HHS Public Access

Author manuscript

Br J Haematol. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

Br J Haematol. 2012 September ; 158(6): 700–711. doi:10.1111/j.1365-2141.2012.09225.x.

Epigenetic induction of adaptive immune response in multiple myeloma: sequential azacitidine and lenalidomide generate cancer testis antigen-specific cellular immunity

Amir A. Toor[1], **Kyle K. Payne**[2], **Harold M. Chung**[1], **Roy T. Sabo**[3], **Allison F. Hazlett**[1], **Maciej Kmieciak**[2], **Kimberly Sanford**[4], **David C. Williams**[4], **William B. Clark**[1], **Catherine H. Roberts**[1], **John M. McCarty**[1], and **Masoud H. Manjili**[2]

[1]Bone Marrow Transplant Program, Department of Internal Medicine, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA

[2]Department of Microbiology and Immunology, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA

[3]Department of Biostatistics, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA

[4]Department of Pathology, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA

Summary

Patients with multiple myeloma (MM) undergoing high dose therapy and autologous stem cell transplantation (SCT) remain at risk for disease progression. Induction of the expression of highly immunogenic cancer testis antigens (CTA) in malignant plasma cells in MM patients may trigger a protective immune response following SCT. We initiated a phase II clinical trial of the DNA hypomethylating agent, azacitidine (Aza) administered sequentially with lenalidomide (Rev) in patients with MM. Three cycles of Aza and Rev were administered and autologous lymphocytes were collected following the 2nd and 3rd cycles of Aza-Rev and cryopreserved. Subsequent stem cell mobilization was followed by high-dose melphalan and SCT. Autologous lymphocyte infusion (ALI) was performed in the second month following transplantation. Fourteen patients have

Correspondence: Amir A. Toor and Masoud H. Manjili, Massey Cancer Center, Virginia Commonwealth University, 1300 E Marshall Street, Richmond, VA 23298, USA. atoor@vcu.edu, mmanjili@vcu.edu.

Conflict of interest disclosure

Amir A Toor: Celgene corporation, research funding. Kyle K Payne: no conflicts. Harold M Chung: no conflicts. Roy T Sabo: no conflicts. Allison F Hazlett: no conflicts. Maciej Kmieciak: no conflicts. Kimberly Sanford: no conflicts. David C Williams Jr: no conflicts. William B Clark: no conflicts. Catherine Roberts: no conflicts. John McCarty: Celgene corporation, research funding. Masoud H Manjili: Celgene corporation, research funding.

This work is dedicated to the memory of the late Taylor Roberts (1984–2003).

Supporting Information

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

Authorship

Amir A Toor: designed research, performed research, analysed data, and wrote the paper. Kyle K Payne: performed research, and wrote the paper. Harold M Chung: performed research, and wrote the paper. Roy T Sabo: analysed data, and wrote the paper. Allison F Hazlett: analysed data. Maciej Kmieciak: performed research. Kimberly Sanford: performed research. David C Williams Jr: performed research. William B Clark: performed research. Catherine Roberts: performed research, analysed data, and wrote the paper. John McCarty: performed research. Masoud H Manjili: designed research, performed research, analysed data, and wrote the paper.

completed the investigational therapy; autologous lymphocytes were collected from all of the patients. Thirteen patients have successfully completed SCT and 11 have undergone ALI. Six patients tested have demonstrated CTA up-regulation in either unfractionated bone marrow ($n = 4$) or CD138+ cells ($n = 2$). CTA (CTAG1B)-specific T cell response has been observed in all three patients tested and persists following SCT. Epigenetic induction of an adaptive immune response to cancer testis antigens is safe and feasible in MM patients undergoing SCT.

Keywords

multiple myeloma; epigenetic induction; cancer testis antigens; adaptive immunity

Allogeneic stem cell transplantation (allo-SCT) is associated with a reduction in relapse rate in patients with multiple myeloma (MM) on the basis of an allo-immune graft vs. myeloma effect, mediated by donor immune cells targeting tumour (myeloma)-specific antigens, resulting in prolonged remission. Allografting is however complicated by graft-versus-host disease and unacceptable treatment-related mortality, obviating the survival benefit particularly if newly diagnosed myeloma patients are considered. On the other hand, patients undergoing high dose therapy with autologous stem cell transplantation (SCT) remain at risk for relapse, despite maintenance and consolidation regimens (Barlogie *et al*, 2006). There are additional toxicities, such as thromboembolic disease and disabling neuropathy, to be considered with such maintenance regimens. An alternative strategy is needed to relieve the burden of treatment toxicity observed in patients with myeloma whilst maintaining and prolonging current treatment efficacy. Immunotherapeutic interventions mimicking graft-versus-myeloma effect in the SCT setting may provide such an option. However efficacious, safe, and widely applicable strategies for immunotherapy remain elusive, limiting this option only to a select number of participants in clinical trials at tertiary cancer centers (Rapoport *et al*, 2009).

Cancer testis antigens (CTA) represent potential targets for immunotherapy in myeloma. These proteins are highly immunogenic with no natural self-tolerance because, under normal circumstances, they are only expressed in ‘immunologically privileged’ germ cells, and in the placenta (Simpson et al, 2005). Aberrant CTA expression has been observed in both solid tumours and in haematological malignancies, particularly in MM (Meklat *et al*, 2007). This often elicits a broad range of cellular and humoral immune responses. In myeloma, several reports have described sporadic over-expression of CTA and accompanying CTA-specific T cell and B cell responses (Lim *et al*, 2001; Wang *et al*, 2003; Jungbluth *et al*, 2005; Van Rhee *et al*, 2005; Condomines *et al*, 2007). Induced CTA alloreactivity has also been reported in MM patients undergoing allografting, possibly associated with freedom from relapse (Atanackovic *et al*, 2007). It is noteworthy that CTA expression is regulated by methylation of CpG islands in the promoters of these genes, which are mostly located on the X chromosome. There is evidence suggesting that therapy with azacitidine (Aza), a potent DNA methyl-transferase inhibitor, increases the expression of various CTA in a variety of *in vitro* and *in vivo* tumour models (Coral *et al*, 2002, 2006; Guo *et al*, 2006).

We hypothesized that *in vivo* induction of CTA by Aza may induce a CTA-specific T cell response if it is sequentially administered with an immuno-modulatory agent, lenalidomide (Rev), which is widely used in the therapy of MM. Rev works in part by increasing T cell and NK cell tumour cytotoxicity *in vitro* (LeBlanc *et al*, 2004; Hayashi *et al*, 2005). Additionally, Rev stimulates T cell proliferation and secretion of interleukin 2 (IL2) and γ-interferon (IFNG) in T cell co-stimulation assays (Corral *et al*, 1999; Davies *et al*, 2001). Further, CTA-specific T cells generated by this combination of Aza and Rev, when adoptively transferred to autologous SCT recipients, could expand *in vivo* and provide robust protection from disease progression. In this setting, SCT produces both a minimal residual disease state, as well as lympho-depletion, promoting the preferential proliferation of adoptively transferred CTA-specific T cells, setting the scene for effective adaptive immunotherapy (Rapoport *et al*, 2005).

In 2009, our programme initiated a multi-step phase II study to determine the feasibility of generating CTA-specific T cells in MM patients and their application in post-transplant maintenance (NCT01050790). MM patients received sequential Aza and Rev (Aza-Rev) to induce the expression of immunogenic CTA on malignant plasma cells and elicit a CTA-specific cellular immune response. The patients had autologous lymphocytes collected and cryopreserved following the second and third cycle of this regimen. After completion of the investigational regimen patients underwent stem cell mobilization and eventually SCT. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was administered post-transplant to facilitate haematopoietic recovery and augment dendritic cell (DC) function. The autologous lymphocytes were adoptively transferred to the patients in the second month after transplant. We report the promising early results of this clinical trial demonstrating the feasibility of administering Aza-Rev to MM patients prior to SCT, as well as the feasibility of collecting and reinfusing autologous lymphocytes following SCT. Further, we demonstrate induction of the expression of CTA in bone marrow of MM patients and an increase in CTA reactive T cell responses monitored by using human recombinant CTAG1B (NY-ESO-1) *in vitro*.

Methods

Patients

Patients were enrolled on a prospective phase II clinical trial approved by the Virginia Commonwealth University (VCU) institutional review board. MM patients referred to VCU's bone marrow transplant programme had to meet the following eligibility criteria; presence of residual disease, with either quantifiable serum or urinary M protein or free light chains, in the presence of a positive immunofixation or clonal bone marrow plasma cells; age between 18 and 70 years; able to undergo high dose therapy and SCT; adequate performance status, marrow (absolute neutrophil count of >1·5 × 10^9/l, platelet count >100 × 10^9/l) and end organ function. Patients refractory to or progressing on therapy with lenalidomide were excluded. Patients with high β2-microglobulin (≥ 0·055 g/l) and adverse cytogenetic changes were offered tandem SCT, whereas those with standard risk disease underwent a single autograft.

Investigational regimen

Patients with MM who were in a partial remission or plateau phase underwent 3 cycles of therapy with Aza 75 mg/m^2 given subcutaneously from day 1–5 (Vidaza; Celgene Corporation, Summit, NJ, USA), and Rev 10 mg daily given orally, from day 6–21 (Revlimid; Celgene Corporation). The 3 cycles of therapy were administered at 4-week intervals prior to blood stem cell mobilization. No planned corticosteroids were administered during this therapy. Following 3 weeks of therapy in the second and third cycles of Aza-Rev, autologous lymphocytes were collected by a single, 18-litre lymphapheresis procedure and cryopreserved (Figure S1). Aspirin was administered for thromboembolic prophylaxis.

After completion of the third cycle of Aza-Rev, peripheral blood stem cell mobilization was performed using granulocyte colony-stimulating factor (G-CSF; filgrastim) 10 μg/kg/day subcutaneously from day 1 until the end of apheresis, either with or without plerixafor (0·24 mg/kg subcutaneously from day 4 until the end of apheresis). Patients then went on to receive high dose melphalan (either 140 or 200 mg/m^2) on day -2, and underwent autologous SCT on day 0. GM-CSF (sargramostim 5 μg/kg/day) was administered from day 4 post-transplant for haematopoietic engraftment. Standard antimicrobial prophylaxis was administered.

Autologous lymphocyte infusion (ALI) was performed between day +30 to +60 of the SCT (following second transplant in tandem SCT recipients), after resolution of regimen-related toxicities and in the absence of active infections. Autologous lymphocytes collected following cycles 2 and 3 were infused together (Figure S1). Diphenhydramine and acetaminophen were administered for infusion reaction prophylaxis. Corticosteroid administration was avoided as much as possible following SCT and ALI. Patients were not given any routine maintenance therapy for myeloma following ALI, except for bisphosphonates when indicated. Periodic myeloma restaging was performed to monitor disease status.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the detection of CTA expression

Patient underwent bone marrow aspiration and biopsy before and after investigational therapy for standard histological studies. qRT-PCR was performed as previously described by our group (Ascierto *et al*, 2012). Both pre- and post- Aza-Rev treatment bone marrow samples were used to determine the expression of 10 different human CTA transcripts [*MAGEA3, MAGEA4, MAGEA5, MAGEA6, MAGEC1, CTAG1B (NY-ESO-1), SPACA3 (SLLP1), AKAP4, SPA17, SPANXB1 & SPANXB2*], using CTA-specific primers and human *GAPDH* (Table S1). Initially, qRT-PCR was performed using RNA isolated from unfractionated Ficoll-Hypaque separated marrow mononuclear cells. Subsequent patients had CD138$^+$ cells isolated from marrow mononuclear cells using an EasySep human CD138 positive selection kit, as instructed by the manufacturer (STEMCELL Technologies, Tukwila, WA, USA) followed by qRT-PCR analysis of CD138$^+$ plasma cells and CD138$^-$ fractions.

IFNG enzyme-linked immunosorbent assay (ELISA) for the detection of CTA-specific T cell responses

Blood samples, autologous lymphocytes and stem cells from the patients were evaluated for the presence of T and NK cells, as well as for CTA (CTAG1B)-specific T cells, before and after Aza-Rev therapy and after SCT. CTAG1B was selected as the antigenic target to be studied, because of its frequent up-regulation in the majority of patients (4/6) following Aza therapy and the availability of recombinant protein for performing IFNG ELISA, as previously described (Kmieciak *et al*, 2011a,b). Briefly, cellular co-cultures were developed in which autologous lymphocytes or stem cell product (4×10^5 cells/well) was cultured with autologous DCs (2:1) in the presence or absence of recombinant CTAG1B (8 μg/ml; 3H Biomedical; Uppsala, Sweden). Supernatants were collected after 20 h and subjected to IFNG ELISA (BD Biosciences, Franklin Lakes, NJ, USA).

Immuno-phenotypic analysis of the blood and stem cell apheresis product for measuring cellular immune parameters was performed, using a dual-platform technique on a CytomicsTM FC500 flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Antibodies to CD3, CD4, CD8, CD34, and CD56 (Beckman Coulter, Inc.) were employed to enumerate T cell subsets, haematopoietic stem cell and NK cells.

Study design

This was a phase II study designed to test the feasibility of safely giving Aza-Rev to patients with MM, followed by the collection and administration of ALI. The study was designed with the expectation that ≥70% of the patients would be able to mobilize a cell dose of 10^7 mononuclear cells/kg and that ≥ 80% of the patients would be able to receive the ALI following SCT. The required sample size, $n = 19$, was based on an independent sample χ^2 test with a 0·05 one-sided significance level and 80% power to detect that 70% of the patients would be able to mobilize the minimal cell dose against the null hypothesis of 40% (aim 1), and also to detect that 80% of the patients will be able to get the infusions post transplant against the null hypothesis of 50% (aim 2). Drop-out was assumed to be 30%. Early stopping criteria for haematopoietic toxicity, were defined as grade 4 neutropenia or thrombocytopenia lasting beyond 7 d, and for non-haematopoietic toxicity, as any unexpected grade 4 toxicity attributable to the Aza-Rev regimen or ALI, with >30% incidence deemed unacceptable. Disease progression, while on investigational therapy, with a progression rate of >30% prior to SCT was also unacceptable as was the inability of >30% of the patients to proceed onto SCT. Disease response and progression were as defined by the International Myeloma Working Group (Lecoutre *et al*, 1995).

Fourteen subjects have been enrolled to date. Temporal changes in $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$ cell subsets at different time points, as well as differences in CTA expressions before and after Aza-Rev therapy were tested using the Wilcoxon signed-rank test. All tests were one-sided, and CTA expression increases were deemed significant for P-values < 0·05. The likelihoods for observing significant outcomes for study aims 1 and 2 are estimated using Bayes methods (Durie *et al*, 2006). We assigned conservative and pessimistic prior beta distributions for each success rate where the modes matched the null hypothesized success rates for each aim (40% for aim 1; 50% for aim 2), and assumed that the accumulated

success rates were binomial processes based on n observed subjects. The predictive probability that we would observe efficacious outcomes for both aims (>70% mobilization for ALI and stem cells; >80% receive infusion) after observing the remaining (19–n) patients into the study would then be estimated using beta-binomial distributions.

Results

Patient demographics

Between February 2010 and February 2012, 14 patients with a median age of 60 years (range 40–69) were enrolled (Table I). Nine patients were African American, and seven were female. International Myeloma Working Group stage at diagnosis was I ($n = 2$), II ($n = 6$) and III ($n = 6$). Six had chromosomal abnormalities, largely consistent with high-risk disease. A median of two prior regimens had been administered (range 1–2) and eight had prior therapy with Rev. A median of 10·6 months had elapsed from diagnosis to start of Aza-Rev therapy and 10 cycles of therapy administered before study therapy was initiated.

Response to Aza-Rev and autologous lymphocyte collection

All 14 patients completed the 3 cycles of Aza-Rev and underwent both autologous lymphocyte (AL) collections. Circulating T cell counts were well preserved following Aza-Rev therapy (P = >0·06) resulting in comparable yield of AL at a median 21 d following cycles 2 and 3 of Aza-Rev, with 0·87 ± 0·38 and 0·82 ± 0·29 × 10^8 $CD3^+$ cells/kg (mean ± SD; $n = 14$, $P = 0·20$) respectively, with the first and second procedures (Table II). There was no significant difference in the circulating T cell subset and NK cell counts between the two cycles of Aza-Rev therapy (T test P = NS) (Fig 1).

Following Aza-Rev, three patients had further disease response (all very good partial remission; VGPR), while nine met the criteria for stable disease and two developed disease progression, representing a non-significant median 23% increase in paraprotein levels before and after therapy (T-test $P = 0·90$) (Fig 2A,B). Notably, two patients with high-risk cytogenetics (Patients 6 and 8) had further improvement in their response status. However, two other patients with high-risk cytogenetics (Patients 12 and 14) developed disease progression, with one requiring salvage chemotherapy. Two patients developed non-sustained grade 3 neutropenia following Aza-Rev. Patient 11 was noted to have an abnormal carbon monoxide diffusing capacity following Aza-Rev, and developed grade 3 pneumonitis following stem cell mobilization with G-CSF + plerixafor. This responded promptly to therapy with antibiotics and low dose corticosteroids.

Stem cell mobilization and transplantation

So far, 14 patients have undergone stem cell mobilization with G-CSF alone ($n = 6$), or G-CSF + plerixafor ($n = 7$), or G-CSF + chemotherapy ($n = 1$), and collected 9·3 ± 3·6 × 10^6 $CD34^+$ cells/kg body weight (Table II) on average. Stem cell mobilization was performed in a median 3 d of collection, and 3·5 weeks following completion of last cycle of Aza-Rev. Notably, the stem cell product had a $CD3^+$ cell dose of 6·0 ± 2.8 × 10^8/kg.

Thirteen patients have undergone SCT to date (tandem SCT in 4). The average stem cell dose infused for the first SCT was 4·7 ± 1·5 × 10^6 $CD34^+$ cells/kg body weight. Neutrophil engraftment was observed at median of 14 d (10–18) following SCT. One patient developed atrial fibrillation followed by hypoxia and pulmonary oedema coincident with engraftment, which resolved with corticosteroid therapy; another developed colonic ileus following engraftment. Both patients had resolution of their symptoms with supportive care.

Autologous lymphocyte infusion and disease status

Currently, 11 patients have received ALI at a median 6 weeks following SCT. Aside from one patient with a prior history of hypertension developing grade 2 elevation in blood pressure, no other toxicities were observed with ALI. The cell dose infused was 2·0 ± 1·0 × 10^8 $CD3^+$ cells/kg. With a median follow up of 7 months post-transplant, 10 of the 11 ALI recipients remain progression-free, with either complete remission ($n = 4$), VGPR ($n = 5$) or stable disease ($n = 1$), without further consolidation or maintenance therapy beyond the ALI (Table II). One (Patient 6) developed fulminant disease progression with extra-medullary relapse and died 6 months following SCT.

Blood $CD3^+$ cell count was significantly increased 2 weeks post-ALI compared with the pre-ALI value ($P = 0·04$), as was the $CD8^+$ cell count ($P < 0·02$) (Fig 3). $CD4^+$ and $CD56^+$ cell counts, although generally higher at 2 weeks following ALI, did not reach significance when compared with the pre-ALI values ($P > 0·06$).

Likelihood of autologous lymphocyte mobilization and infusion

Bayesian analyses were used to estimate the likelihood of successfully achieving study goals for both aims 1 and 2 by the end of the trial. The first study goal was to determine whether >70% of all patients were able to mobilize (AL and stem cells) against the null hypothesized value of 40%. All 14 of the recruited subjects were able to mobilize AL, leading to a predictive probability of 99%, indicating a high likelihood of adequate lymphocyte collection with this regimen. Of the 14 recruited subjects, 13 were able to mobilize adequate stem cells to proceed on to transplantation, again leading to a high likelihood (94%) of low potential for long-term marrow toxicity from this regimen. The second study aim was to ensure that >80% of all patients would be able to proceed on to receive ALI against the null hypothesized value of 50%. To date, of the 14 recruited subjects, 11 have advanced to the point where they are eligible to receive infusions, and all have done so, leading to a predictive probability of 87%.

In vivo induction of CTA expression with *Aza-Rev* in MM

Quantitative RT-PCR evaluating a panel of 10 human CTA in unfractionated bone marrow specimens collected before and after Aza-Rev from four patients demonstrated the induction of 6–8 discrete CTA in each patient (Fig 4A). Notably, the number of plasma cells infiltrating the marrow was similar in the pre- and post-Aza-Rev therapy samples in these patients, indicating that the overexpression was associated with disease. However, in order to determine cellular source of the CTA expression, bone marrow cells from two independent patients were fractionated into $CD138^+$ plasma cells and CD138− cells. As shown in Fig 4B, $CD138^+$ cells were major source of Aza-induced CTA expression.

Of the CTA, *MAGEA4* and *AKAP4* were expressed in bone marrow cells of all patients after investigational therapy ($n = 6$). Expression of other CTAs included *MAGEA3* (3/6), *MAGEA5* (5/6), *MAGEA6* (5/6), *MAGEC1* (4/6), *CTAG1B* (4/6), *SPACA3* (2/6), *SPA17* (4/6) and *SPANXB1* & *SPANXB2* (1/6). When compared to pre-therapy expression in the cohort tested, the level of expression was significantly increased in 4 of 10 CTA as follows: *MAGEA4* ($P = 0{\cdot}02$), *MAGEA6* ($P = 0{\cdot}02$), *SPA17* ($P = 0{\cdot}03$) and *AKAP4* ($P = 0{\cdot}02$). The increase in *MAGEA3* ($P = 0{\cdot}09$), *MAGEA5* ($P = 0{\cdot}08$), *MAGEC1* ($P = 0{\cdot}13$), *CTAG1B* ($P = 0{\cdot}07$), *SPACA3* ($P = 0{\cdot}13$) and *SPANXB1*& *SPANXB2*($P = 0{\cdot}50$) expression, however, did not reach statistical significance.

CTA-specific T cell response following Aza-Rev therapy

Autologous lymphocytes and blood mononuclear cells obtained from three patients were subjected to IFNG ELISA, in the presence or absence of recombinant CTAG1B, utilizing autologous monocyte-derived DCs. As shown in Fig 5, in Patients 1 and 4, CTAG1B-reactive T cells appeared after the administration of the 1st cycle Aza-Rev and peaked following the 3rd cycle. We used T cells and DCs in the absence of the antigen as negative control. T cells of each patient showed different levels of IFNG production in response to CTAG1B *in vitro*, ranging from 5000 pg/ml in Patient 1, to 250 pg/ml in Patient 4 (Fig 5). CTAG1B-specific T cell response was also detected at high levels in the stem cell product (Figure S2). The patient (Patient 4) with low level CTAG1B induction in bone marrow showed similar low level CTAG1B-reactive T cell responses, suggesting that the magnitude of CTA expression is directly correlated with the robustness of T cell response. One patient (Patient 2) showed pre-existing T cell response towards CTAG1B, which persisted after Aza-Rev.

The immune response against CTAG1B was detectable in all the patients 2 months following ALI, but was no longer demonstrable in the two patients who were in complete remission 1 year after transplantation. Significantly, these patients (2 and 4) recorded further improvement in their response from VGPR to complete remission at 8 and 10 months following ALI. Patient 1, however, had a persistent weak CTAG1B-reactive T cell response 1-year post transplant, in the presence of stable minimal residual disease (MRD). This finally disappeared by 15 months post-transplantation (data not shown).

Discussion

This clinical trial demonstrated the *in vivo* epigenetic induction of highly immunogenic CTAs in patients with MM. This induction was associated with a subsequent cell-mediated immune response, which was transferable at the time of SCT and persisted following transplant. This was accomplished with the administration of a well-tolerated regimen of chemo-immunotherapy, which is easy to deliver and widely applicable making such adaptive immunotherapy available wherever SCT is performed for MM.

At the present time the only known curative therapy for patients with MM is allo-SCT. The graft-versus-myeloma effect observed following allografting has been linked to the emergence of tumour antigen-specific cellular and humoral immune response (such as against B cell maturation antigen), particularly following donor lymphocyte infusion

(Bellucci *et al*, 2004, 2005). Similarly, antibodies against HY antigens in male recipients of female donor stem cells are correlated with freedom from relapse following allo-SCT (Miklos *et al*, 2005). A guiding principle in understanding such graft-versus-tumour and graft-versus-host responses is the allo-reactivity of donor T cells to 'non-self' minor histocompatibility antigens, oligopeptides that differ between human leucocyte antigen (HLA)-matched donors and recipients (Shlomchik, 2007), such as the HY antigens in the above example. Unfortunately, the recognition of such non-self antigens also triggers graft-versus-host disease, which erodes the benefit observed in terms of relapse protection following allografting. Therefore, if the paradigm of immune recognition of 'non-self' can be extended to the autologous setting, one may then observe the graft-versus-tumour benefit without the risk of graft-versus-host disease.

CTA offer such a target, and have been subject of intense study because of their aberrant expression in a variety of tumours. In patients with malignant melanoma, *CTAG1B* reactive T cells have been isolated and expanded *ex vivo* and re-infused into autologous recipients with dramatic responses recorded (Hunder *et al*, 2008). Recently, *in vitro* evidence of CTA overexpression by epigenetic modification and an adaptive T cell response has been demonstrated against *MAGEA4* in patients with Hodgkin lymphoma treated with decitabine (Cruz *et al*, 2011). Similar findings have been reported with acute myeloid leukaemia and myelodysplasia, where therapy with Aza and valproic acid (a histone deacytelase inhibitor), has led to the emergence of cytotoxic T cells reactive to MAGEA1, MAGEA2, MAGEC2 and RA-GEA1 peptides over the course of treatment (Goodyear *et al*, 2010). This synergy was also demonstrated in myeloma cells lines with Aza and MGCD-0103, up-regulating the expression of *MAGEA3* in myeloma cell lines, which in turn stimulated cytokine responses from MAGEA3-specific cytotoxic T cells (Moreno-Bost *et al*, 2011). Our finding of CTA up-regulation in bone marrow and plasma cells from myeloma patients treated with Aza-Rev *in vivo*, corroborates these *in vitro* findings. Evaluation of the expression of a limited panel of CTA showed over-expression of multiple CTA in each patient tested following hypomethylating therapy with Aza, suggesting a broad-spectrum effect of such epigenetic modification. Such poly-antigen overexpression should in theory overcome the limitation of differential immunogenicity of different CTA. Further, it is likely that, depending on the patients HLA repertoire, one may see a polyclonal antigen-driven T cell response against a number of antigens, facilitating more effective tumour control compared with situations where single antigens are targeted using "ex vivo" vaccine generation.

Certain CTA transcripts have rarely been identified in normal tissues other than testis, albeit at a low level. These are termed testis selective (*MAGEA3* to *A6*, *AKAP4, SPA17* in our panel) as opposed to testis restricted (*MAGEC1, CTAG1B, SPACA3, SPANXB1, SPANXB2*) CTA (Hofmann *et al*, 2008). This implies that the CTA overexpression seen in unfractionated marrow may derive from normal haematopoietic elements. However, when tested in fractionated marrow cell populations, CTA induction following Aza-Rev in our patients appears to be limited to $CD138^+$ plasma cells, which could be primed to overexpress these antigens, as opposed to normal haematopoietic cells. This may be related to the abnormal regulation of, or activity of epigenetic modifiers, such as DNA methyltransferase, in cancer cells. Recently, altered histone methylation with a more open chromatin structure has been demonstrated in patients with chromosomal translocation

(4:14) in myeloma, related to aberrant MMSET activity (Martinez-Garcia *et al*, 2011). A similar mechanism may be invoked in explaining the differential response to Aza between normal and malignant marrow elements. Particularly intriguing is the observation of disease responses in two patients with abnormal cytogenetics following investigational therapy, despite a very low dose of lenalidomide being used, suggesting a drug sensitization effect of epigenetic modification on the malignant clone in selected patients. Aza is known to promote susceptibility to apoptosis in myeloma cell lines by restoring the expression of proteins such as RASD1, (Nojima *et al*, 2009) DAPK1 (Chim *et al*, 2007) and SPI1 (PU1) (Tatetsu *et al*, 2007). In such instances Aza has shown restoration of dexamethasone or doxorubicin sensitivity in resistant cell lines (Kiziltepe *et al*, 2007). A similar mechanism may be invoked in the responses we have observed.

Aside from the augmenting the direct cytotoxicity of lenalidomide against myeloma cells, synergy may be observed in terms of immuno-modulation. Hypomethylating therapy may facilitate autologous anti-tumour immune response by augmenting HLA class I expression on antigen-presenting cells as well as tumour cells in a variety of tumour models (Coral *et al*, 2006; Fonsatti *et al*, 2007; Natsume *et al*, 2008). Immuno-modulation by hypo-methylating therapy was demonstrated when decitabine + interferon-γ treated target neuroblastoma cell lysis was accomplished in an HLA class I-restricted context by CTA-specific cytotoxic T cells derived from normal donors (Bao *et al*, 2011). Such synergy may be partly derived from demethylation of HLA class II transcriptional activator (CII TA) gene promoters augmenting HLA class II expression in response to extraneous interferon- γ (De Lerma Barbaro *et al*, 2008). These data support the validity of our immunotherapeutic approach combining Aza with Rev.

CTA reactivity has been invoked as a possible mechanism for graft-versus-leukaemia responses in allogeneic SCT recipients (McLarnon *et al*, 2010). We tested CTAG1B reactivity by incubating peripheral blood lymphocytes and stem cell products with recombinant CTAG1B pulsed autologous DCs. CTAG1B-reactive T cells were observed in 3 patients tested, with reactivity correlating with level of *CTAG1B* expression observed post Aza-Rev therapy, and being maintained for 2–11 months following SCT. The post-transplant maintenance of CTAG1B-reactivity was protracted in one patient (Patient 1) with high levels of *CTAG1B* expression and MRD. Additionally two other patients (Patients 2 and 4) improved their response from VGPR to CR in the months following ALI. This suggests ongoing disease suppressive activity in the patient with MRD and possible maintenance of *CTAG1B* (and other CTA) expression in clonal plasma cells. Further, the CTA-specific T cell response elicited may be polyclonal and synergistic, targeting several different CTA simultaneously providing redundancy in terms of protective capability.

CTA-reactive T cells were identified in both the stem cell product as well as the ALI, which were given relatively early after transplantation to utilize the cytokine-rich milieu present with lymphocyte depletion following high dose therapy (Klebanoff *et al*, 2005; Rapoport *et al*, 2005, 2009). The absence of competing lymphocyte populations could, in this setting, lead to preferential proliferation of oligoclonal T cells in the ALI. Additionally we used GM-CSF for haematopoietic reconstitution rather than G-CSF to avoid the T-helper cell type 2 skewing and T cell hypo-responsiveness reported with the use of G-CSF (Sloand *et al*,

2000). GM-CSF also promotes DC differentiation, potentially triggering T cell reactivity against clonal plasma cells over-expressing CTA in the MRD state. Whether this results in a higher rate of CR achievement and improved survival will not be known definitively until randomized trials are conducted, comparing standard high dose and maintenance therapy with the current regimen. Further survival advantage may be observed if immunomodulatory drugs, particularly Rev, are used following SCT in conjunction with ALI to augment and prolong CTA-specific T cell reactivity. This will be explored in future patients enrolled on a follow up clinical trial.

At the current time we do not have adequate follow-up, nor the number of patients necessary to define the value of this immunotherapeutic approach in maintaining remission in patients with MM. When compared with the current standard of consolidation and maintenance therapy reported by many of the larger groups performing myeloma trials, ALI, if proven comparable in a larger cohort of patients, would represent a paradigm shift in the management of myeloma. This will be particularly true for patients with standard risk disease and may allow the management of these patients without protracted maintenance therapy with its own inherent toxicities.

Two issues that arose during this trial have been the problem of inadequate disease control by this regimen, and the potential unmasking of auto-immune toxicities. The protocol as it is currently executed safeguards against the former by incorporating this immunotherapy in a stem cell transplant scheme, using the ALI to target MRD following SCT. On the other hand, the potential unmasking of auto-immune toxicities is a matter that will need protracted follow up and early detection through careful follow up of patients undergoing this immunotherapy. Additionally, antigen loss as a mechanism of tumour escape will also need to be monitored for, by evaluating post-transplant persistence of CTA expression on plasma cells in bone marrow to determine the durability of this epigenetic modification.

In conclusion, we demonstrate the safety and feasibility of epigenetic modification resulting in over-expression of antigenic targets in MM. This may then be exploited in formulating adaptive immunotherapy protocols in these patients. Adoptively transferred cells may maintain long-term surveillance against malignant plasma cells in patients with MM and translate into prolonged freedom from progression in this otherwise incurable disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge the contribution of the nurses, transplant coordinators, pharmacists, nurse practitioners, and physician’s assistants in the BMT programme at VCU, particularly Mr. Richard Edwards PA-C and Ms. Christina McLaughlin in the VCU stem cell laboratory and Ms. Jennie Anderson in therapeutic apheresis. We also acknowledge Dr. Kevin Hogan for his critical review of our manuscript and Ms. Cheryl Jacocks-Terrell for help in preparing the manuscript. We gratefully acknowledge research support and the funding of this clinical trial by Celgene Corporation. We also thank VCU Massey Cancer Center and the Commonwealth Foundation for Cancer Research for support.

References

Ascierto ML, Kmieciak M, Idowu MO, Manjili R, Zhao Y, Grimes M, Dumur C, Wang E, Ramakrishnan V, Wang XY, Bear HD, Marincola FM, Manjili MH. A signature of immune function genes associated with recurrence-free survival in breast cancer patients. Breast Cancer Research and Treatment. 2012; 131:871–880. [PubMed: 21479927]

Atanackovic D, Arfsten J, Cao Y, Gnjatic S, Schnieders F, Bartels K, Schilling G, Faltz C, Wolschke C, Dierlamm J, Ritter G, Eiermann T, Hossfeld DK, Zander AR, Jungbluth AA, Old LJ, Bokemeyer C, Kröger N. Cancer-testis antigens are commonly expressed in multiple myeloma and induce systemic immunity following allogeneic stem cell transplantation. Blood. 2007; 109:1103–1112. [PubMed: 17023585]

Bao L, Dunham K, Lucas K. MAGE-A1, MAGE-A3, and NY-ESO-1 can be upregulated on neuroblastoma cells to facilitate cytotoxic T lymphocyte-mediated tumor cell killing. Cancer Immunology, Immunotherapy. 2011; 60:1299–1307. [PubMed: 21626030]

Barlogie B, Tricot G, Anaissie E, Shaughnessy J, Rasmussen E, Van Rhee F, Fassas A, Zangari M, Hollmig K, Pineda-Roman M, Lee C, Talamo G, Thertulien R, Kiwan E, Krishna S, Fox M, Crowley J. Thalidomide and hematopoietic-cell transplantation for multiple myeloma. New England Journal of Medicine. 2006; 354:1021–1030. [PubMed: 16525139]

Bellucci R, Wu CJ, Chiaretti S, Weller E, Davies FE, Alyea EP, Dranoff G, Anderson KC, Munshi NC, Ritz J. Complete response to donor lymphocyte infusion in multiple myeloma is associated with antibody responses to highly expressed antigens. Blood. 2004; 103:656–663. [PubMed: 14563636]

Bellucci R, Alyea EP, Chiaretti S, Wu CJ, Zorn E, Weller E, Wu B, Canning C, Schlossman R, Munshi NC, Anderson KC, Ritz J. Graft-versus-tumor response in patients with multiple myeloma is associated with antibody response to BCMA, a plasma-cell membrane receptor. Blood. 2005; 105:3945–3950. [PubMed: 15692072]

Chim CS, Liang R, Fung TK, Choi CL, Kwong YL. Epigenetic dysregulation of the death-associated protein kinase/p14/HDM2/p53/Apaf-1 apoptosis pathway in multiple myeloma. Journal of Clinical Pathology. 2007; 60:664–669. [PubMed: 17557868]

Condomines M, Hose D, Raynaud P, Hundemer M, De Vos J, Baudard M, Moehler T, Pantesco V, Moos M, Schved J, Rossi J, Reme T, Goldschmidt H, Klein B. Cancer/testis genes in multiple myeloma: expression patterns and prognosis value determined by microarray analysis. The Journal of Immunology. 2007; 178:3307–3315. [PubMed: 17312182]

Coral S, Sigalotti L, Altomonte M, Engelsberg A, Colizzi F, Cattarossi I, Maraskovsky E, Jager E, Seliger B, Maio M. 5-aza-2 ‘-deoxycytidine-induced expression of functional cancer testis antigens in human renal cell carcinoma: immunotherapeutic implications. Clinical Cancer Research. 2002; 8:2690–2695. [PubMed: 12171902]

Coral S, Sigalotti L, Colizzi F, Spessotto A, Nardi G, Cortini E, Pezzani L, Fratta E, Fonsatti E, Di Giacomo AM, Nicotra MR, Natali PG, Altomonte M, Maio M. Phenotypic and functional changes of human melanoma xenografts induced by DNA hypomethylation: immunotherapeutic implications. Journal of Cellular Physiology. 2006; 207:58–66. [PubMed: 16252259]

Corral L, Haslett P, Muller G, Chen R, Wong L, Ocampo CJ, Patterson RT, Stirling DI, Kaplan G. Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogues that are potent inhibitors of TNF-alpha. The Journal of Immunology. 1999; 163:380–386. [PubMed: 10384139]

Cruz CR, Gerdemann U, Leen AM, Shafer JA, Ku S, Tzou B, Horton TM, Sheehan A, Copeland A, Younes A, Rooney CM, Heslop HE, Bollard CM. Improving T-cell therapy for relapsed EBV-negative Hodgkin lymphoma by targeting upregulated MAGE-A4. Clinical Cancer Research. 2011; 17:7058–7066. [PubMed: 21908573]

Davies F, Raje N, Hideshima T, Lentzsch S, Young G, Tai Y, Lin B, Podar K, Gupta D, Chauhan D, Treon SP, Richardson PG, Schlossman RL, Morgan GJ, Muller GW, Stirling DI, Anderson KC. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood. 2001; 98:210–216. [PubMed: 11418482]

De Lerma Barbaro A, De Ambrosis A, Banelli B, Li Pira G, Aresu O, Romani M, Ferrini S, Accolla RS. Methylation of CIITA promoter IV causes loss of HLA-II inducibility by IFN-gamma in promyelocytic cells. International Immunology. 2008; 20:1457–1466. [PubMed: 18829986]

Durie BG, Harousseau JL, Miguel JS, Durie BG, Harousseau JL, Miguel JS, Bladé J, Barlogie B, Anderson K, Gertz M, Dimopoulos M, Westin J, Sonneveld P, Ludwig H, Gahrton G, Beksac M, Crowley J, Belch A, Boccadaro M, Cavo M, Turesson I, Joshua D, Vesole D, Kyle R, Alexanian R, Tricot G, Attal M, Merlini G, Powles R, Richardson P, Shimizu K, Tosi P, Morgan G, Rajkumar SV. International Myeloma Working Group. International uniform response criteria for multiple myeloma. Leukemia. 2006; 20:1467–1473. [PubMed: 16855634]

Fonsatti E, Nicolay HJ, Sigalotti L, Calabrò L, Pezzani L, Colizzi F, Altomonte M, Guidoboni M, Marincola FM, Maio M. Functional up-regulation of human leukocyte antigen class I antigens expression by 5-aza-2'-deoxycytidine in cutaneous melanoma: immunotherapeutic implications. Clinical Cancer Research. 2007; 13:3333–3338. [PubMed: 17545540]

Goodyear O, Agathanggelou A, Novitzky-Basso I, Siddique S, McSkeane T, Ryan G, Vyas P, Cavenagh J, Stankovic T, Moss P, Craddock C. Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. Blood. 2010; 116:1908–1918. [PubMed: 20530795]

Guo Z, Hong J, Irvine K, Chen AG, Spiess PJ, Liu Y, Zeng G, Wunderlich JR, Nguyen DM, Restifo NP, Schrump DS. De novo induction of a cancer/testis antigen by 5-aza-2 '-deoxycytidine augments adoptive immunotherapy in a murine tumor model RID A-5713-2008. Cancer Research. 2006; 66:1105–1113. [PubMed: 16424047]

Hayashi T, Hideshima T, Akiyama M, Podar K, Yasui H, Raje N, Kumar S, Chauhan D, Treon SP, Richardson P, Anderson KC. Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. British Journal of Haematology. 2005; 128:192–203. [PubMed: 15638853]

Hofmann O, Caballero OL, Stevenson BJ, Chen YT, Cohen T, Chua R, Maher CA, Panji S, Schaefer U, Kruger A, Lehvaslaiho M, Carninci P, Hayashizaki Y, Jongeneel CV, Simpson AJ, Old LJ, Hide W. Genome-wide analysis of cancer/testis gene expression. Proceedings of the National Academy of Sciences of the USA. 2008; 105:20422–20427. [PubMed: 19088187]

Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R, Jungbluth A, Gnjatic S, Thompson JA, Yee C. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. New England Journal of Medicine. 2008; 358:2698–2703. [PubMed: 18565862]

Jungbluth A, Ely S, DiLiberto M, Niesvizky R, Williamson B, Frosina D, Chen Y, Bhardwaj N, Chen-Kiang S, Old LJ, Cho HJ. The cancer-testis antigens CT7 (MAGE-C1) and MAGE-A3/6 are commonly expressed in multiple myeloma and correlate with plasma-cell proliferation. Blood. 2005; 106:167–174. [PubMed: 15761016]

Kiziltepe T, Hideshima T, Catley L, Raje N, Yasui H, Shiraishi N, Okawa Y, Ikeda H, Vallet S, Pozzi S, Ishitsuka K, Ocio EM, Chauhan D, Anderson KC. 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. Molecular Cancer Therapeutics. 2007; 6:1718–1727. [PubMed: 17575103]

Klebanoff CA, Khong HT, Antony PA, Palmer DC, Restifo NP. Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. Trends in Immunology. 2005; 26:111–117. [PubMed: 15668127]

Kmieciak M, Basu D, Payne KK, Toor A, Yacoub A, Wang X, Smith L, Bear HD, Manjili MH. Activated NKT cells and NK cells render T cells resistant to myeloid-derived suppressor cells and result in an effective adoptive cellular therapy against breast cancer in the FVBN202 transgenic Mouse. The Journal of Immunology. 2011a; 187:708–717. [PubMed: 21670315]

Kmieciak M, Payne KK, Idowu MO, Grimes MM, Graham L, Ascierto M, Wang E, Wang X, Bear HD, Manjili M. Tumor escape and progression of HER-2/neu negative breast cancer under immune pressure. Journal of Translational Medicine. 2011b; 9:35. [PubMed: 21453513]

LeBlanc R, Hideshima T, Catley L, Shringarpure R, Burger R, Mitsiades N, Mitsiades C, Cheema P, Chauhan D, Richardson PG, Anderson KC, Munshi NC. Immunomodulatory drug costimulates T cells via the B7-CD28 pathway. Blood. 2004; 103:1787–1790. [PubMed: 14512311]

Lecoutre B, Derzko G, Grouin JM. Bayesian predictive approach for inference about proportions. Statistics in Medicine. 1995; 14:1057–1063. [PubMed: 7569500]

Lim SY, Laxmanan S, Stuart G, Ghosh SK. Anti-lymphoma immunity: relative efficacy of peptide and recombinant DNA vaccine. Cancer Detection and Prevention. 2001; 25:470–478. [PubMed: 11718453]

Martinez-Garcia E, Popovic R, Min DJ, Sweet SMM, Thomas PM, Zamdborg L, Heffner A, Will C, Lamy L, Staudt LM, Levens DL, Kelleher NL, Licht JD. The MMSET histone methyl transferase switches global histone methylation and alters gene expression in t(4;14) multiple myeloma cells. Blood. 2011; 117:211–220. [PubMed: 20974671]

McLarnon A, Piper KP, Goodyear OC, Arrazi JM, Mahendra P, Cook M, Clark F, Pratt G, Craddock C, Moss PAH. CD8 (+) T-cell immunity against cancer-testis antigens develops following allogeneic stem cell transplantation and reveals a potential mechanism for the graft-versus-leukemia effect. Haematologica. 2010; 95:1572–1578. [PubMed: 20460638]

Meklat F, Li Z, Wang Z, Zhang Y, Zhang J, Jewell A, Lim SH. Cancer-testis antigens in haematological malignancies. British Journal of Haematology. 2007; 136:769–776. [PubMed: 17223912]

Miklos DB, Kim HT, Miller KH, Guo L, Zorn E, Lee SJ, Hochberg EP, Wu CJ, Alyea EP, Cutler C, Ho V, Soiffer RJ, Antin JH, Ritz J. Antibody responses to H-Y minor histocompatibility antigens correlate with chronic graft-versus-host disease and disease remission. Blood. 2005; 105:2973–2978. [PubMed: 15613541]

Moreno-Bost A, Szmania S, Stone K, Garg T, Hoerring A, Szymonifka J, Shaughnessy J, Barlogie B, Prentice HG, Van Rhee F. Epigenetic modulation of MAGE-A3 antigen expression in multiple myeloma following treatment with the demethylation agent 5-azacitidine and the histone deacetlyase inhibitor MGCD0103. Cytotherapy. 2011; 13:618–628. [PubMed: 21171821]

Natsume A, Wakabayashi T, Tsujimura K, Shimato S, Ito M, Kuzushima K, Kondo Y, Sekido Y, Kawatsura H, Narita Y, Yoshida J. The DNA demethylating agent 5-aza-2'-deoxycytidine activates NY-ESO-1 antigenicity in orthotopic human glioma. International Journal of Cancer. 2008; 122:2542–2553. [PubMed: 18240144]

Nojima M, Maruyama R, Yasui H, Suzuki H, Maruyama Y, Tarasawa I, Sasaki Y, Asaoku H, Sakai H, Hayashi T, Mori M, Imai K, Tokino T, Ishida T, Toyota M, Shinomura Y. Genomic screening for genes silenced by DNA methylation revealed an association between RASD1 inactivation and dexamethasone resistance in multiple myeloma. Clinical Cancer Research. 2009; 15:4356–4364. [PubMed: 19549772]

Rapoport AP, Stadtmauer EA, Aqui N, Badros A, Cotte J, Chrisley L, Veloso E, Zheng Z, Westphal S, Mair R, Chi N, Ratterree B, Pochran MF, Natt S, Hinkle J, Sickles C, Sohal A, Ruehle K, Lynch C, Zhang L, Porter DL, Luge S, Guo C, Fang H, Blackwelder W, Hankey K, Mann D, Edelman1 R, Frasch C, Levine BL, Cross A, June CH. Restoration of immunity in lymphopenic individuals with cancer by vaccination and adoptive T-cell transfer. Nature Medicine. 2005; 11:1230–1237.

Rapoport AP, Stadtmauer EA, Aqui N, Vogl D, Chew A, Fang H, Janofsky S, Yager K, Veloso E, Zheng Z, Milliron T, Westphal S, Cotte J, Huynh H, Cannon A, Yanovich S, Akpekl G, Tan M, Virts K, Ruehle K, Harris C, Phillip S, Vonderheide RH, Levine BL, June CH. Rapid Immune Recovery and graft-versus-host disease-like engraftment syndrome following adoptive transfer of costimulated autologous T cells. Clinical Cancer Research. 2009; 15:4499–4507. [PubMed: 19509133]

Shlomchik WD. Graft-versus-host disease. Nature reviews. Immunology. 2007; 7:340–352. [PubMed: 17438575]

Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. Nature Reviews Cancer. 2005; 5:615–625. [PubMed: 16034368]

Sloand EM, Kim S, Maciejewski JP, Van Rhee F, Chaudhuri A, Barrett J, Young NS. Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo. Blood. 2000; 95:2269–2274. [PubMed: 10733495]

Tatetsu H, Ueno S, Hata H, Yamada Y, Takeya M, Mitsuya H, Tenen DG, Okuno Y. Down-regulation of PU.1 by methylation of distal regulatory elements and the promoter is required for myeloma cell growth. Cancer Research. 2007; 67:5328–5336. [PubMed: 17545613]

Van Rhee F, Szmania S, Zhan F, Gupta SK, Pomtree M, Lin P, Batchu RB, Moreno A, Spagnoli G, Shaughnessy J, Tricot G. NY-ESO-1 is highly expressed in poor-prognosis multiple myeloma and

induces spontaneous humoral and cellular immune responses. Blood. 2005; 105:3939–3944. [PubMed: 15671442]

Wang Z, Zhang Y, Liu H, Salati E, Chiriva-Internati M, Lim SH. Gene expression and immunologic consequence of SPAN-Xb in myeloma and other hematologic malignancies. Blood. 2003; 101:955–960. [PubMed: 12393489]

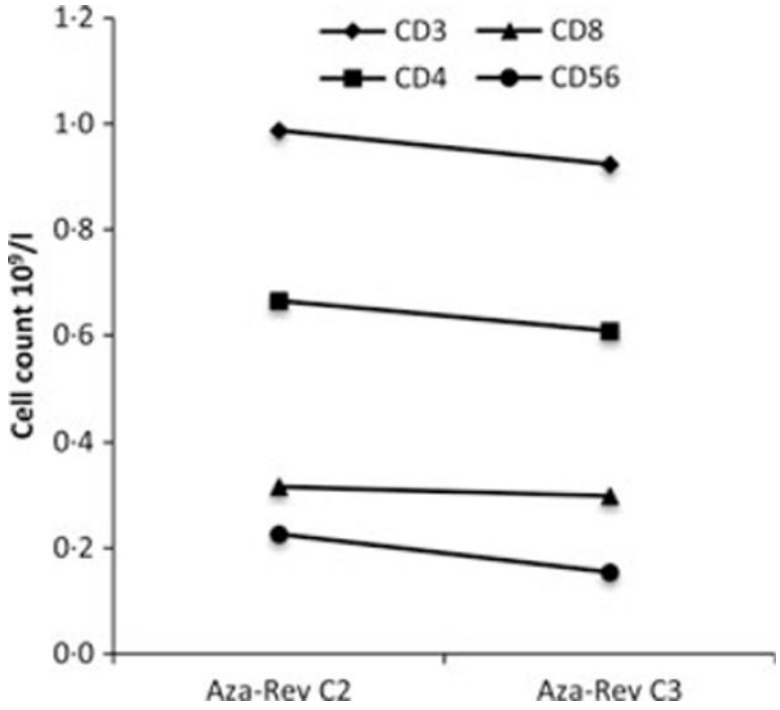

Fig 1.

Blood T cell subset and NK cell counts (mean) following investigational therapy. Counts prior to lymphaphereses 1 and 2, following cycles 2 (C2) and 3 (C3) of Aza-Rev.

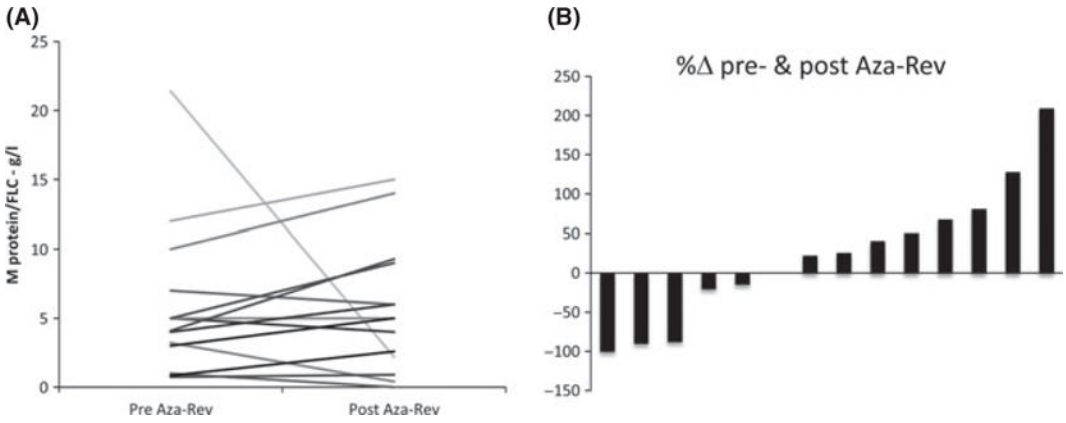

Fig 2.

Disease response to investigational therapy. (A) Paraprotein levels before and after 3 cycles of Aza-Rev. (B) Percent change in paraprotein levels, before and after 3 cycles of Aza-Rev.

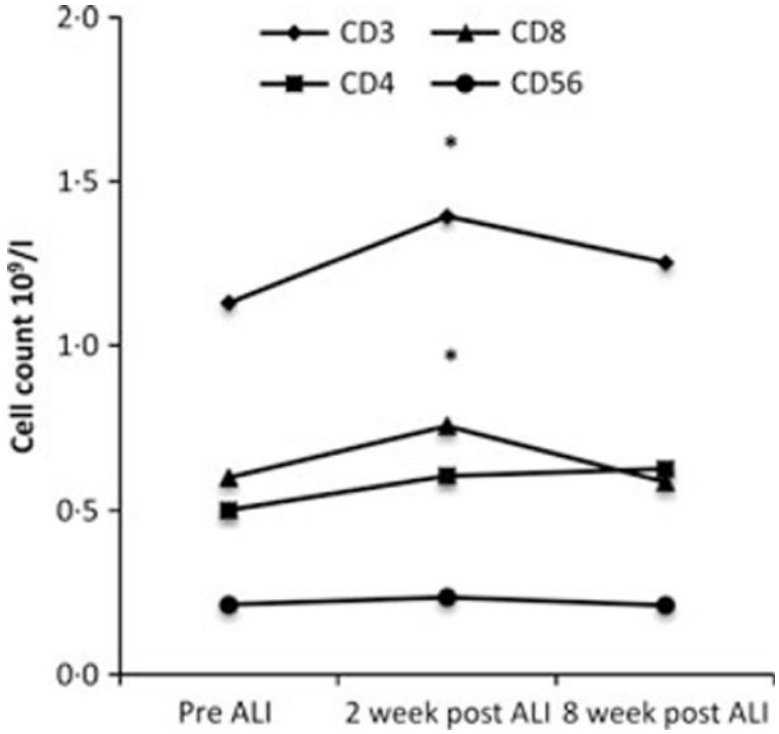

Fig 3.

Blood T cell subset and NK Cell counts (mean) following SCT. Counts pre-ALI, 2 and 8 weeks following ALI. * Signifies statistically significant increase over earlier time point.

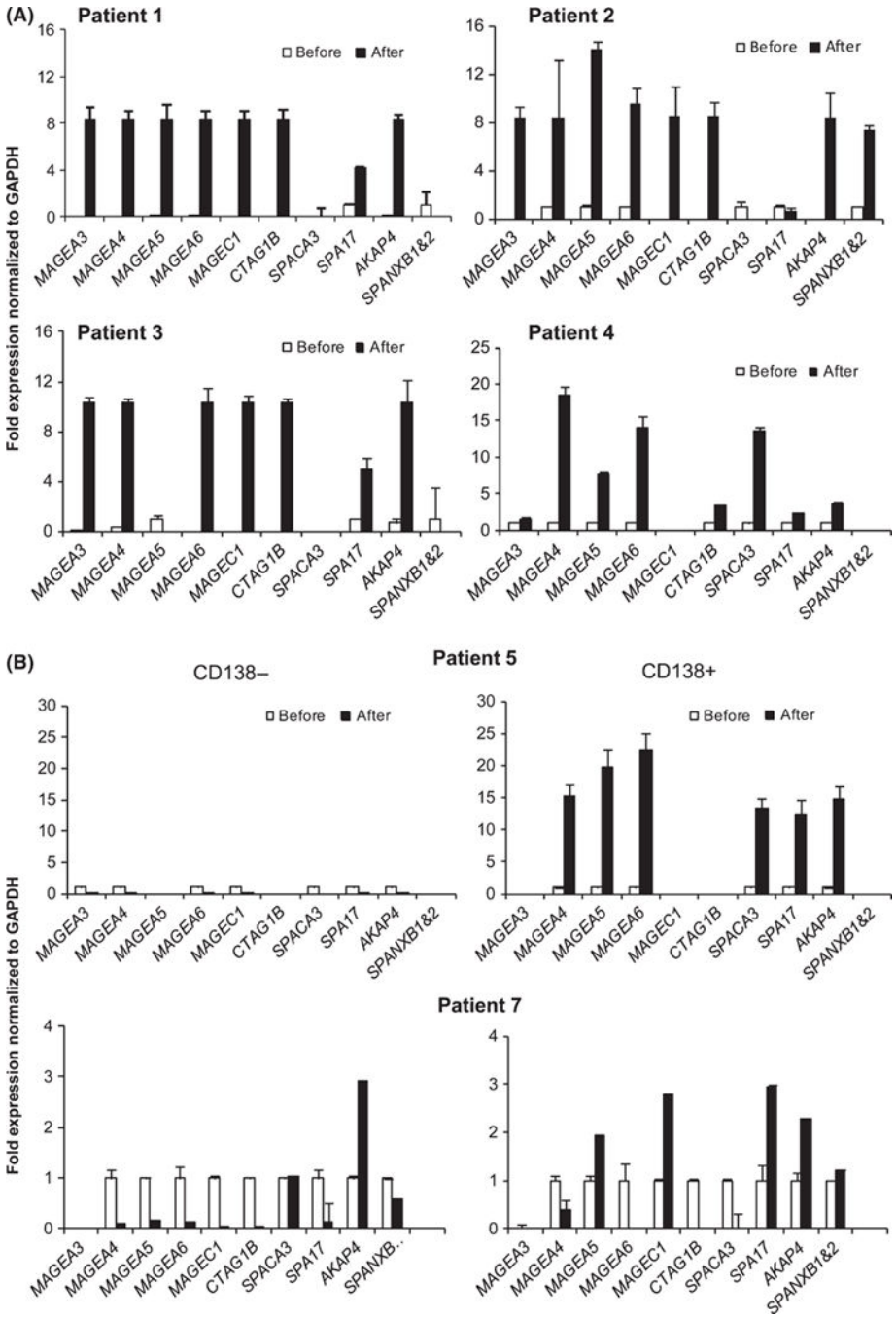

Fig 4.

Induction of a panel of CTA expression in bone marrow of patients with MM following a 3-cycle administration of Aza-Rev. (A) Fold expression of CTA prior to (before) or following 3 cycles of Aza-Rev (after) in unfractionated bone marrow cells. Bone marrow biopsy % clonal plasma cell infiltration, pre- and post Aza-Rev in individual patients: Patient 1: 15% and 20%; Patient 2: 5–10% and 5%; Patient 3: 10% and 10%; Patient 4: 5% and 5%. (B) Fold expression of CTA in fractionated CD138− and $CD138^{+}$ bone marrow cells of two patients. Data were normalized to human *GAPDH*.

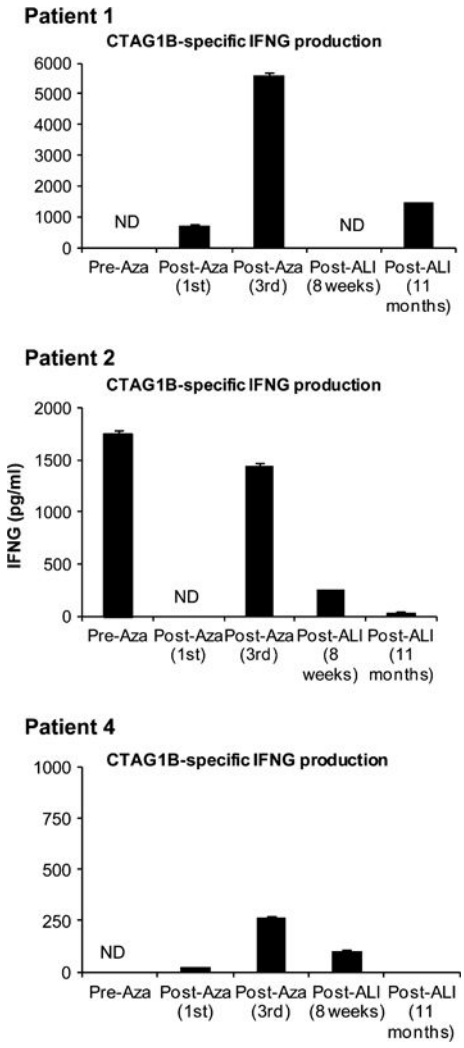

Fig 5.

Induction of CTAG1B-reactive T cell responses in patients with MM. Lymphocytes were prepared from blood samples prior to, after 1st cycle or 3rd cycles Aza-Rev administration as well as 8 weeks or 11 months after autologous lymphocyte infusion (ALI). Lymphocytes were co-cultured with autologous dendritic cells (DCs) in 2:1 ratios in the presence or absence of recombinant CTAG1B for 20 h. γ-interferon (IFNG) production was detected in the supernatant. Data are presented after subtracting background IFNG production by T cells plus DCs in the absence of the antigen. ND: not done.

Table I

Patient characteristics.

UPN	Age (years)	MM subtype	Stage at diagnosis	Cytogenetics	Prior therapy	Number of cycles prior Rx	Response to prior Rx
12430-01	60	IgG λ	III*	N	TD	11	VGPR
12430-02	64	IgG κ	II	N	TD × 1; RD	12	PR
12430-03	63	IgG κ	II	A[1]	TD; VD	8	PR
12430-04	68	IgG κ	II	N	RD	6	PR
12430-05	55	IgG κ	II	N	RD	10	VGPR
12430-06	53	IgG κ	III*	A[2]	VDoxD	7	VGPR
12430-07	40	IgG κ	II	A[3]	D; VD (4)	5	VGPR
12430-08	61	κ	III	A[4]	VD	10	PR
12430-09	69	IgA λ	I*	N	VD	9	PR
12430-10	59	κ	III	N	VDox; RD	12	PR
12430-11	60	κ	I	N	V; RD	11	VGPR
12430-12	54	κ	II	A[5]	RD	7	SD
12430-13	65	κ	II	A[6]	RD; VD	12	VGPR
12430-14	52	IgG κ	III	A[7]	RD	3	PR

UPN, unique patient number; MM, multiple myeloma; Rx, therapy; N, Normal; A, abnormal; TD, Thalidomide + dexamethasone; RD, Revlimid (lenalidomide) + dexamethasone; VD, Velcade (bortezomib) + dexamethasone; D, dexamethasone; VDox, Velcade + doxorubicin; SD, stable disease; PR, Partial remission; VGPR, Very good partial remission; REL, Relapse. Abnormal Cytogenetic/Fluorescent *in situ* hybridization results- 1: (+3, +7, +11, −13, +15, +17), 2: (−13, 17, *FGFR3:IGH@* translocation), 3: (−13, 14q32 translocation), 4: (46–51, X,−X, del 6q13, +11,−13, del 16q24, +19; +11q23/*ATM*,−13, *IGH@* translocation), 5: (−6q21/*MYB*,−13), 6: [−13q, t(11:14) *CCND1: IGH@*], 7: [t(11:14) *CCND1: IGH@*].

*Durie-Salmon staging, diagnostic β2m not available. All other diagnosis staging used the International Myeloma Working Group criteria.

Table II

Cell collection and clinical outcome following SCT and ALI.

	CD3$^+$ cell yield						
UPN	Apheresis 1	Apheresis 2	PBSC (CD34$^+$)	PBSC (CD3$^+$)	Time SCT to ALI (days)	Follow-up post -SCT (month)	Last disease status
12430-01*	0·58	0·26	8·65	3·08	39	19	VGPR
12430-02	1·03	0·99	8·72	5·5	52	17	CR
12430-03	0·95	0·82	10·71	6·22	46	15	VGPR
12430-04	0·51	0·48	10·28	5·26	49	12	CR
12430-05*	0·76	0·82	8·64	3·07	31	7	CR
12430-06*	0·94	1·01	14·57	4·57	32	7	REL
12430-07	1·57	1·44	16·9	6·48	49	6	SD
12430-08*	0·31	0·63	7·34	6·54	39	5	VGPR
12430-09	0·77	0·86	8·73	3·82	32	5	CR
12430-10	1·55	1·06	8·93	12·79	45	5	VGPR
12430-11	0·71	1·03	11·29	8·31	51	2	VGPR
12430-12	0·75	0·59	1·1	–	–	–	SD
12430-13†	1·46	0·84	6·44	9·07	–	<1	–
12430-14†	0·63	0·65	8·9	2·84	–	<1	–

SCT, autologous stem cell transplantation; PBSC, Peripheral blood stem cells; Cell dose reported: CD3$^+$ cells- $\times 10^8$ cells/kg; CD34$^+$ cells- $\times 10^6$ cells/kg; ALI, autologous lymphocyte infusion; SD, stable disease; CR, complete remission; VGPR, Very good partial remission; REL, Relapse. (–) Not evaluable.

*Tandem transplantation prior to ALI.

†ALI not infused to date.