owing to treatment-emergent AEs: one patient in Group A due to an injection site ulcer (grade 2) and one patient in Group B with mood alteration (grade 1) who discontinued Cy but not tecemotide. Three (18%) patients in Group A and 5 (29%) in Group B reported ≥1 grade 3/4 treatment-emergent AE. In one instance in Group B – a case of encephalitis with secondary symptoms of aphasia, cerebral hemorrhage, hypoxia, loss of consciousness and status epilepticus – this was assessed as possibly related to study medication. The patient was initially diagnosed with stage II MM and previous treatment with several regimens had included high-dose melphalan followed by autologous stem cell transplantation and maintenance chemotherapy with bortezomib (discontinued prior to study entry). The event occurred during the 6-weekly treatment phase after the patient had received 13 tecemotide injections and 7 Cy infusions, and started 27 and 24 d after the last administrations of Cy and tecemotide,

Table 2. Summary of on-treatment induction of overall MUC1-specific immune responses

		Patients with overall on-treatment immune response, n/N (%)
All patients		15/32 (47)
Treatment group	Group A: single Cy dosing	8/17 (47)
	Group B: repeated Cy dosing	7/15 (47)
Baseline MUC1-specific immune response	Present	8/17 (47)
	Absent	7/15 (47)
Disease stage	Untreated asymptomatic stage I/II	7/13 (54)
	Previously treated stage II/III	8/19 (42)



Group A: Tecemotide + single Cy dosing

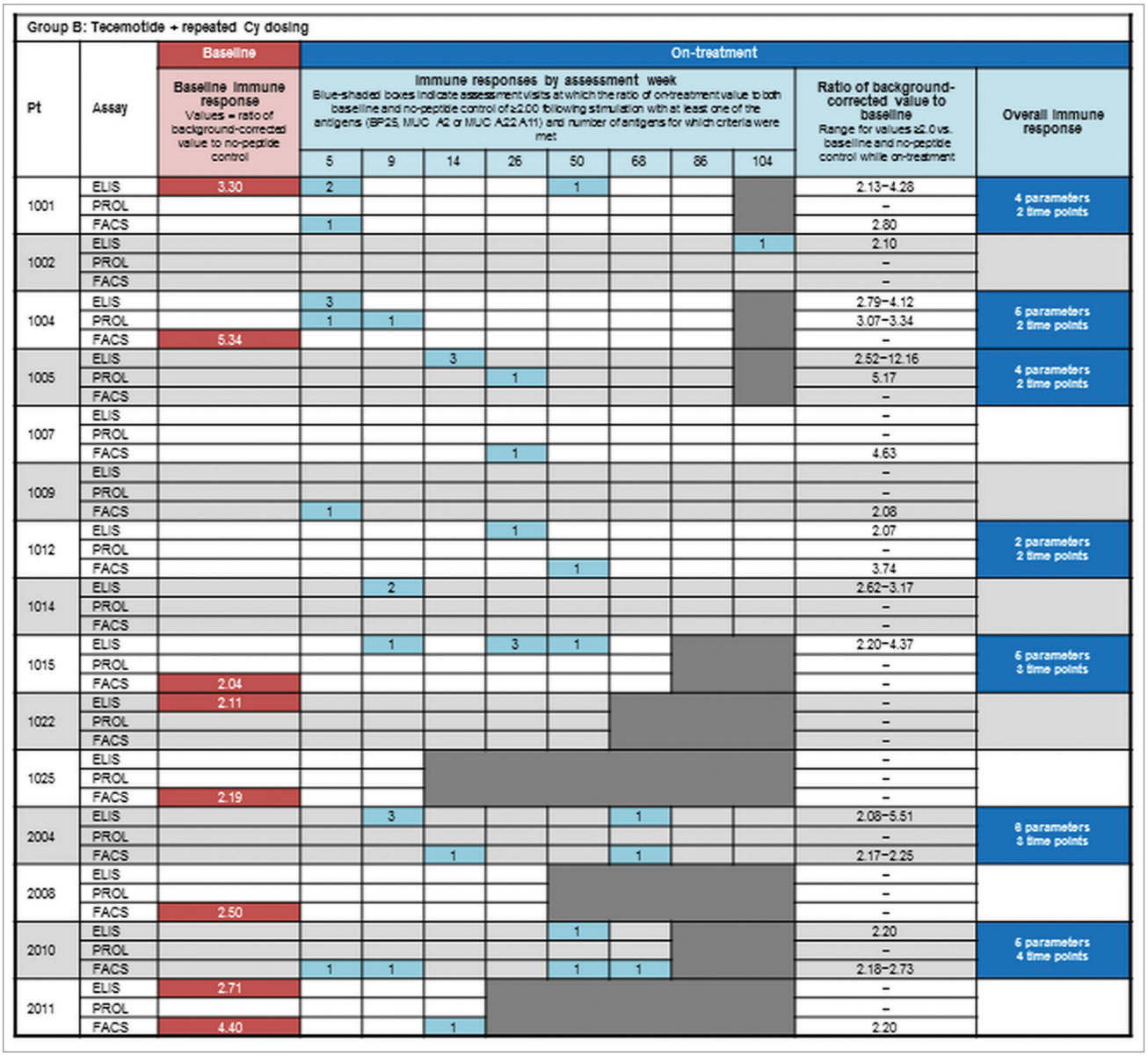
Pt	Assay	Baseline: Baseline immune response (Values = ratio of background-corrected value to no-peptide control)	On-treatment: Immune responses by assessment week (Blue-shaded boxes indicate assessment visits at which the ratio of on-treatment value to both baseline and no-peptide control of ≥2.00 following stimulation with at least one of the antigens (BP25, MUC A2 or MUC A22 A11) and number of antigens for which criteria were met)								On-treatment: Ratio of background-corrected value to baseline (Range for values ≥2.0 vs. baseline and no-peptide control while on-treatment)	Overall immune response
			5	9	14	26	50	68	86	104		
1003	ELIS										–	
	PROL										–	
	FACS							1			2.14	
1006	ELIS								1		12.93	
	PROL										–	
	FACS										–	
1010	ELIS										–	
	PROL			1							5.50	
	FACS										–	
1011	ELIS	2.16				1					3.40	2 parameters 2 time points
	PROL										–	
	FACS							1			3.49	
1013	ELIS		1	2							2.20–4.23	6 parameters 4 time points
	PROL										–	
	FACS	2.55	1						1	1	2.29–5.57	
1016	ELIS	2.95									–	
	PROL					1					3.02	
	FACS										–	
1017	ELIS				1	1					7.94–10.92	7 parameters 4 time points
	PROL		1	1	1	1					2.51–3.44	
	FACS	2.40		1							2.20	
1018	ELIS	3.80						1			2.31	2 parameters 2 time points
	PROL										–	
	FACS								1		5.80	
1019	ELIS	4.43									–	
	PROL										–	
	FACS		1								3.51	
1021	ELIS										–	
	PROL										–	
	FACS										–	
1023	ELIS	3.56									–	
	PROL				3						3.45–4.70	
	FACS	2.16									–	
1024	ELIS	3.00	2	1							2.10–3.77	8 parameters 3 time points
	PROL		1	1	1						2.52–2.98	
	FACS										–	
2001	ELIS								3		2.84–6.73	8 parameters 3 time points
	PROL								3		2.59–2.80	
	FACS		1	1							9.94–11.74	
2002	ELIS	2.44									–	
	PROL										–	
	FACS	4.53									–	
2003	ELIS			1							3.27	3 parameters 2 time points
	PROL										–	
	FACS		2								2.91–6.14	
2005	ELIS		1								5.20	4 parameters 3 time points
	PROL										–	
	FACS			2			1				2.64–6.67	
2012	ELIS	2.81									–	
	PROL										–	
	FACS										–	

Figure 2. Baseline MUC1 immune responsiveness and on-treatment induction of MUC1-specific immune responses. (**A**) Group A: tecemotide with single Cy dosing and (**B**) Group B: tecemotide with repeated Cy dosing. Baseline MUC1-specific immune response (■) defined as a ≥2-fold increase over no peptide control in at least one assay at baseline. On-treatment assessment time points at which there was a ≥2-fold increase over baseline and no peptide control are highlighted (■) for each patient and assay (ELISPOT, proliferation and FACS). Immune responses were assessed following stimulation with the MUC1-derived peptides BP25, MUC A2 or MUC A22 A11, and values in the shaded boxes indicate the number of antigens for which there was a ≥2-fold increase for each assay and time point. On-treatment responses were defined as a ≥2-fold increase over baseline and no peptide control in at least one of the assays (overall immune response, ■) on at least 2 occasions while on study treatment. Cy: cyclophosphamide; ELIS: enzyme-linked immunosorbent spot (ELISpot); FACS: fluorescence-activated cell sorting; PROL: proliferation. Pt: patient.

respectively. Study treatment was discontinued but the patient died approximately 2 months after the onset of neurologic symptoms. Post-mortem assessments were inconclusive as to the cause. The event led to the clinical hold after which repeated dosing of Cy was halted.

Clinical effects

There were no objective clinical responses according to Bladé criteria.[23] Disease progression occurred in 9 of 17 (53%) patients in Group A and 5 of 15 (33%) patients in Group B (IDA Set). Median time to tumor progression (TTP) was 15.2 months in



Group B: Tecemotide + repeated Cy dosing

Pt	Assay	Baseline	On-treatment									
		Baseline immune response Values = ratio of background-corrected value to no-peptide control	Immune responses by assessment week Blue-shaded boxes indicate assessment visits at which the ratio of on-treatment value to both baseline and no-peptide control of ≥2.00 following stimulation with at least one of the antigens (BP2S, MUC A2 or MUC A22.A11) and number of antigens for which criteria were met								Ratio of background-corrected value to baseline Range for values ≥2.0 vs. baseline and no-peptide control while on-treatment	Overall immune response
			5	9	14	26	50	68	86	104		
1001	ELIS	3.30	2				1				2.13–4.28	4 parameters 2 time points
	PROL										–	
	FACS		1								2.80	
1002	ELIS									1	2.10	
	PROL										–	
	FACS										–	
1004	ELIS		3								2.79–4.12	6 parameters 2 time points
	PROL		1	1							3.07–3.34	
	FACS	5.34									–	
1005	ELIS				3						2.52–12.16	4 parameters 2 time points
	PROL					1					5.17	
	FACS										–	
1007	ELIS										–	
	PROL										–	
	FACS					1					4.63	
1009	ELIS										–	
	PROL										–	
	FACS		1								2.08	
1012	ELIS					1					2.07	2 parameters 2 time points
	PROL										–	
	FACS						1				3.74	
1014	ELIS			2							2.62–3.17	
	PROL										–	
	FACS										–	
1015	ELIS			1		3	1				2.20–4.37	6 parameters 3 time points
	PROL										–	
	FACS	2.04									–	
1022	ELIS	2.11									–	
	PROL										–	
	FACS										–	
1025	ELIS										–	
	PROL										–	
	FACS	2.19									–	
2004	ELIS			3				1			2.08–5.51	8 parameters 3 time points
	PROL										–	
	FACS				1			1			2.17–2.25	
2008	ELIS										–	
	PROL										–	
	FACS	2.50									–	
2010	ELIS						1				2.20	6 parameters 4 time points
	PROL										–	
	FACS		1	1			1	1			2.18–2.73	
2011	ELIS	2.71									–	
	PROL										–	
	FACS	4.40			1						2.20	

Figure 2. Continued.

Group A and was not reached in Group B (hazard ratio [HR] = 0.378; 95% CI 0.121, 1.180; p = 0.08), although the small number of events should be noted.

The frequency of progressive disease was 33% (5 of 15 patients) among patients with an overall induced immune response vs. 53% (9 of 17 patients) of those without. Median TTP was not reached for immune responders and was 15.9 months for non-responders (HR = 0.486; 95% CI 0.162, 1.458, p = 0.19).

The slope of M-protein concentration over time was analyzed comparing per patient the slope before enrolment to the slope after study entry. A down-shift in slope was assessed as reflecting reduced tumor activity. While receiving tecemotide the M-protein slope was down-shifted compared to that prior to study enrolment in 13 of 29 (45%) evaluable patients (**Fig. 5A**). On-treatment reduction in the M-protein slope occurred in 9 of 12 (75%) patients with chemotherapy-naïve MM stage I/II and 4 of 17 (24%) patients with previously treated MM stage II/III. On-treatment changes in M-protein concentration did not differ significantly between Groups A and B. The presence of a treatment-induced MUC1 response was not associated with M-protein changes during treatment. However, the median area under the curve (AUC) of M-protein concentration changes up to week 26 of treatment (M-protein AUC26) was positive (increasing) in patients with, but negative (decreasing) in patients without, a pre-existing baseline MUC1 response (p = 0.01; **Fig. 5B**). Appendix S7 shows the time course of M-protein changes for a representative patient with an on-treatment slope reduction.

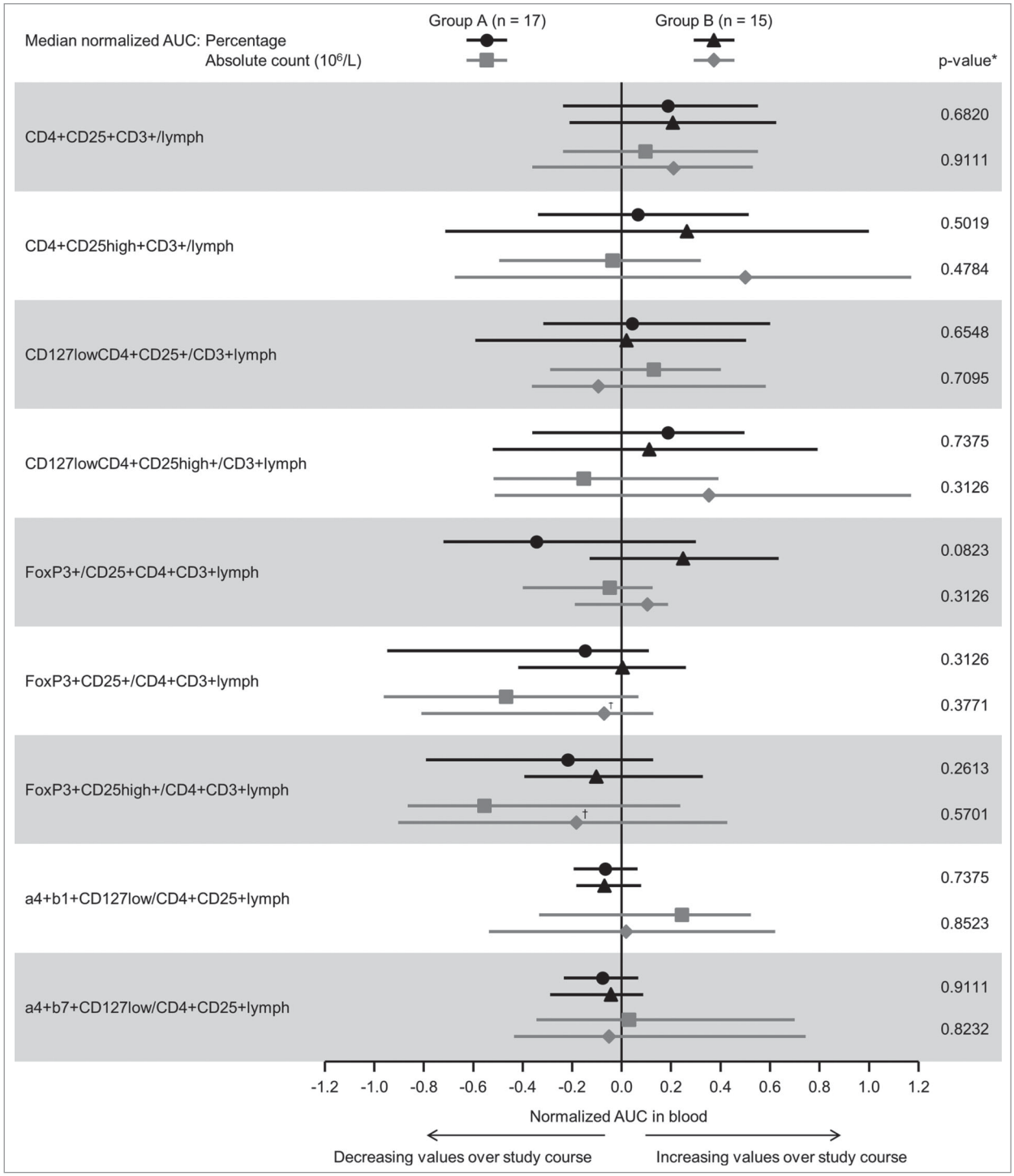


Figure 3. Median normalized AUC values for T cell subsets in blood. Comparison of median normalized AUC values of percentages and absolute counts for (**A**) Treg cells in Groups A and B; and, (**B**) naïve, effector and memory CD4+ and CD8+ T cells for patients with and without an immune response. Bars = interquartile range. AUC: area under the curve. *Mann-Whitney U-test testing similarity of distribution of AUC values regarding treatment arms. †n = 14.

Discussion

This is the first exploratory trial of the antigen-specific cancer immunotherapy (ASCI) tecemotide in patients with MM. The primary objective was to determine whether tecemotide induced a MUC1-specific T cell response. We also sought to characterize the nature of any immune response, and to obtain preliminary insights into the effects of single versus repeated low-doses of Cy

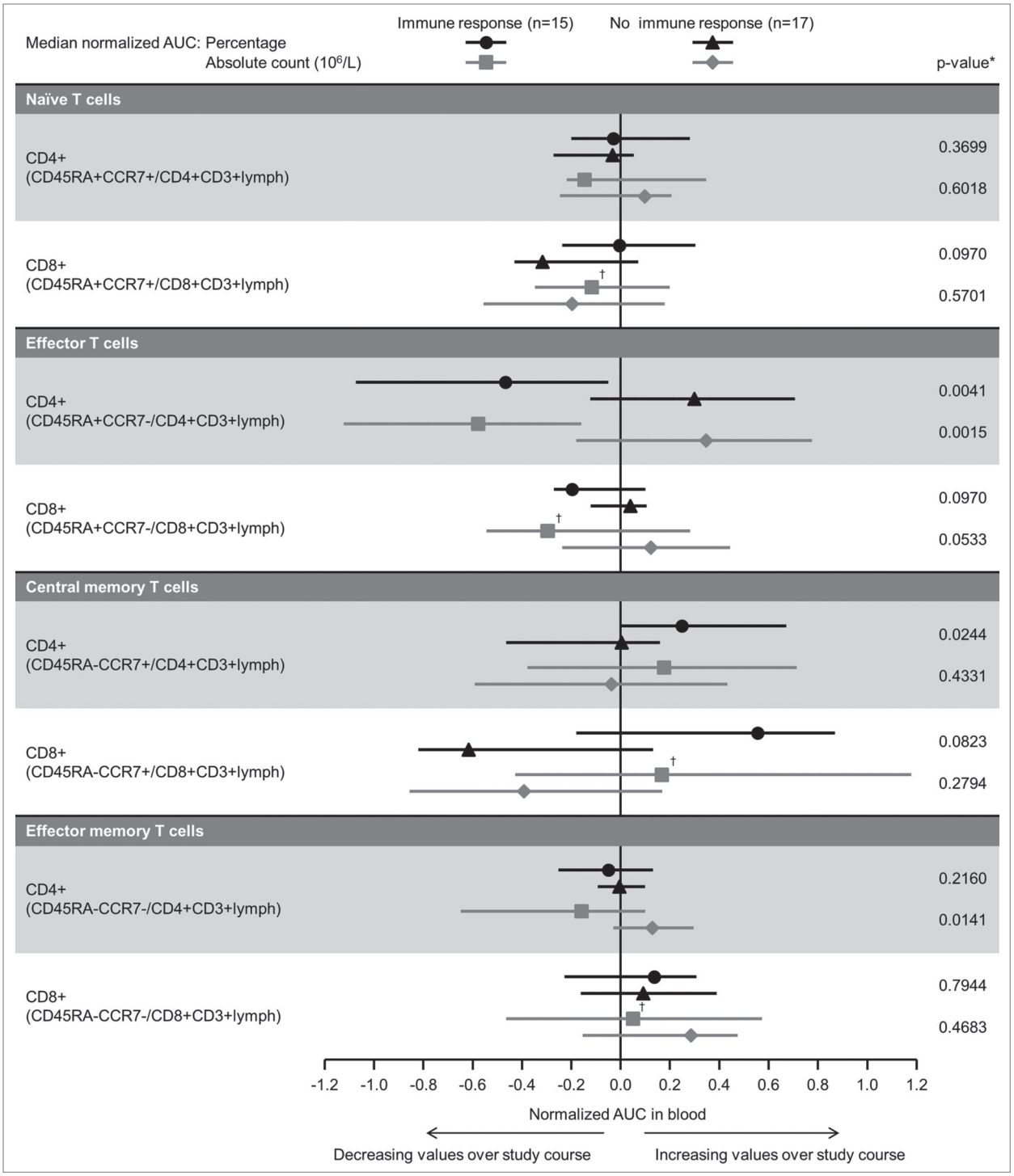
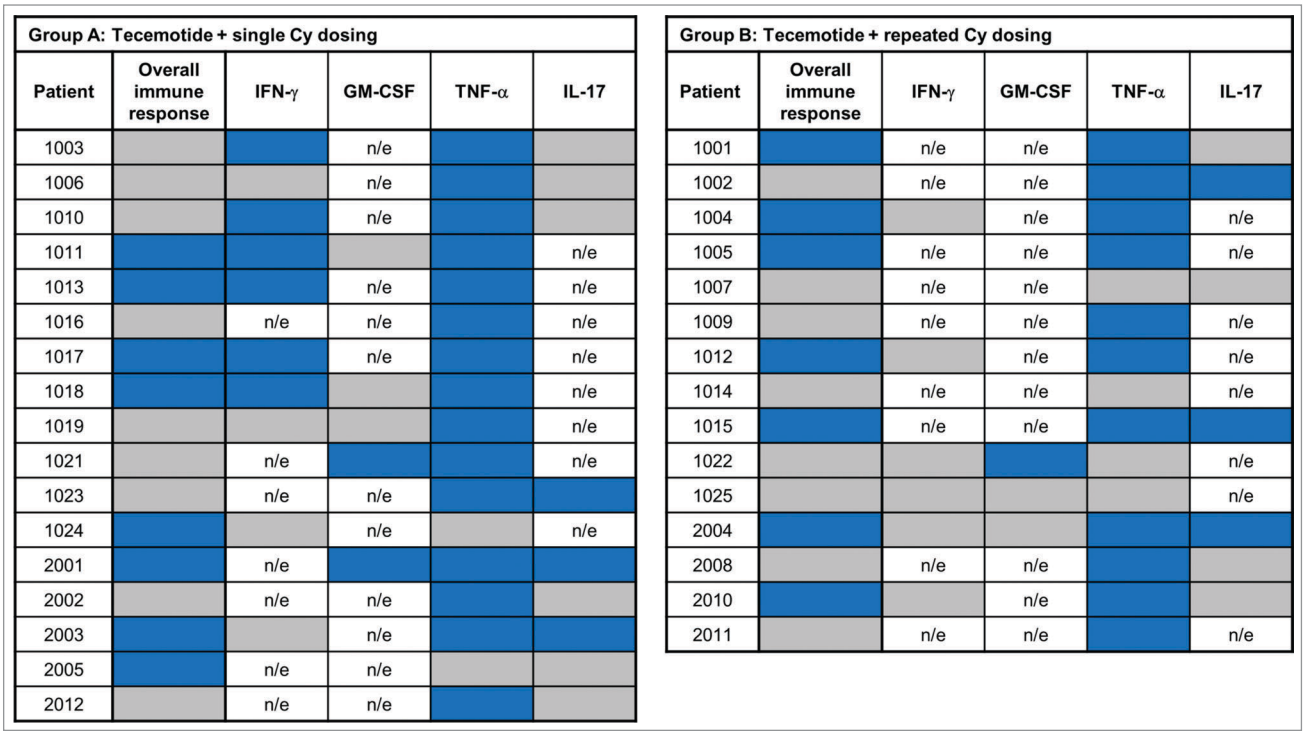


Figure 3. Continued.

on Treg levels and the response to immunotherapy, as well as to gain a preliminary assessment of the possible clinical, disease-stabilizing effects of tecemotide.

MUC1 expression is present in ~40–60% of patients with MM,[25] including those with newly diagnosed disease.[26] This study included only patients with MUC1-expressing MM cells. The frequency of spontaneous MUC1-specific T cell responses prior to starting study treatment suggested that the immune system was often already primed to tumor-derived MUC1 as a result of host immunosurveillance.

Our results support the ability of tecemotide immunotherapy to elicit MUC1-specific responses in a substantial proportion of people with MM, with ~50% of patients in the study showing an induced or augmented response during the study. The rate of



Group A: Tecemotide + single Cy dosing

Patient	Overall immune response	IFN-γ	GM-CSF	TNF-α	IL-17
1003			n/e		
1006			n/e		
1010			n/e		
1011					n/e
1013			n/e		n/e
1016		n/e	n/e		n/e
1017			n/e		n/e
1018					n/e
1019					n/e
1021		n/e			n/e
1023		n/e	n/e		
1024			n/e		n/e
2001		n/e			
2002		n/e	n/e		
2003			n/e		
2005		n/e	n/e		
2012		n/e	n/e		

Group B: Tecemotide + repeated Cy dosing

Patient	Overall immune response	IFN-γ	GM-CSF	TNF-α	IL-17
1001		n/e	n/e		
1002		n/e	n/e		
1004			n/e		n/e
1005		n/e	n/e		n/e
1007		n/e	n/e		
1009		n/e	n/e		n/e
1012			n/e		n/e
1014		n/e	n/e		n/e
1015		n/e	n/e		
1022					n/e
1025					n/e
2004					
2008		n/e	n/e		
2010			n/e		
2011		n/e	n/e		n/e

Figure 4. Immunotherapy-induced production of non-disease-related cytokines by PBMC. Cytokine production determined by Luminex® assay following *in vitro* stimulation of PBMC with MUC1-derived peptides (BP25, MUC-A2 and MUC-A22-A11). Induction (■) or no induction (■) of IFN-γ, GM-CSF, TNF-α and IL-17 production (≥50% increase on one occasion) as well as overall immune response (≥2-fold on 2 occasions, see supplementary data Appendix S3) for patients in the Immunologic Diagnostic Analysis Set. n/e = not evaluable. Cy: cyclophosphamide; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN-γ: interferon-γ; IL-17: interleukin-17; TNF-α: tumor necrosis factor-α.

treatment-induced MUC1-specific immune responses was similar in both treatment groups and in patients with or without a pre-existing MUC1 immune response. However, immune responses were generally weak with poor durability and did not correlate with a reduction in M-protein, possibly due to the poor magnitude of the immune response. Given the lack of previous studies investigating immune responses to peptide-based immunotherapies at multiple timepoints, it is difficult to speculate on the reasons for poor durability. However, poor immunogenicity of active immunotherapies has often been recognized as a limitation of this therapeutic strategy,[27] and is likely to be explained, at least in part, by the up-regulation of immunosuppressive entities within the tumor microenvironment.[28] As such, combination therapies that enhance immune effector mechanisms may be of value to achieve maximum clinical benefit. Cytokines such as interleukins, interferons and granulocyte-macrophage colony-stimulating factor (GM-CSF) are potential immune stimulants. Of note, GM-CSF has been used effectively in combination with several active immunotherapies.[29-31] Toll-like receptors have been shown to potentiate the immune response to vaccination in preclinical studies, and clinical trials are now warranted to investigate this strategy further.[27] Immune checkpoint inhibitors represent another promising area of combination immunotherapy research.[32]

The assessment of leukocyte populations over time is difficult to interpret and may have been impacted by the clinical hold. However, on-treatment induction of a MUC1-specific immune response appeared to be associated with reduced CD4+ effector/effector memory T cells and to involve a Th1/T-cytotoxic 1-like response.

Repeated administration of low-dose Cy was halted after the clinical hold, confounding the secondary objective of comparing the 2 Cy dosing schedules. Consequently, any impact of repeated vs. single low doses of Cy remains unclear. Results from before the hold show that repeated low doses of Cy reduced Treg levels in the days immediately following each infusion in some patients. However, in the long-term, Tregs tended to increase. While studies in humans have shown that low intravenous doses of Cy can decrease Treg number and function,[33] this observation is sometimes limited to a subset (~50%) of patients[34] and others report no such effect.[35] These differences may be explained by the considerable variation in Cy pharmacokinetics between individuals. Genetic factors,[36] weight,[37,38] age[39] and organ dysfunction[40,41] have all been shown to influence Cy pharmacokinetics.

The study was exploratory and was not designed or powered to provide a definitive assessment of clinical safety and efficacy. However, the preliminary findings are compatible with a potential treatment effect of tecemotide in multiple myeloma. Study treatment was generally well-tolerated, consistent with experience in other malignancies.[19,42-45] The adverse events (AEs) most frequently reported as possibly related to tecemotide administration were injection-site reactions and flu-like symptoms. Few serious AEs were reported and, with one exception, were not considered

Table 3. Treatment-emergent adverse events (Safety Analysis Set). (A) Treatment-emergent AEs of any grade reported in ≥15% of patients in either treatment group. (B) All grade 3/4 treatment-emergent AEs

Adverse event, n (%)	Group A Single Cy dosing (n = 17)	Group B Repeated Cy dosing (n = 17)
A) Treatment-emergent AEs reported in ≥15% of patients in either treatment group		
Back pain	10 (59)	7 (41)
Fatigue	9 (53)	11 (65)
Nausea	7 (41)	12 (71)
Upper respiratory tract infection	7 (41)	9 (53)
Nasopharyngitis	7 (41)	7 (41)
Cough	5 (29)	2 (12)
Injection site nodule	4 (24)	7 (41)
Chest pain	4 (24)	4 (24)
Arthralgia	4 (24)	2 (12)
Depression	4 (24)	2 (12)
Pain in extremity	3 (18)	5 (29)
Pyrexia	3 (18)	5 (29)
Hypertension	3 (18)	4 (24)
Injection site erythema	3 (18)	4 (24)
Rash	3 (18)	4 (24)
Headache	3 (18)	3 (18)
Myalgia	3 (18)	2 (12)
Injection site pruritus	3 (18)	0 (0)
Constipation	2 (12)	8 (47)
Diarrhea	2 (12)	3 (18)
Contusion	1 (6)	6 (35)
Vertigo	1 (6)	3 (18)
Alopecia	0 (0)	3 (18)
Anemia	0 (0)	3 (18)
B) All treatment-emergent grade 3/4 events		
At least one grade 3/4 event	3 (18)	5 (29)
Not related to study treatment*	Pt 1006: deep vein thrombosis, erysipelas Pt 2001: retinal detachment (2 reports) Pt 2005: breast cancer	Pt 1007: wound infection Pt 1009: neck pain Pt 1015: atrial fibrillation, bacterial arthritis, cholecystitis, sepsis Pt 2008:dysphagia, pneumonia (2 reports), hypoalbuminemia Pt 2011: pyrexia, sepsis
Possibly related to study treatment*	–	Pt 2008: fatal encephalitis

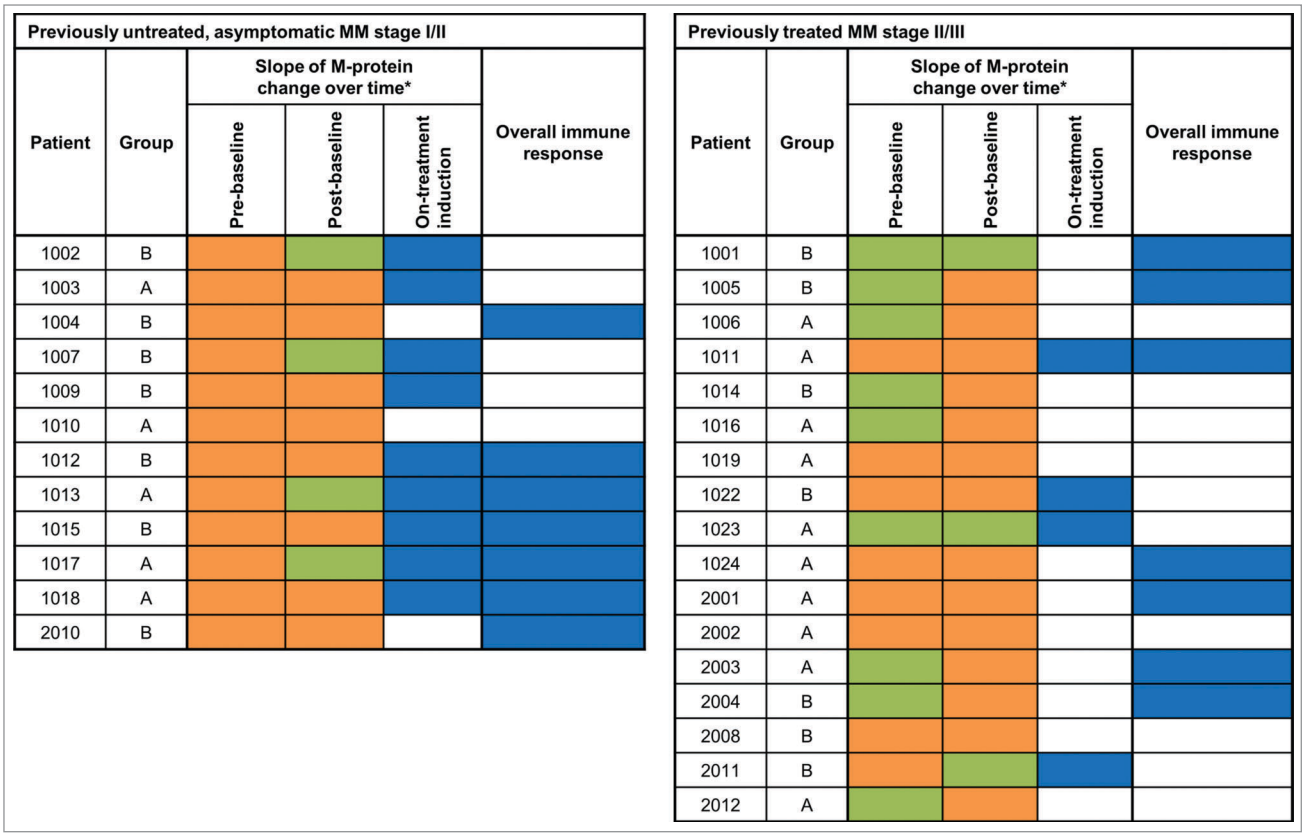
AE: adverse event; Pt: patient.
*As assessed by the investigator.

as related to study treatment. The underlying cause of the case of fatal encephalitis could not be clearly established, even after autopsy.

No objective clinical responses were seen according to Bladé criteria. This was not unexpected as, based on the mechanism of action of tecemotide, disease stabilization might be anticipated but not objective tumor regressions. It is now widely recognized that the standard response criteria for assessing the effects of cytotoxic chemotherapy have important limitations when applied to immunotherapeutic agents and do not provide a complete description of their effects.[46] Larger controlled trials are needed to explore the possibility of prolonged TTP in patients with an induced/augmented MUC1-specific immune response after treatment with tecemotide. The results from the START trial of tecemotide in unresectable stage III NSCLC, in which prolonged survival with tecemotide was observed in a subgroup of patients following concurrent but not sequential chemoradiotherapy,[45] support the potential for clinical benefit with tecemotide in selected patients and are consistent with the findings from this study.

M-protein concentrations were determined as a biochemical indicator of disease stabilization, with reduction over time thought to reflect reduced tumor activity.[23] The on-treatment reductions in M-protein changes over time in nearly 50% of patients provide early support for a possible clinical benefit of tecemotide particularly in early stage MM patients. We found no clear evidence that repeated Cy administration explained the downshift in M-protein concentration changes over time.

The reductions in M-protein concentration (as shown by negative AUC) in the majority of patients without a pre-existing MUC1-specific immune response are consistent with a disease-stabilizing effect. There was no evidence of disease stabilization among the patients with spontaneous MUC1-specific immune responses prior to treatment. The significance of a pre-treatment immune response for patient outcomes is likely to be complex. Our results and especially the subgroup results should be interpreted with caution given the small sample size but could reflect previous escape of the tumor from immune surveillance, rendering it less susceptible to a subsequent treatment-induced immune



Previously untreated, asymptomatic MM stage I/II

Patient	Group	Slope of M-protein change over time*			Overall immune response
		Pre-baseline	Post-baseline	On-treatment induction	
1002	B				
1003	A				
1004	B				
1007	B				
1009	B				
1010	A				
1012	B				
1013	A				
1015	B				
1017	A				
1018	A				
2010	B				

Previously treated MM stage II/III

Patient	Group	Slope of M-protein change over time*			Overall immune response
		Pre-baseline	Post-baseline	On-treatment induction	
1001	B				
1005	B				
1006	A				
1011	A				
1014	B				
1016	A				
1019	A				
1022	B				
1023	A				
1024	A				
2001	A				
2002	A				
2003	A				
2004	B				
2008	B				
2011	B				
2012	A				

Figure 5. M-protein concentration changes. (**A**) Comparison of the slope of M-protein changes over time before and during study treatment. Negative M-protein slope/decrease (■); Positive M-protein slope/increase (■); On-treatment reduction in M-protein slope* / Overall induced MUC1-specific immune response (■). (**B**) On treatment changes in M-protein concentration over time (AUC_{26}) according to the presence or absence of a spontaneous, pre-treatment or induced, on-treatment MUC1-specific immune response. Values are medians, with Q1-Q3 interquartile ranges (boxes) and minimum-maximum ranges (bars). P-value are from Mann-Whitney U-test testing similarity of AUC value distributions between groups. MM: multiple myeloma; MUC1: mucin 1. *Difference in M-protein slope post- versus pre-baseline <0; analysis excludes patients with <5 pre- or <5 post-baseline values; analyzed with 2 separate linear regression models on pre- and post- baseline values for each patient vs. treatment day, including intercept, "slope" refers to value of regression coefficient of slope parameter.

response. Other studies reporting baseline immune responses have shown variable effects on patient outcome. In a phase I study of patients with stage III/IV melanoma treated with PD1 blockade and a multipeptide vaccine, high numbers of pre-treatment MART-1 tetramer positive $CD8^+$ T cells were associated with disease progression, while lower numbers were associated with treatment response or stable disease. However, there was considerable variability in T cell numbers among the patients in each group.[47] Moreover, a phase I study of peptide vaccination in hepatocellular carcinoma indicated that those with a baseline immune response were more likely to experience stable disease than disease progression.[48] Thus it appears unlikely that pre-existing immunity can reliably predict patient outcomes.

Tecemotide has been evaluated in epithelial cancers with preliminary evidence of clinical benefit.[45] By demonstrating for the first time induction/augmentation of MUC1-specific immune responses in a substantial proportion of patients with MM, a malignancy frequently associated with cellular and humoral immunodeficiency, our findings suggest that, in principle, the clinical utility of tecemotide may extend to haematological malignancies. However, the immune responses we observed were weak and of poor durability. Our results provide preliminary evidence that the most robust responses and greatest clinical benefit may be seen in patients with early stage disease, and suggest that future investigations of ASCI should focus on this subgroup of patients. Furthermore, it appears that combining ASCI with appropriate immunomodulatory therapy, for example to overcome immunosuppressive mechanisms deployed in the tumor microenvironment, is critical to optimizing immune responses directed against tumor antigens and that exploration of the most effective combinations should be a priority for future studies.

The study had a number of limitations. It did not include a non-treated control or tecemotide-only arm. The inclusion criteria for the study appear wide, with both pre-treated and treatment-naïve patients being recruited. However, the patients had a similar clinical presentation with none showing an emerging need for therapy as evidenced by the large proportion of patients that continued into an extended immunization protocol. There was an imbalance between study groups in the MM disease stage at baseline, which limits conclusions that can be drawn regarding the differences in TTP between the 2 groups. The comparison of single versus repeated Cy dosing on

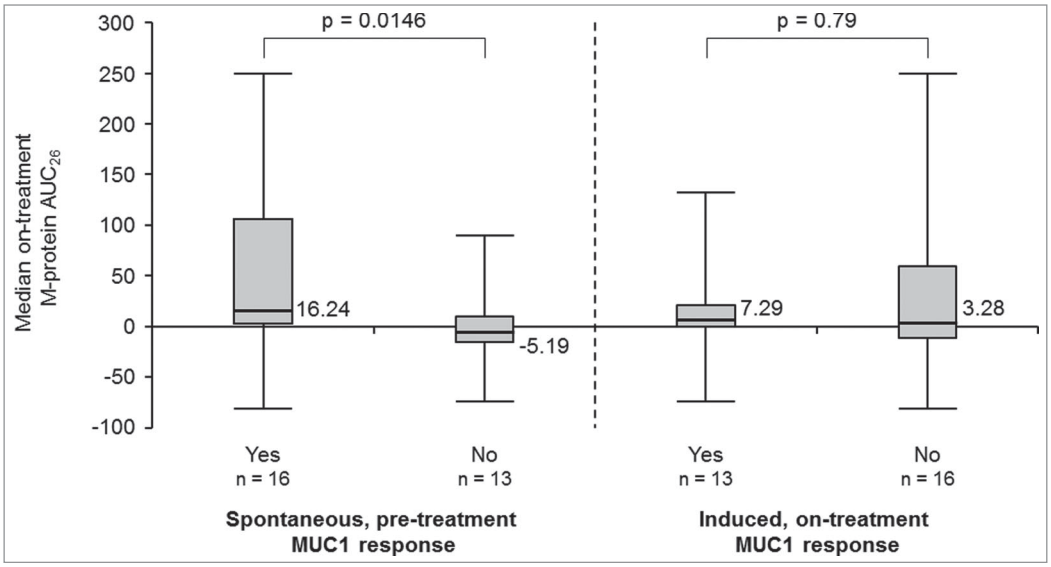


Figure 5. Continued.

immune effects including Treg frequency was also compromised due to the clinical hold. A further confounding effect is the interruption to all study treatments for the duration of the clinical hold, which occurred at different points in the 6-weekly tecemotide treatment phase for individual patients. Due to the exploratory nature of the study, all findings need to be confirmed in further studies.

In summary, MUC1-specific immune responses were induced or augmented in a substantial proportion of patients with MUC1-expressing MM cells during this study of tecemotide and Cy, and preliminary evidence is consistent with clinical stabilization in a subset of these patients.

Patients and Methods

Patients

Eligible patients had MUC1-expressing MM cells (MUC1 expression by ≥10% of plasma MM cells, estimated by immunostaining of clot sections of formalin-fixed, paraffin-embedded bone marrow biopsies using monoclonal antibodies against CD138 (clone MI15; Dako A/S, Copenhagen, Denmark) and against CD227 (MUC1; clone HMPV; BD Biosciences - PharMingen, San Diego CA, USA)) and either:

- Previously untreated, slowly progressive, asymptomatic stage I/II MM with rising monoclonal protein (M-protein) concentrations (≥10%) on 2 occasions separated by ≥4 weeks within the last 18 months.

Or:

- Stage II/III MM with a stable response/plateau phase (Bladé criteria[23]) and a treatment-free interval of ≥3 months after prior anti-tumor therapy.

These patient groups were selected because of the relatively stable/slowly progressive state of MM, which may allow for better response to immunotherapy, and because of feasibility considerations for this single-center trial. Patients were ≥18 years, with Eastern Cooperative Oncology Group performance status ≤1 and life expectancy ≥6 months. Other inclusion criteria included platelet count ≥100 × 10^9/L, white blood cell count ≥2.5 × 10^9/L, hemoglobin ≥90 g/L, total bilirubin ≤1.5 × the upper reference range (URR), aspartate aminotransferase ≤2.5 × URR and serum creatinine ≤2 × URR.

Key exclusion criteria included previous exposure to MUC1-targeting therapy, radiotherapy, immunotherapy or any investigational drug in the preceding 30 d and presence of any pre-existing medical condition requiring chronic steroid or immunosuppressive therapy other than maintenance therapy with prednisone ≤10 mg/day or equivalent. Medical conditions excluding participation included hereditary/congenital immunodeficiencies, autoimmune disease that could compromise patient safety, clinically significant cardiac disease, other previous malignancy within 5 y (excluding basal cell carcinoma of the skin, *in situ* carcinoma of the uterine cervix or gastrointestinal intramucosal carcinoma), known hepatitis B and/or C, and splenectomy.

Study design and conduct

All patients in this single-center study received tecemotide and were randomly allocated (1:1 ratio) to single (Group A) or repeated (Group B) dosing of Cy using a randomization list with permutated blocks of randomly varying block sizes. Tecemotide was given as 8 consecutive weekly subcutaneous administrations followed by 6-weekly administration until disease progression requiring anti-tumor therapy (Appendix S1). Each tecemotide dose was administered as 4 injections to different anatomical sites, each containing one quarter of the total dose (806 μg lipopeptide, Appendix S2). In Group A, a single intravenous infusion of Cy (300 mg/m^2; maximum total dose of 600 mg) was administered 3 d before the first tecemotide injection. In Group B, Cy was additionally administered 3 d prior to the tecemotide injection at week 5, and 3 d before each injection during the 6-weekly treatment phase starting at week 14.

Patients were enrolled between January 2008 and February 2010, and the primary analysis was performed in January 2011 after the last patient on treatment had reached Week 50. The study was impacted by a clinical hold due to a serious AE of fatal

encephalitis in a patient in Group B that was possibly treatment-related. At the time of the clinical hold, patient recruitment had been completed and all patients had completed at least the weekly treatment phase. Treatment was interrupted for 5 months at various stages for individual patients. Repeated dosing of Cy in Group B was stopped when the study resumed.

Recruitment of 15 patients per treatment arm was considered sufficient for the exploratory analysis of immunologic effects and power calculations were not performed.

Ethics statement

All patients gave written, informed consent. The study was conducted in compliance with the principles of the International Conference on Harmonisation guidelines on Good Clinical Practice, the Declaration of Helsinki and local regulatory requirements, and was approved by the Karolinska Ethical Committee Review Board (EPN 2009/1765–32) and the Swedish Medical Products Agency (151:2007/52348). The trial was registered at EudraCT as #2006–001810–33 and at www.clinicaltrials.gov as #NCT01094548.

Immunomonitoring

The primary objective was to evaluate the MUC1-specific immune response to tecemotide in patients with MM. Peripheral blood mononuclear cells (PBMC) were collected at baseline, before the first Cy infusion, prior to tecemotide administration at week 5, one week after the last weekly treatment (week 9), at weeks 14, 26 and 50 during the 6-weekly treatment phase, and every 18 weeks thereafter.

The primary target variable was the specific anti-MUC1 T cell response. The following parameters were considered after short-term (5 d for ELISpot/lymphoproliferation assays, 6 hours for intracellular interferon-γ [IFN-γ] staining) in vitro stimulation of PBMC with MUC1-derived peptides:

- ELISpot: *in vitro* stimulation with either BP25, MUC-A2 or MUC-A11
- Proliferation assay: stimulation with either BP25, MUC-A2 or MUC-A11
- Intracellular cytokine staining by FACS:
    - IFN-γ+CD69+/CD8+CD3+lymphocytes following stimulation with BP25
    - IFN-γ+CD69+/CD4+CD3+lymphocytes following stimulation with BP25.

BP25 is a synthetic MUC1 peptide consisting of 25 amino acids (STAPPAHGVTSAPDTRPAPGSTAPP). It differs from lipopeptide BLP25 by the absence of a palmitoyl side chain that facilitates binding of BP25 to a liposome. MUC-A2[11] and MUC-A22-A11[24] are synthetic MUC1 peptides consisting of 9 amino acids (MUC-A2: TSAPDTRPA; MUC-A22-A11: STAPPAHGV) and known to bind to HLA-A2 and HLA-A11, respectively. These were used to assess immune responses to peptides with different HLA specificities. The main criteria for assessing MUC1-specific immune responses were the following:

- ***Overall induced immune response:*** for at least 2 timepoints: at least one parameter in at least one assay with ratio to background ≥2, and ratio of background-corrected value to baseline ≥2
- ***Early increase of MUC1-specific immune response:*** for at least one parameter and timepoint up to Week 9: ratio to background ≥2, and ratio of background-corrected value to baseline ≥2
- ***MUC1-specific immune response at baseline:*** for at least one parameter and at least one baseline sample: ratio to background ≥2

Further details on the criteria for immune responses can be found in Appendix S3.

Determination of Treg frequencies and further T-cell phenotyping in blood was performed by FACS at baseline, prior to the first Cy infusion, prior to tecemotide administration at weeks 5 and 9, and throughout the 6-weekly treatment phase. Naive and memory, central and effector T cells were characterized using flow cytometry by their surface expression of CD45RA and CCR7, as initially proposed by Sallusto.[49] AUC of the different cell frequencies over time was calculated to assess increases (positive AUC) or decreases (negative AUC) of cell populations. Induction of T helper (Th) 1 cytokine secretion by PBMC was assessed by Luminex® assay (Luminex, Austin TX, USA) following short-term *in vitro* stimulation of PBMC with the MUC1-derived peptides. Induction of cytokine production following stimulation by MUC1-derived peptides was retrospectively defined as a 50% increase over baseline for the specified cytokine on ≥1 occasion throughout immunomonitoring.

Immunomonitoring and tumor cell analyses were performed with freshly isolated cells at the Cancer Center Karolinska, Stockholm, Sweden. Data management was performed by Quintiles, Illkirch, France and statistical analysis by PRA International, Mannheim, Germany, both under the supervision of Merck KGaA, Darmstadt, Germany.

Safety and tolerability

Safety and tolerability were assessed in terms of incidence, severity and relatedness of AEs, including injection site reactions, physical examination findings, vital signs, and laboratory and other assessments. Vital signs were monitored before each tecemotide administration and for 1 hour and 0.5 hours after each administration in the weekly and 6-weekly treatment phases, respectively. Injection sites were inspected at baseline and at each tecemotide administration visit.

Clinical efficacy

Clinical efficacy assessments included response and TTP determined using Bladé criteria,[23] as well as time to anti-tumor therapy and M-protein concentrations.

Statistical analysis

The main analysis of immunologic response, safety and efficacy was performed after all patients had reached week 50 of treatment or had discontinued study medication. Immunologic

parameters were analyzed in the IDA Set comprising patients with complete data during the primary treatment phase (i.e., baseline, week 5 and week 9) for ≥1 of the MUC1-specific immunomonitoring assays. The Safety Analysis Set comprised all subjects that received ≥1 dose of study treatment.

M-protein linear regression coefficients prior to enrolment vs. on study were calculated for each patient to explore changes in M-protein over time. M-protein concentration was measured pre-screening, at week 9, prior to every 6-weekly tecemotide administration and at the end of treatment visit. M-protein concentrations prior to enrolment were used to assess historical MM status. Slope analysis of M-protein data excluded patients with <5 pre- or <5 post-baseline values and used 2 separate linear regression models on pre- and post-baseline values for each patient versus treatment day, including intercept. 'Slope' refers to the regression coefficient for the slope parameter with negative slope indicating decreasing M protein concentrations over time and positive slope indicating rising M protein concentrations.

On-treatment changes over time were assessed as area under the curve (AUC), normalized to account for differences in treatment duration (Appendix S4). Between-group comparisons of AUC were based on the Mann-Whitney U-test. Time to progression was analyzed with the Kaplan-Meier method. Univariate Cox proportional hazards regression for the estimation of hazard ratios and their 95% confidence intervals, as well as the log-rank test were used for between-group comparisons.

All statistical analyses were performed separately for the 2 treatment arms, unless otherwise specified. The study was not powered to detect differences in responses between the arms. Statistics for continuous variables include means, medians, ranges and appropriate measures of variability. Qualitative variables were summarized by means of counts and percentages. Unless otherwise stated the calculation of proportions included the missing category. Statistical analyses are considered as descriptive even when statistical tests were conducted. No adjustment for multiplicity was performed.

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Clinical Research Organization: Quintiles

Disclosure of Potential Conflicts of Interest

AÖ received research funding from Merck KGaA. AS and AvH held employment positions at, and stock in, Merck KGaA, and AS holds stock in Oncothyreon. HM acted as a consultant for, and received research funding and honoraria from, Merck KGaA. UF held an employment position at Merck Serono S.A. Geneva. AC, EL and ER do not have any conflicts of interest to disclose.

Acknowledgments

The authors would like to thank patients, investigators and the study teams. Medical writing assistance was provided by Ian Faulkner, International Medical Press, UK.

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