

# Tumour cell/dendritic cell fusions as a vaccination strategy for multiple myeloma

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Multiple myeloma (MM) remains an incurable haematological malignancy despite advances in the biology and therapeutics of this disease. It affects more than 14 400 new individuals in the United States annually and accounts for 2% of cancer-related deaths (Jemal *et al*, 2003). The introduction of high-dose chemotherapy in the early 1980s was a milestone in the treatment of MM, as it resulted in higher rates of complete remission (CR), based on clinical and laboratory criteria (McElwain & Powles, 1983). A large volume of data has since accumulated, both in the allogeneic and autologous transplant setting (Attal *et al*, 1996; Schlossman & Anderson, 1999; Child *et al*, 2003; Corradini *et al*, 2003). In a prospective randomized trial, the Intergroupe Francais du Myeloma (Attal *et al*, 1996) and more recently, the Medical Research Council Myeloma VII trial (Child *et al*, 2003) have clearly demonstrated the benefits of autologous peripheral blood (PB) stem cell transplantation over conventional chemotherapy in previously untreated patients. Exciting data have emerged with the use of novel agents like Thalidomide, CC-5013 and Bortezomib (Raje &

## Summary

Multiple myeloma (MM) cells express certain tumour-associated antigens (TAAs) that could serve as targets for active-specific immunotherapy. The aim of the present study was to test the MM/dendritic cell (DC) fusion as a vaccination strategy. We fused MM cells with DC to generate fusion cells (FCs) and tested their antigen presenting cell (APC) function in mixed lymphocyte reactions and cytotoxicity assays. First, the HS Sultan and SK0-007 HAT sensitive human MM cell lines and DCs generated from peripheral blood of normal donors were fused in the presence of 50% polyethylene glycol to form FCs. Next, tumour cells freshly isolated from patients were similarly fused with autologous DCs to generate FCs. The FCs demonstrated a biphenotypic profile, confirmed both by flow-cytometry and dual immunofluorescence microscopy. These FCs induced MM-specific cytotoxicity. FCs, but not MM cells or DCs alone, were potent stimulators of autologous patient T cells. More importantly, FC-primed autologous peripheral blood mononuclear cells demonstrated major histocompatibility complex-restricted MM-specific cytotoxicity. These studies therefore demonstrated that MM/DC FC can trigger an autologous immune response to MM cells and formed the framework for a clinical trial currently underway.

**Keywords:** immunotherapy, autologous, dendritic cells, vaccines, cytotoxicity.

Anderson, 1999; Singhal *et al*, 1999; Richardson *et al*, 2002, 2003) in patients with relapsed and refractory MM, affording more options of treatments to patients. However, most responses are not durable, underlying the importance of developing new therapeutic strategies for MM patients.

Most tumours evade immune surveillance due to the failure of antigen presentation, related to their lack of major histocompatibility complexes (MHCs) (Garrido *et al*, 1993) or co-stimulatory molecule expression (Antonia *et al*, 1995), as well as the presence of cytokines (Antonia *et al*, 1995; Qin *et al*, 1997; Letterio & Roberts, 1998) that negatively modulate the immune system. Current immunotherapy approaches in cancer attempt to repair these defects and generate antigen-specific immunity. Vaccination strategies mainly exploit the presence of tumour-associated antigens (TAAs) to elicit immune responses by various manipulations of the antigens and/or the tumour cells. Dendritic cells (DCs) are the most potent known professional antigen presenting cells (APCs) capable of priming naive cytotoxic T lymphocytes (CTLs) and

inducing antigen-specific CTLs *in vivo* (Szabolcs *et al*, 1995, 1997; Yi *et al*, 2002). In an effort to generate tumour-specific immunity, DCs have been manipulated to present tumour antigen by pulsing with whole tumour antigen (Hsu *et al*, 1996), naked DNA (Condon *et al*, 1996; Syrengelas *et al*, 1996; King *et al*, 1998; Stevenson *et al*, 1999), or whole tumour RNA (Condon *et al*, 1996; Lokhorst *et al*, 1997; Massaia *et al*, 1999), fused with tumour cells (Gong *et al*, 1997, 1998, 2000, 2002), or genetically modified prior to vaccination (Song *et al*, 1997; Specht *et al*, 1997). These modified DCs have already induced antigen-specific, MHC-restricted CTL responses in animal studies, with associated anti-tumour activity in both prophylaxis and treatment models. MM would appear to be an ideal tumour for such novel immune therapeutic strategies, as *in vitro*, animal, and early clinical trials have demonstrated that it may be possible to induce both allogeneic and autologous immune responses to MM cells (Kwak *et al*, 1995; Lokhorst *et al*, 1997; Alyea *et al*, 1998). More importantly, MM cells express certain TAAs, like Mucin 1 (MUC1) (Treon *et al*, 1999) and the idiotype protein (Massaia *et al*, 1999; Reichardt *et al*, 1999; Osterroth *et al*, 2000; Wen *et al*, 2002; Zeis *et al*, 2002), which could serve as targets for the generation of active-specific immunity. We have also previously demonstrated the induction of myeloma-specific CTLs using DCs primed with apoptotic MM cells (Hayashi *et al*, 2003).

We (Raje *et al*, 1999) and others (Pfeiffer *et al*, 1997; Tarte *et al*, 1998) have previously demonstrated that functional DCs can be easily generated from either bone marrow (BM) or PB of MM patients. In the present study, we have fused MM cells with DC to generate fusion cells (FCs) and tested their APC function in mixed lymphocyte reaction and CTL assays. The objective was to generate antigen-specific immune responses to both known and unknown TAAs. The FCs had a typical biphenotypic profile (i.e. MM and DC), confirmed by dual immunofluorescence microscopy and flow-cytometry. These FCs induced tumour-specific lysis by CTLs. We also fused five MM patients' tumour cells with autologous DCs. These patients' FCs expressed a biphenotypic profile and stimulated autologous patient T-cell proliferation, whereas either patient MM cells or DCs alone failed to induce T-cell proliferation. More importantly, autologous peripheral blood mononuclear cells (PBMCs) primed with autologous FC-triggered, MHC-restricted, MM-specific lysis. These studies are a demonstration of an autologous *in vitro* T-cell response to patient MM cells triggered by FC. They form the framework for a clinical trial to test whether FC vaccinations can achieve *in vivo* anti-tumour immunity and related clinical response in patients with MM.

## Materials and methods

### Cell lines

The HS Sultan (CRL-1484) and SK0-007(J3) (CRL-8033-2) MM cell lines were obtained from American Type Culture

Collection (ATCC; Rockville, MD, USA). These two human MM cell lines were used on the basis of HAT sensitivity. HS Sultan cells were cultured in 90% Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine supplemented with 25 IU/ml penicillin, 25 µg/ml streptomycin, 5 mmol/l L-glutamine (all from GIBCO BRL, Cheshire, UK), and 10% fetal bovine serum (FBS; Sigma Diagnostics, St Louis, MO, USA). SK0-007(J3) cells were maintained in RPMI 1640 medium supplemented with 15% FBS, 1 mmol/l sodium pyruvate (Sigma Diagnostics), and 2 mmol/l L-glutamine. To prevent the outgrowth of HAT-resistant revertants, the media routinely contained 20 µg/ml of 6-thioguanine (Burroughs Wellcome, Research Triangle Park, NC, USA).

### Patient cells

Bone marrow aspirate samples were obtained from six MM patients after written informed consent was obtained according to Institutional Guidelines. Bone marrow mononuclear cells were isolated by centrifugation on Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ, USA). Tumour cells were selected either by flow-cytometry or enriched by a negative selection protocol using immunomagnetic beads, as previously described (Tai *et al*, 2000). Purified tumour cells (>95% CD38<sup>+</sup> CD45RA<sup>-</sup> and/or CD138<sup>+</sup>) were used for fusion with autologous DCs.

### Culture of DCs

Peripheral blood mononuclear cells were isolated by centrifugation on Ficoll-Paque (Pharmacia Biotech) from PB of six MM patients and from normal donors, as previously described (Raje *et al*, 1999). Briefly, PBMCs were suspended in RPMI 1640 medium containing 10% heat-inactivated human AB serum, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (10% RPMI); plated on 6-well, flat-bottomed tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA); and incubated for 2 h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Non-adherent cells were harvested, and fresh 10% RPMI medium containing granulocyte-macrophage colony stimulating factor (GM-CSF; 1000 units/ml) and interleukin (IL)-4 (800 units/ml) (Genzyme, Boston, MA, USA) was added to the adherent layer. After 7 d, loosely adherent cells were harvested and used for fusion and further studies.

### Fusion of DC to MM cells

**Cell lines.** The HS Sultan and SK0-007 MM cells were fused with normal donor-derived DCs in the presence of polyethylene glycol (PEG) 1500 (Boehringer Mannheim, Indianapolis, IN, USA) using the standard protocol for production of hybridoma cells (Lane *et al*, 1986). Briefly, DCs and MM cells were washed with plain RPMI 1640 prewarmed to 37°C, mixed in a ratio of 5:1, and centrifuged for 5 min at 400 g. The supernatant was removed using a

pipette, and the cell pellet was gently broken-up by tapping the bottom of the Falcon tube. The tube was placed in a water bath at 37°C and 1 ml of 50% PEG was slowly added to the pellet over 1 min. The cell pellet was resuspended by stirring with the end of the pipette for a further minute, followed by the addition of 1 ml of plain RPMI 1640 over 1 min, and 9 ml over 2 min, with continuous stirring. The cell suspension was incubated for a further 5 min at 37°C, centrifuged and the supernatant discarded. The fused cells were resuspended in RPMI 1640 medium containing 10% human AB serum, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate and 1X HAT medium supplement (Boehringer Mannheim) to prevent the overgrowth of tumour–tumour fusions.

*Patient cells.* Patient MM cells were similarly fused to autologous DCs and resuspended in RPMI 1640 medium containing 10% human AB serum, 2 mmol/l L-glutamine, and 1 mmol/l sodium pyruvate, without the addition of HAT medium supplement.

In both cases, the FCs were plated in 24-well fibronectin-coated plates (Becton Dickinson).

#### Characterization of FC phenotype

*Dual immunofluorescence.* Dual staining of cytopins of FCs, DCs and MM cells was performed to assay for DC lineage antigens (CD40, CD80, CD86 and HLA-DR; Pharmingen, San Diego, CA, USA), MHC class I and CD83 (Beckman Coulter, Fullertown, CA, USA) as well as MM-associated antigens (CD38, CD19 and CD138; Beckman Coulter). Briefly, cytopins were dried at room temperature, and cells were fixed with 5% acetic acid/ethanol for 20 min at –20°C. Slides were washed three times with phosphate-buffered saline (PBS) and placed in a moist chamber. Cytopins were blocked with goat sera for 20 min, as the secondary antibody used was a goat anti-mouse antibody. Slides were washed three times in PBS and incubated with the first antibody for 1 h at room temperature. The slides were then washed three times with PBS and incubated with the second antibody at room temperature for 1 h. Slides were washed three times with PBS and covered with Fluorsave reagent (Calbiochem, La Jolla, CA, USA) and cover slips before viewing under a fluorescent microscope (Olympus, Melville, NY, USA); images were saved as Oncor images.

*Flow cytometry.* Indirect immunofluorescence flow-cytometry using the Coulter Epics XL (Coulter Corp., Miami, FL, USA) was performed to assay for the expression of DC lineage antigens (CD40, CD80, CD86 and HLA-DR; Pharmingen), MHC class I and CD83 (Beckman Coulter) as well as MM-associated antigens (CD38, CD19 and CD138; Beckman Coulter). Cells were washed in PBS and incubated in PBS with 20% human AB serum at room temperature for 20 min to eliminate non-specific Fc receptor binding. After washing with PBS, cells were incubated with primary murine monoclonal antibodies reactive with the above antigens for 30 min on ice.

After several washes, the cells were developed with goat anti-murine antibody conjugated with fluorescein isothiocyanate. Cells were then washed, fixed with 2% paraformaldehyde, and evaluated by flow-cytometry.

#### Generation of CTL lines

Peripheral blood mononuclear cells from normal donors ( $1 \times 10^7$ ) and one MM patient were suspended in RPMI 1640 medium containing 10% heat-inactivated human AB serum, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. PBMCs were stimulated weekly using  $1 \times 10^6$  irradiated (1.5 Gy) FCs. PBMCs were also stimulated with either MM cells or DCs alone as controls. IL-2 (20 units/ml) was added after the first 48 h of culture of the CTL lines. All cell line experiments were repeated at least three times.

#### Cytotoxicity assays

Effector cells generated as above were tested *in vitro* for their MM-specific cytotoxicity in a standard 4 h  $^{51}\text{Cr}$ -release assay at weekly intervals. Briefly,  $1 \times 10^6$  target cells (DC pulsed with either HS Sultan or SK0 007 apoptotic MM cells in the case of normal donors or autologous MM cells in patient studies) were labelled with 3.7 GBq of  $^{51}\text{Cr}$  (New England Nuclear, Boston, MA, USA) for 60 min at 37°C and washed three times to remove unincorporated isotope. The labelled targets were added to 96-well U-bottom plates ( $1 \times 10^4$  cells/well) and incubated with varying ratios of effector cells for 4 h at 37°C in a 5% CO<sub>2</sub> atmosphere. Assays were performed in triplicate. The supernatants were assayed for  $^{51}\text{Cr}$  release in a gamma counter. Spontaneous release of  $^{51}\text{Cr}$  was assessed by incubation of targets in the absence of effectors, and maximum release of  $^{51}\text{Cr}$  was determined by incubation of targets in 0.1% Triton X-100. The percentage of specific  $^{51}\text{Cr}$  release was determined by the following equation:

$$\text{Percentage-specific lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100.$$

To confirm whether the CTLs were class I and/or class II restricted, anti-MHC class I (W6/32) and anti-class II [9–49 (I3)] blocking antibodies (Pharmingen) were added to the target at a concentration of 20 µg/ml and incubated for 60 min on ice prior to  $^{51}\text{Cr}$  release assay.

#### Autologous T-cell proliferation assays

We next examined the function of FCs derived from MM patients as APCs in autologous T-cell proliferation assays. Graded numbers of irradiated (1.5 Gy) FCs were added to  $2 \times 10^5$  autologous T cells in 96-well U-bottomed culture plates for 5 d. T cells were obtained from PBMCs using T-cell enrichment columns (R&D Systems, Minneapolis, MN, USA) via high affinity negative selection. T-cell proliferation was

determined by measuring the uptake of tritiated thymidine ( $^3\text{H}$ ]TdR) by cells pulsed with 3.7 kBq of  $^3\text{H}$ ]TdR (925 GBq/mmol; Du Pont-New England Nuclear, Wilmington, DE, USA) for the last 12 h of 5 d cultures. Results were expressed as mean counts per minute  $\pm$  standard deviation in triplicates. Patient-derived DCs and MM cells served as control stimulator cells.

## Results

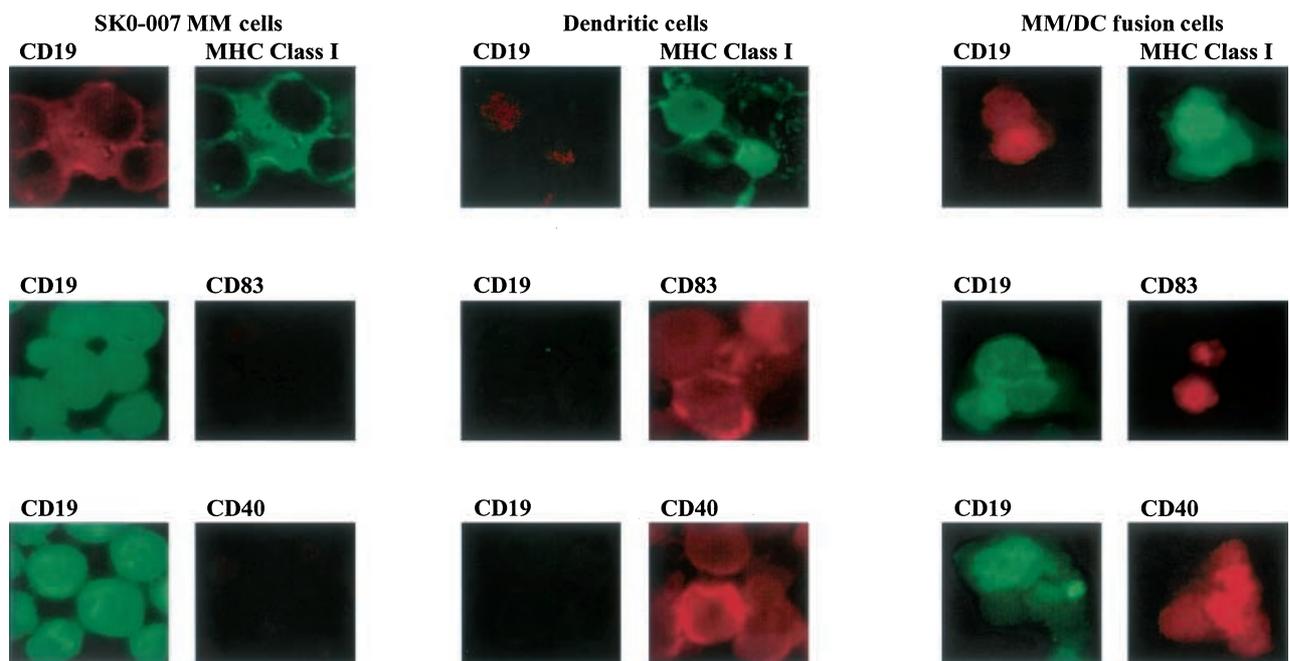
### *FCs demonstrated a biphenotypic profile*

Dual staining of cytopins of FCs, DCs and MM cells was performed to assay the expression of DC lineage antigens (CD40, CD80, CD86, HLA-DR, MHC class I and CD83) as well as MM-associated antigens (CD38, CD19 and CD138) on days 5, 15 and 20 following fusion. The MM/DC FC demonstrated a typical biphenotypic profile, with cell surface expression of both DC antigens and MM-associated antigens. By days 5–7, fusions of either MM cell lines alone or of MM patient cells alone were no longer viable. A count of 10–30% cells expressed a DC phenotype and 70–90% a biphenotypic (MM and DC) cell surface phenotype. By day 15, 70–90% of FC expressed both MM- and DC-associated antigens, as shown for SK0-007(J3) MM cell/DC FCs in Fig 1. Specifically, immunofluorescence microscopy showed that the MM cells expressed MHC class I and CD19, but lacked the CD40 and

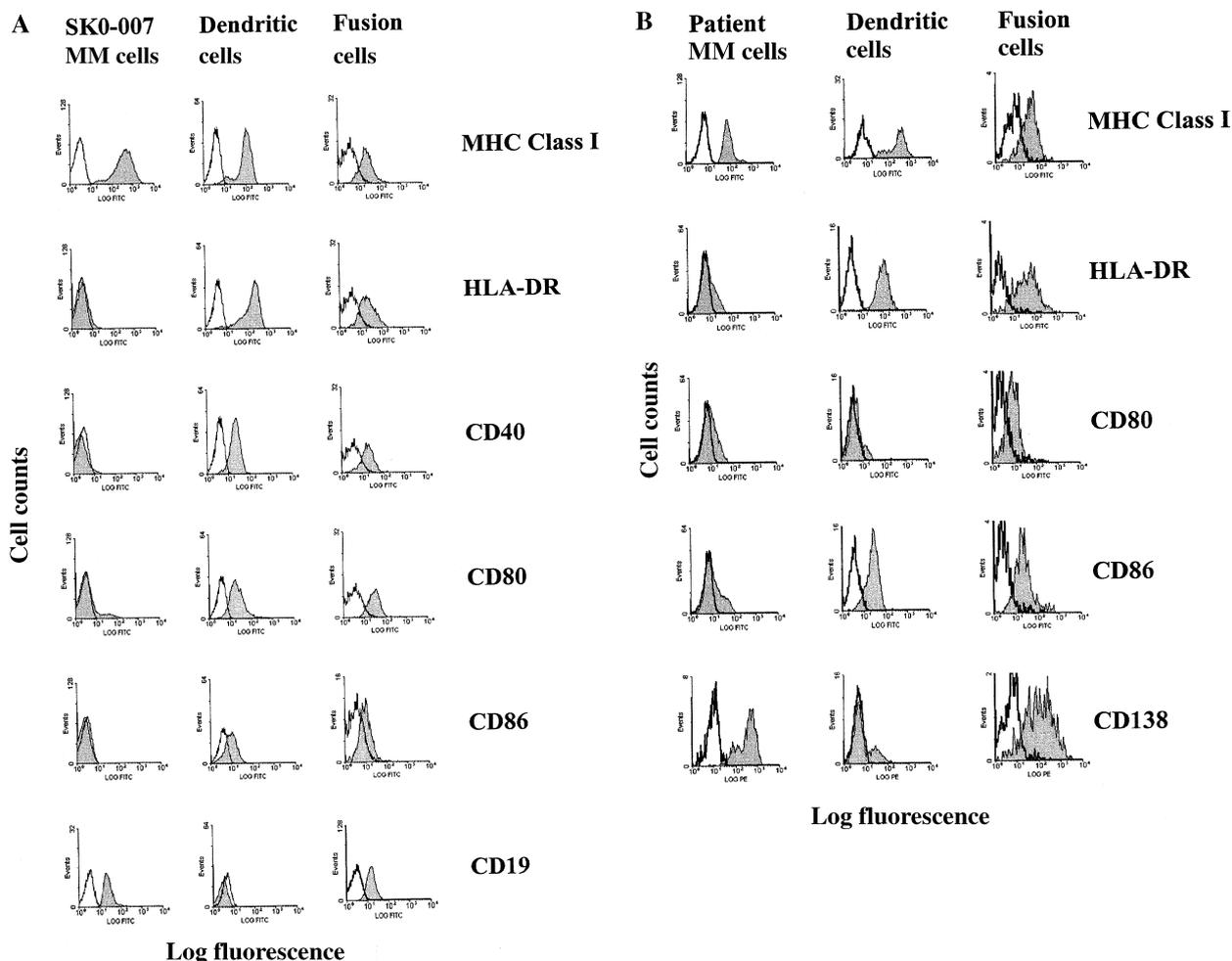
CD83 DC lineage-specific antigens. Conversely, DCs expressed CD40 and CD83 but lacked CD19. Importantly, the FC expressed MHC class I, CD19, CD40 and CD83 antigens, confirming their biphenotypic profile. FCs of DCs with either MM cell lines or MM patient cells were 60–90% homogenous after 3 weeks of culture.

This biphenotypic profile of MM/DC FCs was further confirmed by flow-cytometry. As shown in Fig 2A, the SK0-007 expressed MHC class I and CD19, but lacked HLA-DR, CD40, CD80, and CD86 staining. On the other hand, the DCs expressed cell surface MHC class I, HLA-DR, CD40, CD80 and CD86, but lacked CD19 staining. The FCs expressed CD19, in addition to DC-associated antigens and co-stimulatory molecules. Similar results were obtained with the HS Sultan FCs (data not shown).

Before testing the function of patient-derived MM/DC FC, their biphenotypic profile was confirmed by immunofluorescent microscopy and flow-cytometric staining for DC-associated antigens and CD38/138 MM-associated antigens. As noted in MM cell line/DC FCs, 60–70% of the patient MM cells/DC FCs expressed both DC lineage (CD80, CD86, MHC class I, and MHC class II and CD38) and/or CD138 by day 15 of culture. FCs of MM cells alone failed to proliferate in culture and did not survive beyond 5–7 d. The remaining 30–40% of cells mainly expressed a DC phenotype. Representative results of one such patient are shown in Fig 2B.



**Fig 1.** MM/DC FCs express a biphenotypic profile by immunofluorescence microscopy. Dual staining of cytopins of FCs, DCs and SK0-007 MM cells was performed to assay for the expression of DC lineage antigens (CD40, CD80, CD86, HLA-DR, MHC class I and CD83) as well as MM-associated antigens (CD38 and CD19). Immunofluorescence microscopy demonstrated the expression of MHC class I and CD19, but lack of CD40 and CD83 on SK0-007 MM cells. In contrast, the DCs expressed CD40 and CD83 strongly but lacked CD19. The MM/DC FCs demonstrated a biphenotypic profile with abundant expression of MHC class I, CD19, CD40 and CD83.



**Fig 2.** FCs express a biphenotypic profile by flow-cytometry. Indirect immunofluorescence flow-cytometry was performed for FCs, DCs and SK0-007 (MM cells). MHC class I and CD19, but no HLA-DR, CD40, CD80 and CD86 were expressed on SK0-007 MM cells. The DCs expressed MHC class I, HLA-DR, CD40, CD80 and CD86, but lacked CD19. The MM/DC FCs expressed both MM-restricted (CD19) and DC-associated antigens and co-stimulatory molecules (A). Similarly, patient-generated FC expressed DC-associated antigens (MHC class I, HLA-DR, CD80 and CD86) in addition to the MM-associated antigen (CD138) (B).

*FCs resulted in autologous T-cell proliferation and MM-specific immunity*

Patient MM cells/DC FCs were next tested as stimulators in autologous T-cell proliferation assays. Patient-derived DCs or MM cells alone served as control stimulator cells. As shown in Fig 3, patient MM cell/DC FCs were potent stimulators of autologous T cells, while either MM cells or DCs alone failed to induce significant T-cell proliferation. These data suggest that FCs resulted in immune recognition and MM-specific T-cell proliferation, which was further confirmed in CTL assays.

*FCs resulted in generation of MM-specific cytotoxicity*

T-cell lines were generated from PBMCs of those donors whose DCs were fused with either the SK0-007 or the HS Sultan MM cell lines. PBMCs ( $1 \times 10^7$ ) were stimulated weekly using  $1 \times 10^6$  irradiated (1.5 Gy) FCs, and with either

MM cells or DCs alone as controls. DCs pulsed with either SK0-007 or HS Sultan MM cell apoptotic bodies were used as targets. PBMCs primed with FC, but not unprimed PBMCs or PBMCs primed with either DC or MM cells alone resulted in 48% and 55% specific lysis, respectively, of MM cell line-pulsed DC targets. Fig 4A shows the DCs before and after pulsing with apoptotic MM cells, which were used as targets in a  $^{51}\text{Cr}$  release assay. As shown in Fig 4B, PBMCs primed with FCs induced a 55% MM-specific cell kill. The natural killer (NK) cell target K562 was used as a cold target to control for non-specific NK activity.

Similar results were observed with patient samples. Specifically,  $1 \times 10^7$  patient PBMCs were stimulated weekly using  $1 \times 10^6$  irradiated (1.5 Gy) autologous FCs, and with either MM cells or DCs alone as controls; autologous MM cells served as targets. FC-primed patient PBMCs mediated a 46% tumour-specific lysis (Fig 5A), while no significant killing was

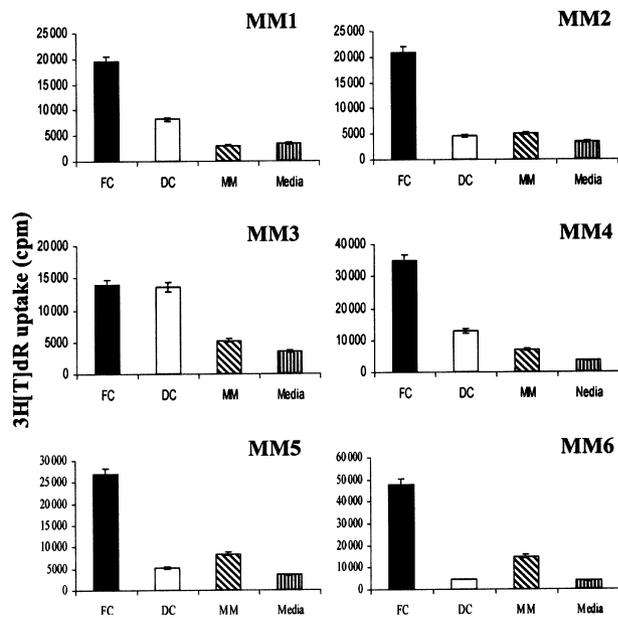


Fig 3. Patient MM/DC FCs stimulate autologous T-cell proliferation. MM/DC FCs from six patients were used as stimulators in autologous T-cell proliferation assays. MM patient/DC FC (■), but not MM cells (▨) or DCs (□) alone induced autologous T-cell proliferation.

triggered in unprimed PBMCs or PBMCs primed with either MM cells or DCs alone in one representative patient. Blocking experiments with MHC class I and with MHC class II antibodies demonstrated a 58% and 45% inhibition of killing, respectively, suggesting that the CTLs were MHC restricted and that both MHC classes I and II contributed to tumour-specific lysis (Fig 5B).

## Discussion

Most vaccination approaches have focussed on generating immunity to a specific tumour antigen (Condon *et al*, 1996; Hsu *et al*, 1996; Syrengelas *et al*, 1996; King *et al*, 1998; Stevenson *et al*, 1999; Zhou *et al*, 1999; Milazzo *et al*, 2003). A major disadvantage to this approach is the possibility of developing tolerance to other known and unknown antigens, which may then contribute to the resurgence of disease. To circumvent this problem, whole tumour cell vaccines are currently under investigation. In this respect, irradiated tumour cells have been transfected with cytokine genes or co-stimulatory molecules using various vector systems or the gene gun (Condon *et al*, 1996; Stewart *et al*, 1998; Turner *et al*, 1998; Tarte *et al*, 1999). Autologous tumour cells transfected with immunomodulatory genes (i.e. IL-2, IL-12, B7-1) using adenoviral vectors have resulted in both the eradication of established malignancy and protection from rechallenge with unmodified tumour cells in animal models (Addison *et al*, 1995; Putzer *et al*, 1997). MM cell lines, and to a lesser extent primary plasma cells, have been infected with adenoviral vectors carrying marker genes, including IL-2 (AdCAIL-2), or

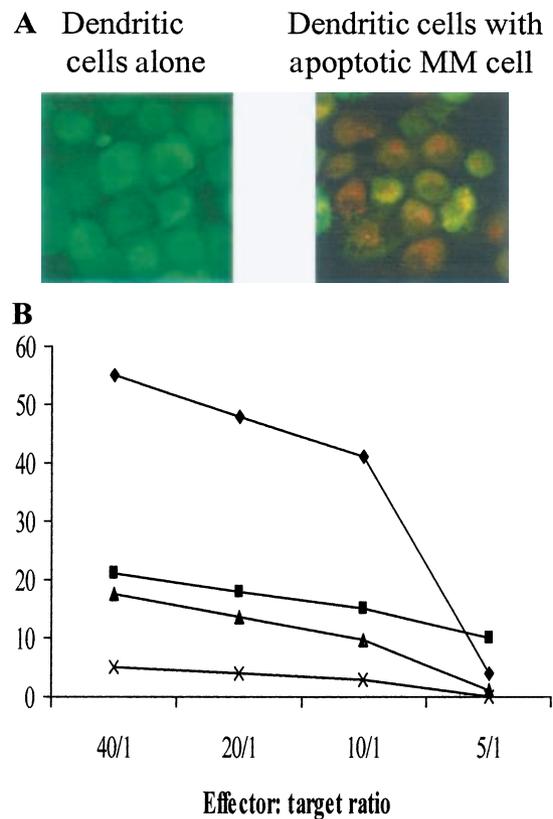
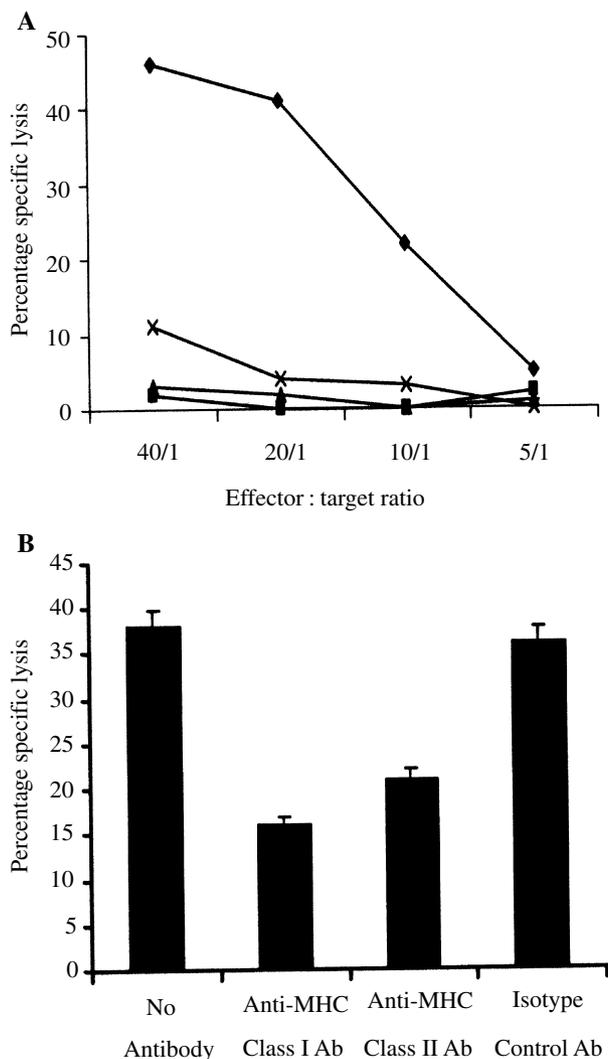


Fig 4. Cell line-generated FCs induce MM-specific cytotoxicity. A count of  $1 \times 10^7$  normal donor PBMCs were stimulated weekly using  $1 \times 10^6$  irradiated (1.5 Gy) FCs. PBMCs were also stimulated or not with MM cells or DCs alone. (A) DCs from the same donor (green tracker) pulsed with apoptotic MM cells (red tracker) were used as targets in the  $^{51}\text{Cr}$  assay. (B) The FC-primed (◆) but not the unprimed (×), MM-primed (■), or DC-primed (▲), T cells resulted in 55% MM-specific cytotoxicity.

IL-12 and B7-1 (AdSH12.B7) (Stewart *et al*, 1998). Tarte *et al* (1999) have shown high levels of B7-1 expression on human myeloma cells using transduction with a B7-1 retrovirus, resulting in stimulation of allogeneic CD8<sup>+</sup>, but not CD4<sup>+</sup>, T-cell proliferation. For one patient with advanced disease, B7-1 gene transfer made it possible to amplify autologous cytotoxic T cells that killed autologous MM cells in an HLA class I-restricted manner, but not autologous phytohaemagglutinin (PHA)-stimulated blasts. Other approaches have included the use of DCs pulsed with whole tumour cell lysates (Nestle *et al*, 1998) and the use of apoptotic tumour cell bodies incorporated into DCs (Hayashi *et al*, 2003), resulting in the presentation of multiple TAA in a class I-restricted manner.

We have taken the approach of fusing whole tumour cells to DC. In this way, hybridomas are constructed which express both tumour-derived antigens and DC-derived co-stimulatory molecules. The fusion of DC and malignant cells to produce a tumour vaccine offers several distinct advantages over other methods of immunization. Multiple TAA are presented by both MHC classes I and II molecules, allowing stimulation of



**Fig 5.** Patient-generated FCs induce MHC-restricted, MM-specific CTLs. A count of  $1 \times 10^7$  PBMCs were stimulated weekly using  $1 \times 10^6$  irradiated (1.5 Gy) FCs. PBMCs were also stimulated or not with MM cells or DCs alone. (A) The FC primed (◆) but not the unprimed (×), MM primed (■), or DC primed (▲), T cells resulted in MM-specific cytotoxicity. PBMCs cells primed by the MM/DC FCs mediated a 46% MM-specific lysis of autologous MM cells. (B) Blocking experiments demonstrated a 58% and 45% inhibition of killing with MHC class I and MHC class II antibodies (Ab), respectively, suggesting that the CTLs were MHC-restricted and that both MHC classes I and II contribute to tumour-specific lysis.

both CD8<sup>+</sup> and CD4<sup>+</sup> effector cells and thereby increasing the probability of an effective cell-mediated anti-tumour response. Fusion of DCs to tumour cells offers other advantages, as DCs can be readily generated from either PB or BM. FCs have been generated and have been successfully used as vaccines in the prevention and treatment of breast carcinoma cells metastatic to the lung (Gong *et al*, 1997). Subsequent vaccination studies of fusion hybrids of DCs and MUC1 have demonstrated their ability to reverse tolerance to the MUC1 antigen and induce anti-tumour responses against human breast cancer (Gong

*et al*, 1998, 2000). We have also used a similar strategy in a mouse model using murine MM cells and demonstrated that IL-12 potentiated anti-myeloma immunity with the use of these FCs (Gong *et al*, 2002). In the present study, we have successfully performed fusions of MM cells to DCs and have induced MM-specific CTL responses. These CTLs were MHC restricted, as confirmed by blocking studies using neutralizing anti-MHC antibodies. Most recently, allogeneic DCs have been fused to renal cell carcinoma and achieved remarkable responses when used as vaccines to treat metastatic renal cell carcinoma (Kufe, 2000; Kugler *et al*, 2000).

The optimal timing of vaccination is currently unknown. The rationale for adjuvant idiotype vaccination after high-dose therapy for MM has been discussed by Reichardt *et al* (1997). In a similar adjuvant setting, Bendandi *et al* (1999) recently used idiotype vaccination in 20 patients with follicular lymphoma to eradicate residual t(14;18)+ lymphoma cells in chemotherapy-induced first clinical CR. The vaccine consisted of whole immunoglobulin protein isolated from the patient's own tumour and conjugated to the immunogenic carrier keyhole limpet haemocyanin. The antigen was mixed with free GM-CSF, due to its ability to enhance CD8<sup>+</sup> effector cell function. All 11 patients with detectable polymerase chain reaction translocations in their primary tumours had detectable tumour in their blood both at diagnosis and after chemotherapy, despite being in complete clinical remission. Following vaccinations, eight of 11 patients converted to a molecular remission. Tumour-specific cytotoxic CD8<sup>+</sup> and CD4<sup>+</sup> T cells were uniformly found (19 of 20 patients), whereas antibodies were detected but not required for molecular remission. Vaccination was thus associated with clearance of residual tumour cells from blood and long-term disease-free survival. This is the first demonstration of molecular remissions following a vaccination strategy and provides the framework for the use of such immunotherapy approaches in the treatment of minimal residual disease (MRD). MM is a haematological malignancy where high CR rates are documented after high-dose therapies and MRD can be assessed using molecular techniques. MM patients who achieve MRD post-transplant therefore appear to be the ideal candidates in whom to test approaches, such as the FC vaccination strategy amongst others.

The FC cell vaccination approach does not come without limitations. The low proliferative capacity of our FCs demands a high starting number of both MM cells and DCs for generating FCs. In order to induce an *in vivo* immune response, repeated vaccinations with FC may be required, suggesting that repeated fusion procedures may be needed. To circumvent some of these problems, we are evaluating several strategies to enhance the APC function of FCs, i.e. the use of cytokine combinations, such as IL-4 and IL-12, or CD40 activation.

An alternative strategy to circumvent the need for large tumour and DC numbers in the FC vaccine strategy is to generate and expand anti-MM-specific T cells *ex vivo* for

autologous adoptive immunotherapy of MRD in the post-transplant setting. Based upon our observations of enhanced APC function of CD40-activated MM cells, evidenced by the induction of autologous T-cell proliferation, we are using CD40-activated irradiated MM cells to stimulate autologous MM-specific T cells and then *ex vivo* expanding these T cells for adoptive immunotherapy (Schultze *et al*, 2001).

In summary, preclinical studies, animal models and early phase I studies in MM have demonstrated tremendous promise in the area of cancer immunotherapy. The aims of these approaches are to eradicate residual tumour and prolong disease-free survival. The present study demonstrates that an MHC-restricted, MM-specific CTL response can be induced using the FC approach. Importantly, we have also demonstrated that FC can trigger an autologous anti-tumour-specific response. These preclinical studies formed the framework for a phase I study using patient MM/DC FCs in vaccination and adoptive immunotherapy protocols in MM that are currently ongoing.

## Acknowledgments

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## References

- Addison, C.L., Braciak, T., Ralston, R., Muller, W.J., Gaudie, J. & Graham, F.L. (1995) Intratumoral injection of an adenovirus expressing interleukin 2 induces regression and immunity in a murine breast cancer model. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 8522–8526.
- Alyea, E.P., Soiffer, R.J., Canning, C., Neuberg, D., Schlossman, R., Pickett, C., Collins, H., Wang, Y., Anderson, K.C. & Ritz, J. (1998) Toxicity and efficacy of defined doses of CD4(+) donor lymphocytes for treatment of relapse after allogeneic bone marrow transplant. *Blood*, **91**, 3671–3680.
- Antonia, S.J., Munoz-Antonia, T., Soldevila, G., Miller, J. & Flavell, R.A. (1995) B7-1 expression by a non-antigen presenting cell-derived tumor. *Cancer Research*, **55**, 2253–2256.
- Attal, M., Harousseau, J.L., Stoppa, A.M., Sotto, J.J., Fuzibet, J.G., Rossi, J.F., Casassus, P., Maisonneuve, H., Facon, T., Ifrah, N., Payen, C. & Bataille, R. (1996) A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome. *New England Journal of Medicine*, **335**, 91–97.
- Bendandi, M., Gocke, C.D., Kobrin, C.B., Benko, F.A., Sternas, L.A., Pennington, R., Watson, T.M., Reynolds, C.W., Gause, B.L., Duffey, P.L., Jaffe, E.S., Creekmore, S.P., Longo, D.L. & Kwak, L.W. (1999) Complete molecular remissions induced by patient-specific vaccination plus granulocyte-monocyte colony-stimulating factor against lymphoma. *Nature Medicine*, **5**, 1171–1177.
- Child, J.A., Morgan, G.J., Davies, F.E., Owen, R.G., Bell, S.E., Hawkins, K., Brown, J., Drayson, M.T. & Selby, P.J. (2003) High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. *New England Journal of Medicine*, **348**, 1875–1883.
- Condon, C., Watkins, S.C., Celluzzi, C.M., Thompson, K. & Faló, Jr, L.D. (1996) DNA-based immunization by *in vivo* transfection of dendritic cells. *Nature Medicine*, **2**, 1122–1128.
- Corradini, P., Cavo, M., Lokhorst, H., Martinelli, G., Terragna, C., Majolino, I., Valagussa, P., Boccadoro, M., Samson, D., Bacigalupo, A., Russell, N., Montefusco, V., Voena, C. & Gahrton, G. (2003) Molecular remission after myeloablative allogeneic stem cell transplantation predicts a better relapse-free survival in patients with multiple myeloma. *Blood*, **102**, 1927–1929.
- Garrido, F., Cabrera, T., Concha, A., Glew, S., Ruiz-Cabello, F. & Stern, P.L. (1993) Natural history of HLA expression during tumour development. *Immunology Today*, **14**, 491–499.
- Gong, J., Chen, D., Kashiwaba, M. & Kufe, D. (1997) Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nature Medicine*, **3**, 558–561.
- Gong, J., Chen, D., Kashiwaba, M., Li, Y., Chen, L., Takeuchi, H., Qu, H., Rowse, G.J., Gendler, S.J. & Kufe, D. (1998) Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 6279–6283.
- Gong, J., Avigan, D., Chen, D., Wu, Z., Koido, S., Kashiwaba, M. & Kufe, D. (2000) Activation of antitumor cytotoxic T lymphocytes by fusions of human dendritic cells and breast carcinoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 2715–2718.
- Gong, J., Koido, S., Chen, D., Tanaka, Y., Huang, L., Avigan, D., Anderson, K., Ohno, T. & Kufe, D. (2002) Immunization against murine multiple myeloma with fusions of dendritic and plasmacytoma cells is potentiated by interleukin 12. *Blood*, **99**, 2512–2517.
- Hayashi, T., Hideshima, T., Akiyama, M., Raje, N., Richardson, P., Chauhan, D. & Anderson, K.C. (2003) *Ex vivo* induction of multiple myeloma-specific cytotoxic T lymphocytes. *Blood*, **102**, 1435–1442.
- Hsu, F.J., Benike, C., Fagnoni, F., Liles, T.M., Czerwinski, D., Taidi, B., Engleman, E.G. & Levy, R. (1996) Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nature Medicine*, **2**, 52–58.
- Jemal, A., Murray, T., Samuels, A., Ghafoor, A., Ward, E. & Thun, M.J. (2003) Cancer statistics, 2003. *CA: A Cancer Journal for Clinicians*, **53**, 5–26.
- King, C.A., Spellerberg, M.B., Zhu, D., Rice, J., Sahota, S.S., Thompson, A.R., Hamblin, T.J., Radl, J. & Stevenson, F.K. (1998) DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. *Nature Medicine*, **4**, 1281–1286.
- Kufe, D.W. (2000) Smallpox, polio and now a cancer vaccine? *Nature Medicine*, **6**, 252–253.
- Kugler, A., Stuhler, G., Walden, P., Zoller, G., Zobywalski, A., Brossart, P., Trefzer, U., Ullrich, S., Muller, C.A., Becker, V., Gross, A.J., Hemmerlein, B., Kanz, L., Muller, G.A. & Ringert, R.H. (2000) Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. *Nature Medicine*, **6**, 332–336.
- Kwak, L.W., Taub, D.D., Duffey, P.L., Bensinger, W.I., Bryant, E.M., Reynolds, C.W. & Longo, D.L. (1995) Transfer of myeloma idiotype-specific immunity from an actively immunised marrow donor. *Lancet*, **345**, 1016–1020.

- Lane, R.D., Crissman, R.S. & Ginn, S. (1986) High efficiency fusion procedure for producing monoclonal antibodies against weak immunogens. *Methods in Enzymology*, **121**, 183–192.
- Letterio, J.J. & Roberts, A.B. (1998) Regulation of immune responses by TGF-beta. *Annual Review of Immunology*, **16**, 137–161.
- Lokhorst, H.M., Schattenberg, A., Cornelissen, J.J., Thomas, L.L. & Verdonck, L.F. (1997) Donor leukocyte infusions are effective in relapsed multiple myeloma after allogeneic bone marrow transplantation. *Blood*, **90**, 4206–4211.
- McElwain, T.J. & Powles, R.L. (1983) High-dose intravenous melphalan for plasma-cell leukaemia and myeloma. *Lancet*, **2**, 822–824.
- Massaia, M., Borriero, P., Battaglio, S., Mariani, S., Beggiato, E., Napoli, P., Voena, C., Bianchi, A., Coscia, M., Besostri, B., Peola, S., Stiefel, T., Even, J., Novero, D., Boccadoro, M. & Pileri, A. (1999) Idiotype vaccination in human myeloma: generation of tumor-specific immune responses after high-dose chemotherapy. *Blood*, **94**, 673–683.
- Milazzo, C., Reichardt, V.L., Muller, M.R., Grunebach, F. & Brossart, P. (2003) Induction of myeloma-specific cytotoxic T cells using dendritic cells transfected with tumor-derived RNA. *Blood*, **101**, 977–982.
- Nestle, F.O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G. & Schadendorf, D. (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nature Medicine*, **4**, 328–332.
- Osterroth, F., Garbe, A., Fisch, P. & Veelken, H. (2000) Stimulation of cytotoxic T cells against idiotype immunoglobulin of malignant lymphoma with protein-pulsed or idiotype-transduced dendritic cells. *Blood*, **95**, 1342–1349.
- Pfeiffer, S., Gooding, R.P., Apperley, J.F., Goldschmidt, H. & Samson, D. (1997) Dendritic cells generated from the blood of patients with multiple myeloma are phenotypically and functionally identical to those similarly produced from healthy donors. *British Journal of Haematology*, **98**, 973–982.
- Putzer, B.M., Hitt, M., Muller, W.J., Emtage, P., Gaudie, J. & Graham, F.L. (1997) Interleukin 12 and B7-1 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 10 889–10 894.
- Qin, Z., Noffz, G., Mohaupt, M. & Blankenstein, T. (1997) Interleukin-10 prevents dendritic cell accumulation and vaccination with granulocyte-macrophage colony-stimulating factor gene-modified tumor cells. *Journal of Immunology*, **159**, 770–776.
- Raje, N. & Anderson, K. (1999) Thalidomide—a revival story. *New England Journal of Medicine*, **341**, 1606–1609.
- Raje, N., Gong, J., Chauhan, D., Teoh, G., Avigan, D., Wu, Z., Chen, D., Treon, S.P., Webb, I.J., Kufe, D.W. & Anderson, K.C. (1999) Bone marrow and peripheral blood dendritic cells from patients with multiple myeloma are phenotypically and functionally normal despite the detection of Kaposi's sarcoma herpesvirus gene sequences. *Blood*, **93**, 1487–1495.
- Reichardt, V.L., Okada, C.Y., Stockerl-Goldstein, K.E., Bogen, B. & Levy, R. (1997) Rationale for adjuvant idiotypic vaccination after high-dose therapy for multiple myeloma. *Biology of Blood and Marrow Transplantation*, **3**, 157–163.
- Reichardt, V.L., Okada, C.Y., Liso, A., Benike, C.J., Stockerl-Goldstein, K.E., Engleman, E.G., Blume, K.G. & Levy, R. (1999) Idiotype vaccination using dendritic cells after autologous peripheral blood stem cell transplantation for multiple myeloma – a feasibility study. *Blood*, **93**, 2411–2419.
- Richardson, P.G., Schlossman, R.L., Weller, E., Hideshima, T., Mitsiades, C., Davies, F., LeBlanc, R., Catley, L.P., Doss, D., Kelly, K., McKenney, M., Mechlowicz, J., Freeman, A., Deocampo, R., Rich, R., Ryoo, J.J., Chauhan, D., Balinski, K., Zeldis, J. & Anderson, K.C. (2002) Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. *Blood*, **100**, 3063–3067.
- Richardson, P.G., Barlogie, B., Berenson, J., Singhal, S., Jagannath, S., Irwin, D., Rajkumar, S.V., Srkalovic, G., Alsina, M., Alexanian, R., Siegel, D., Orłowski, R.Z., Kuter, D., Limentani, S.A., Lee, S., Hideshima, T., Esseltine, D.L., Kauffman, M., Adams, J., Schenkein, D.P. & Anderson, K.C. (2003) A phase 2 study of bortezomib in relapsed, refractory myeloma. *New England Journal of Medicine*, **348**, 2609–2617.
- Schlossman, R.L. & Anderson, K.C. (1999) Bone marrow transplantation in multiple myeloma. *Current Opinion in Oncology*, **11**, 102–108.
- Schultze, J.L., Anderson, K.C., Gilleece, M.H., Gribben, J.G. & Nadler, L.M. (2001) A pilot study of combined immunotherapy with autologous adoptive tumour-specific T-cell transfer, vaccination with CD40-activated malignant B cells and interleukin 2. *British Journal of Haematology*, **113**, 455–460.
- Singhal, S., Mehta, J., Desikan, R., Ayers, D., Roberson, P., Eddlemon, P., Munshi, N., Anaissie, E., Wilson, C., Dhodapkar, M., Zeddis, J. & Barlogie, B. (1999) Antitumor activity of thalidomide in refractory multiple myeloma. *New England Journal of Medicine*, **341**, 1565–1571.
- Song, W., Kong, H.L., Carpenter, H., Torii, H., Granstein, R., Rafii, S., Moore, M.A. & Crystal, R.G. (1997) Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *Journal of Experimental Medicine*, **186**, 1247–1256.
- Specht, J.M., Wang, G., Do, M.T., Lam, J.S., Royal, R.E., Reeves, M.E., Rosenberg, S.A. & Hwu, P. (1997) Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. *Journal of Experimental Medicine*, **186**, 1213–1221.
- Stevenson, F.K., Link, Jr, C.J., Traynor, A., Yu, H. & Corr, M. (1999) DNA vaccination against multiple myeloma. *Seminars in Hematology*, **36**, 38–42.
- Stewart, A.K., Schimmer, A.D., Bailey, D.J., Dube, I.D., Cappe, D., Moen, R.C., Gaudie, J. & Graham, F.L. (1998) In vivo adenoviral-mediated gene transfer of interleukin-2 in cutaneous plasmacytoma. *Blood*, **91**, 1095–1097.
- Syngelas, A.D., Chen, T.T. & Levy, R. (1996) DNA immunization induces protective immunity against B-cell lymphoma. *Nature Medicine*, **2**, 1038–1041.
- Szabolcs, P., Moore, M.A. & Young, J.W. (1995) Expansion of immunostimulatory dendritic cells among the myeloid progeny of human CD34+ bone marrow precursors cultured with c-kit ligand, granulocyte-macrophage colony-stimulating factor, and TNF-alpha. *Journal of Immunology*, **154**, 5851–5861.
- Szabolcs, P., Gallardo, H.F., Ciocon, D.H., Sadelain, M. & Young, J.W. (1997) Retrovirally transduced human dendritic cells express a normal phenotype and potent T-cell stimulatory capacity. *Blood*, **90**, 2160–2167.
- Tai, Y.T., Teoh, G., Shima, Y., Chauhan, D., Treon, S.P., Raje, N., Hideshima, T., Davies, F.E. & Anderson, K.C. (2000) Isolation and

- characterization of human multiple myeloma cell enriched populations. *Journal of Immunological Methods*, **235**, 11–19.
- Tarte, K., Olsen, S.J., Yang Lu, Z., Legouffe, E., Rossi, J.F., Chang, Y. & Klein, B. (1998) Clinical-grade functional dendritic cells from patients with multiple myeloma are not infected with Kaposi's sarcoma-associated herpesvirus. *Blood*, **91**, 1852–1857.
- Tarte, K., Zhang, X.G., Legouffe, E., Hertog, C., Mehtali, M., Rossi, J.F. & Klein, B. (1999) Induced expression of B7-1 on myeloma cells following retroviral gene transfer results in tumor-specific recognition by cytotoxic T cells. *Journal of Immunology*, **163**, 514–524.
- Treon, S.P., Mollick, J.A., Urashima, M., Teoh, G., Chauhan, D., Ogata, A., Raje, N., Hilgers, J.H., Nadler, L., Belch, A.R., Pilarski, L.M. & Anderson, K.C. (1999) Muc-1 core protein is expressed on multiple myeloma cells and is induced by dexamethasone. *Blood*, **93**, 1287–1298.
- Turner, J.G., Tan, J., Crucian, B.E., Sullivan, D.M., Ballester, O.F., Dalton, W.S., Yang, N.S., Burkholder, J.K. & Yu, H. (1998) Broadened clinical utility of gene gun-mediated, granulocyte-macrophage colony-stimulating factor cDNA-based tumor cell vaccines as demonstrated with a mouse myeloma model. *Human Gene Therapy*, **9**, 1121–1130.
- Wen, Y.J., Min, R., Tricot, G., Barlogie, B. & Yi, Q. (2002) Tumor lysate-specific cytotoxic T lymphocytes in multiple myeloma: promising effector cells for immunotherapy. *Blood*, **99**, 3280–3285.
- Yi, Q., Desikan, R., Barlogie, B. & Munshi, N. (2002) Optimizing dendritic cell-based immunotherapy in multiple myeloma. *British Journal of Haematology*, **117**, 297–305.
- Zeis, M., Frenzke, H., Schmitz, N., Uharek, L. & Steinmann, J. (2002) Idiotype protein-pulsed dendritic cells produce strong anti-myeloma effects after syngeneic stem cell transplantation in mice. *Bone Marrow Transplantation*, **29**, 213–221.
- Zhou, W.Z., Hoon, D.S., Huang, S.K., Fujii, S., Hashimoto, K., Morishita, R. & Kaneda, Y. (1999) RNA melanoma vaccine: induction of antitumor immunity by human glycoprotein 100 mRNA immunization. *Human Gene Therapy*, **10**, 2719–2724.