Idiotype vaccination of multiple myeloma patients using monocyte-derived dendritic cells

VOLKER L. REICHARDT, CATERINA MILAZZO, WOLFRAM BRUGGER, HERMANN EINSELE, LOTHAR KANZ, PETER BROSSART

Background and Objectives. Dendritic cells (DC) pulsed with multiple myeloma (MM) patient-specific idiotype (Id) protein can induce MM-specific T-cell responses.

Design and Methods. We established serum-free culture conditions to generate monocyte-derived DC for clinical use to circumvent anti-xenogenic immune responses with repetitive vaccinations. In a clinical phase I trial twelve patients responsive to high dose chemotherapy (HDT) were vaccinated with autologous Id pulsed DC vaccines followed by Id/keyhole limpet hemocyanin (Id/KLH) booster immunizations co-injected with granulocyte-macrophage colony-stimulating factor as adjuvant.

Results. *In vitro* studies showed that serum-free-generated DC were equally effective in the induction of specific T-cell responses as were DC generated with fetal calf serum. On average 4.5×10^6 DC of >60% purity were generated from peripheral blood monocytes obtained 3-6 months after HDT and autologous stem cell transplantation. Ten of twelve patients received all planned vaccines without serious toxicity. Two patients developed Id-specific T-cell proliferative responses, in one patient an Id-specific cytotoxic T lymphocyte (CTL) response was measured. Id-specific TH1 cytokine secretion was found in one of the T-cell responding patients. All patients who received at least three Id/KLH vaccines mounted strong KLH specific T-cell and delayed antibody responses. Two patients remain in clinical partial response at 25 and 29 months after the start of the vaccination and ten patients have progressed, six of whom have died from progressive disease or infectious complications.

Interpretation and Conclusions. Serum-free DC vaccines induce Id-specific T-cell responses in MM patients.

Key words: immunotherapy, cytotoxic T lymphocytes, T cells, serum-free culture conditions.

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Specific immunotherapy using dendritic cell (DC) vaccines has proven to be a feasible adjuvant therapeutic option for various human malignancies. Phase I clinical trials in low grade non-Hodgkin's lymphoma (NHL), malignant melanoma, multiple myeloma (MM), breast and ovarian cancer have yielded promising results, mostly demonstrating the induction of specific immune responses to tumor-specific or tumor-associated antigens.[1-7] Immature circulating DC from fresh peripheral blood have been successfully utilized for *in vitro* antigen loading and maturation[1,2,6] but also monocyte-derived DC could successfully be used as cellular vaccines after an *in vitro* culture period and *pulsing* with the tumor peptide or protein antigen.[3-5,7]

We generated DC populations using serum-free tissue culture conditions in order to avoid the repetitive stimulation with xenogenic antigens of fetal calf serum (FCS). We started a phase I clinical trial in MM patients responsive to high-dose chemotherapy (HDT) and peripheral blood stem cell transplantation (PBSCT). The specificity of DC vaccination was based upon the unique MM idiotypes (Id) which were isolated and used in a patient- and MM clone-specific manner.[6] The rationale for treating patients in a status of minimal residual disease is based on clear evidence from murine models of myeloma[8,9] and on pivotal clinical studies[10,11] as discussed earlier.[12]

We evaluated the feasibility of generating monocyte-derived DC under serum-free culture conditions from MM patients shortly after HDT and PBSCT. The second goal was to induce Id-specific T-cell responses to potentially add antitumor immune reactivity. Third, we evaluated the immunocompetence of MM patients, post-HDT and PBSCT, to the neoantigen keyhole limpet hemocyanin (KLH), which served as a carrier protein. The use of granulocyte-macrophage colony-stimulating factor (GM-CSF) replaced the use of chemical adjuvants as published earlier.[6] The clinical course of all study patients was monitored.

Design and methods

Patients and HDT with PBSCT

Twelve patients with laboratory, radiological and histopathologic findings consistent with secretory MM who were treated at our institution with either single or double HDT and autologous PBSCT were offered to participate in this immunotherapy trial. The institutional ethical review board approved the study and informed

consent was obtained from all study patients prior to enrollment. Ten patients consented in written and oral form after detailed information, two participants consented orally after thorough information. Serum and bone marrow samples were obtained from participating patients early after diagnosis, preferably before the initiation of cytoreductive therapy. Many of the study patients were heavily pretreated prior to HDT and PBSCT (Table 1) and all patients had stage II or III disease according to the Durie/Salmon classification. Mobilization of autologous PBSC was achieved either by cyclophosphamide at 4 g/m^2 (patients MM01-MM04, MM08, MM11, MM12) or by ifosfamide (2.5 g/m^2, day 1-3), epirubicin (100 mg/m^2, day 1) and etoposide (150 mg/m^2, day 1-3) (patients MM05-MM07, MM09, MM10) followed by granulocyte-colony-stimulating factor (G-CSF). The transplant conditioning regimen consisted of high dose melphalan (200 mg/m^2 given in split doses on two consecutive days) (MEL 200) followed by reinfusion of stored autologous PBSC. Patient MM03 received only 140 mg/m^2 melphalan for transplant conditioning due to pre-existing severe renal insufficiency. Three of the twelve patients enrolled (patients MM05, MM07 and MM10) were treated with two cycles of HD-MEL three months apart, each followed by autologous stem cell rescue. The response to HDT and PBSCT was evaluated immediately prior to initiation of the adjuvant Id vaccination protocol. Complete response (CR) was defined as the repeated absence of the known monoclonal immunoglobulin (Ig) in immunofixation electrophoresis and no evidence of clonal plasma cells in BM cytology and histology. Partial response (PR) was defined as a reduction of serum monoclonal Ig by >50% of the pretransplant level, a lack of Bence-Jones proteinuria and less than 10% plasma cell involvement in BM biopsy. Stable disease (SD) was defined as less than 50% reduction and less than 20% increase of monoclonal Ig as compared to levels in serum samples prior to HDT and PBSCT. During the vaccination protocol only bisphosphonate therapy was given in parallel.

Table 1. MM Id vaccine: patients' characteristics.

Patient	Sex/Age	Id Isotype	Prior therapy (no. of cycles)	Pre-PBSCT conditioning	Post-PBSCT response
MM01	f/47	IgG1κ	MP (×3), Dex	MEL 200	PR
MM02	f/45	IgG1λ	VAD (×3)	MEL 200	PR
MM03	f/59	IgG1κ	CMF (×3)[a], VAD, Dex, VID	MEL 140	PR
MM04	f/52	IgG1κ	Dex, VID (×3)	MEL 200	SD
MM05	m/49	IgG1κ	VID (×4)	MEL 200 (×2)	CR
MM06	m/60	IgG1κ	MP (×6), HDT (Bu/Cy/TMI) +auto PBSCT, VRID (x 4)	MEL 200	PR
MM07	m/57	IgG1κ	ID (×4)	MEL 200 (×2)	PR
MM08	f/49	IgG1κ	Dex, VID (×2)	MEL 200	PR
MM09	f/53	IgG1λ	ID (×4)	MEL 200	PR
MM10	m/58	IgAκ	ID (×4)	MEL 200 (×2)	PR
MM11	f/56	IgG1κ	ID (×6)	MEL 200	PR
MM12	m/44	IgG1κ	ID (×2), Cy (×2)	MEL 200	PR

MP: melphalan, prednisone; Dex: dexamethasone; VAD: vincristine, adriamycin, dexamethasone; VID: vincristine, idarubicin, dexamethasone; ID: idarubicin, dexamethasone; VRID: vinorelbine, idarubicin, dexamethasone; Cy: cyclophospamide; Bu/Cy/TMI: busulfan, cyclophospamide, total marrow irradiation; MEL 140: melphalan at 140 mg/m^2; MEL 200: melphalan at 200 mg/m^2. [a]MM03 was treated with adjuvant CMF: cyclophosphamide, methotrexate and 5-FU for an invasive breast carcinoma diagnosed 9 years before the diagnosis of MM.

Serum-free DC generation

Monocyte-derived DC were generated using either buffy coat preparations of blood from healthy donors, supplied by the Transfusion Medicine department of Tübingen University Hospital, or using freshly drawn whole blood from volunteers or participating MM patients after informed consent had been obtained. Briefly, whole blood or buffy coats were heparinized and diluted with 1 to 2 volumes of phosphate buffered saline pH 7.4 (Invitrogen Corporation, Karlsruhe, Germany). PB mononuclear cells (PBMNC) were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and seeded at 1×10^7 cells per well into 6-well plates (Becton Dickinson Falcon, Heidelberg, Germany). After two hours of incubation at 37°C non-adherent cells were removed by washing thoroughly 3 times. The adherent cells, which were almost exclusively CD14 positive cells, were then cultured in the appropriate tissue culture medium supplemented with GM-CSF at 100 ng/mL (Novartis, Nürnberg, Germany) and interleukin-4 (IL-4) at 1000 U/mL (R&D Systems, Wiesbaden, Germany). No antibiotics were added and GM-CSF and IL-4 were fed to the cultures every other day. Monocyte cultures were achieved by culturing adherent cells without the addition of cytokines and harvesting the cells by gentle treatment with a cell scraper. Serum-free media tested were X-VIVO 10, X-VIVO 15, X-VIVO 20 (Bio Whittaker, Walkersville, MD, USA) and AIM-V (Gibco-BRL, Eggenstein, Germany). Purified protein was added to the immature DC on day 5 of the culture. Tetanus toxoid (TT from Behring, Marburg, Germany) was used at 10 μg/mL if not otherwise stated, KLH (Biosyn GmbH, Fellbach, Germany) was

used at 50 μg/mL and individually purified myeloma Id was used at 100 μg/mL. Tumor necrosis factor α (TNFα from R&D Systems) was added at 10 ng/mL to the DC cultures for the last 48 hours to induce maturation of the protein-pulsed DC. Protein-pulsed DC (day 5) were harvested, washed free of protein and used for *in vitro* or *in vivo* purposes on day 7 or 10 as indicated.

In vitro DC characterization

During DC generation the tissue cultures were monitored by light microscopy every other day for morphology. Allostimulatory capacity of serum-free generated DC was compared to that of DC from the same donor generated in RPMI 1640 (Gibco-BRL) with 10% FCS (Invitrogen) using the same protocol as mentioned above. For protein uptake and processing studies immature DC were pulsed on day 5 of culture with titrated amounts of TT and co-incubated with constant numbers of autologous PBMNC responders for proliferation assays. Serum-free generated DC were compared to monocytes for antigen-specific proliferation using titrated amounts of stimulators pulsed with antigen and a constant number of autologous responders. To test the ability of serum-free-generated DC (X-VIVO 15) to induce peptide-specific cytotoxic T-lymphocyte (CTL) responses *in vitro*, we used the HLA-A2 restricted and mucin 1 (MUC1)-derived peptide M1.1 as previously described.[13] Briefly, peptide-pulsed and irradiated DC (0.5×10^6/mL) from a healthy donor were added to autologous PBMNC (2.5×10^6/mL) in 24-well tissue culture plates (Becton Dickinson Falcon) to induce peptide-specific CTL. After three restimulations with peptide-loaded, irradiated PBMNC, specific lysis of peptide-loaded HLA-A2 target cells (Croft) was evaluated. The Croft cell line is an EBV-immortalized B-cell line and was kindly provided by O.J. Finn (Pittsburgh, PA, USA).

Antibodies and flow cytometry

Immunostaining of DC preparations was performed using commercially available murine monoclonal antibodies conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Antibodies against CD86 and CD40 were from Pharmingen (Hamburg, Germany), antibodies against CD3, CD19, CD80, HLA-DR, CD54, CD14 and mouse IgG isotype controls were from Becton Dickinson Falcon, anti-CD83 was from Coulter-Immunotech Diagnostics, Hamburg, Germany and anti-CD1a was from Dako, Hamburg, Germany. All DC preparations were analyzed on a FACSan Calibur (Becton Dickinson, Seattle, WA, USA).

Id purification

MM patient-specific Id proteins were purified from autologous serum samples obtained before or during initial cytoreductive therapy. The procedures of myeloma isotype identification and the subsequent idiotype purification using affinity chromatography, sequential pH step elution and final anion exchange chromatography were exactly as previously described.[6] The purification of IgA isotype was also achieved as previously described. All purified Id proteins were extensively dialyzed against sterile normal saline, filtered through a 0.45-mm membrane and tested for microbiological contamination prior to use.

Id/DC vaccines

Serum-free-generated (X-VIVO 15) monocyte-derived DC preparations were obtained from adherent PBMNC of MM patients with responsive disease 3 to 6 months following HDT and autologous PBSCT. GM-CSF and IL-4 were added every other day. Purified Id protein was added to DC cultures on day 5 at a final concentration of 100 μg/mL. TNFα was added during the last 48 h prior to harvest on day 10 of culture. The autologous Id-pulsed DC were washed free of protein and cytokines and used as vaccines after taking aliquots for sterility testing and FACS analyses. Id-pulsed DC for vaccination were resuspended in 10 mL PBS with 5% autologous plasma before bolus i.v. administration. A second Id/DC vaccine was given i.v. on day 15 after the beginning of the vaccination schedule.

Id/KLH vaccines

Individual Id protein was conjugated to KLH using glutaraldehyde as previously described.[6] After extensive dialysis, 5 or 6 aliquots of Id/KLH vaccines were stored at –20°C prior to use. Id/KLH vaccines were started 4 weeks after the second Id/DC vaccine and given at 4-week intervals. Five Id/KLH booster injections were administered subcutaneously with co-injection of GM-CSF at 250 $\mu g/m^2$ into the same site. Subcutaneous administration of the adjuvant cytokine, GM-CSF, into the vaccination site was repeated for three consecutive days. The dose of GM-CSF was reduced to 100 $\mu g/m^2$ if local swelling and discomfort developed. The adjuvant GM-CSF was stopped if strong local reactions developed already after the administration of Id/KLH and the first dose of GM-CSF.

If patients progressed during the course of vaccination, the protocol was stopped and salvage chemotherapy was promptly initiated.

T-cell assays

Id-specific T-cell responses were monitored by a T-cell proliferation assay. Heparinized blood samples were obtained before each Id vaccination and after the last Id/KLH vaccine. PBMNC were isolated using Ficoll-Paque density centrifugation, washed and plated at 2×10^5 cells per well in 96-

well U-bottom plates in X-VIVO 15 medium supplemented with titrating amounts of autologous Id protein, isotype matched control Ig, KLH or medium alone. Stimulation was done in triplicate or quadruplicate wells for all protein concentrations. Low dose interleukin-2 (IL-2 from R&D Systems) was added to cultures at 20 IU/mL on day 3. On day 5 of culture, 1 μcurie of ^{3}H-thymidine was added to the wells and the plate was incubated for an additional 16 to 18 hours before the cells were harvested onto filters, washed and counted in a scintillation counter. A stimulation index (SI) of 2 or greater was considered significant.

Cytotoxic T-cell assays were performed using PBMNC of vaccinated patients as effector cells (E) in ^{51}Cr-release assays. PBMNC were freshly isolated at different time points after vaccination and stimulated in bulk cultures at 3×10^{6} cells/mL in X-VIVO 15 medium supplemented with autologous Id at 100 μg/mL, respectively isotype matched Ig or KLH as a control. Low dose IL-2 was added at 20 IU/mL on day 3 and effector cells were restimulated with either Id-pulsed or control protein-pulsed autologous, irradiated (30 Gy) DC on day 8. A second restimulation with irradiated Id pulsed autologous PBMNC was performed on day 15 and cytotoxicity assays were performed on day 5 or 6 after the second restimulation. Target cells were autologous mature DC generated as described above and pulsed with either autologous Id protein on day 5 or pulsed with KLH as control protein. DC targets were labeled with ^{51}Cr (Amersham, Braunschweig, Germany), washed and exposed to titrating amounts of effector cells for an incubation period of 4 hours. Spontaneous release and maximal release were determined by adding either medium alone or 1% Triton-X. The specific lysis was calculated as previously described by Hsu and colleagues.[1]

TNFα secretion

T helper 1 type (TH 1) cytokine responses after Id/KLH vaccination were measured after *in vitro* restimulation of bulk PBMNC with titrating amounts of autologous Id, isotype-matched irrelevant Ig or titrating concentrations of KLH. Briefly, 2×10^{5} PBMNC/well were cultured for proliferation assays as described above in triplicate or quadruplicate wells. On day 5, supernatants of triplicate wells with the same antigenic concentrations were harvested and pooled. Supernatants were measured with a TNFα kit for assaying the concentration of secreted TNFα, representative of a TH 1 cytokine, according to the manufacturer's instructions (Pharmingen). TNFα secretion was considered to be Id specific if values of Id-stimulated PBMNC were more than double the values obtained with medium or isotype-matched irrelevant Id control.

Humoral response to KLH

Humoral immune responses to the xenoantigen KLH, which serves as a strongly immunogenic carrier for the individual Id, were measured using a sandwich enzyme linked immunosorbent assay (ELISA). Briefly, 96-well flat-bottom plates (Nunc maxisorb, Wiesbaden, Germany) were coated with KLH at 5 μg/mL in 50 mM carbonate buffer pH 9.4 and left overnight. After washing and blocking, titrated serum samples obtained before, during and after Id/KLH vaccination were incubated on the plate. The standard was a goat anti KLH antibody (kindly provided by R. Levy, Stanford, USA). After extensive washing the plate was incubated with biotinylated KLH (EZ-Link Sulfo-NHS-LC-Biotinylation Kit, Pierce, Sankt Augustin, Germany) which was detected with HRP-Streptavidin. Plates were read at 405 nm.

Results

In vitro characterization of DC

To obtain DC vaccines under serum-free culture conditions using a standardized protocol, we first compared 4 commercially available serum-free media to analyze the yield of DC, the immunophenotypic profile and *in vitro* DC function.

All media tested allowed the generation of predominantly veiled cells of DC morphology. The number of DC (defined by CD1a and/or CD83 expression and lack of CD14 expression) per 1×10^{7} cells plated in a single well of a 6-well plate varied between 1×10^{5} and 4×10^{5} with no significant superiority of a single medium for DC yield from among all media tested (*data not shown*). Cells of typical DC morphology and of mature DC phenotype were generated with all media, as demonstrated by light microscopy and FACS phenotype. Analysis of the *in vitro* allostimulatory capacity of serum-free generated DC, as detected in the mixed lymphocyte reaction (MLR), revealed that X-VIVO 15 and X-VIVO 10 compared favorably to X-VIVO 20 and AIM-V (Figure 1). In repeated experiments X-VIVO 15 was superior to X-VIVO 10 which led to the decision to use X-VIVO 15 (*data not shown*). Immature DC generated in X-VIVO 15 without TNFα maturation were equally effective as immature DC generated in serum containing RP-10 medium at inducing TT-specific T-cell proliferation in an autologous system (Figure 2). DC generated in X-VIVO 15 were superior to monocytes of the same donor in TT protein uptake and specific T-cell induction (*data not shown*). Finally, X-VIVO 15-generated DC could be used to induce specific cytotoxic T-cell responses *in vitro* against a MUC1-derived peptide (Figure 3).

Using the optimized protocol for X-VIVO 15 we found it necessary to prolong the time of culture to 10 days in order to obtain a higher number of DC loosely adhering to plastic after TNFα maturation.

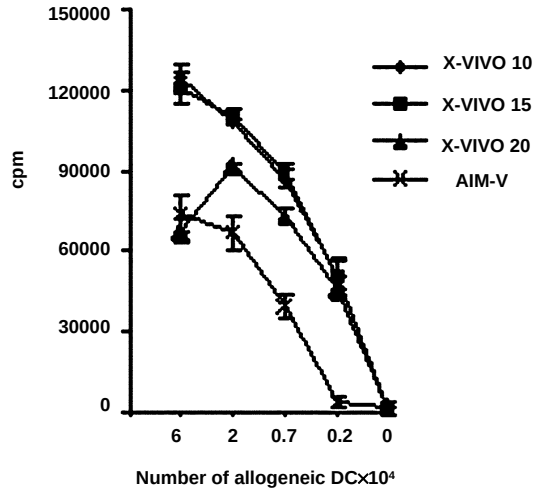

Figure 1. Monocyte-derived dendritic cells generated in serum-free media are potent stimulators of allogeneic lymphocytes. DC were generated in different serum-free tissue culture media (X-VIVO 10, X-VIVO 15, X-VIVO 20 and AIM-V) starting from plastic adherent monocytes of the same donor as described in the methods section. DC were harvested, irradiated with 30 Gy and used as titrated stimulators of allogeneic PBMNC (1×10^5 per well in triplicate). The uptake of ^{3}H thymidine was measured after 6 days of culture. DC generated in X-VIVO 10 and X-VIVO 15 compared favorably to DC grown in X-VIVO 20 and AIM-V.

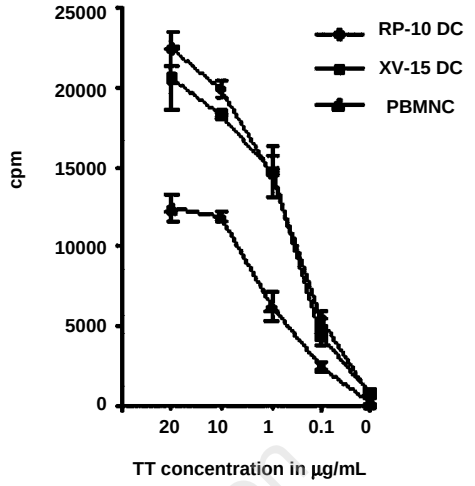

Figure 2. Serum-free-generated DC are equally effective in tetanus toxoid-specific T-cell stimulation as are DC generated in FCS-containing medium. Tetanus toxoid (TT) specific T-cell responses after *in vitro* restimulation were evaluated with *bulk* PBMNC from a healthy individual three weeks after booster immunization with TT in alumn. In a 6-day proliferation assay 1×10^5 PBMNC were stimulated in triplicate either with titrated concentrations of TT protein alone or with the addition of 1×10^4 unpulsed autologous DC. The autologous DC were either generated for 7 days in serum containing medium (RPMI with 10% FCS) or in serum-free medium (X-VIVO 15) without the addition of TNFα. Both DC populations were equivalently effective in augmenting the TT-specific autologous proliferation as measured by ^{3}H thymidine incorporation.

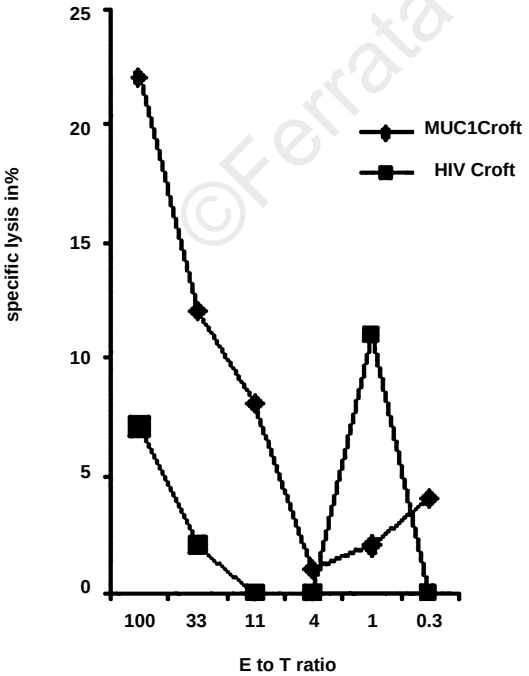

Figure 3. Serum-free-generated DC allow the induction of peptide-specific CTL *in vitro*. DC were generated from the buffy coat of a healthy donor (HLA-A2+) and mature DC (day 10) were pulsed with the MUC1-derived peptide M1.1 as described in the methods section. Autologous PBMNC were induced with peptide-loaded and irradiated DC and were restimulated 3 times with autologous peptide pulsed PBMNC. The induction of *de novo* CTL responses with serum-free-generated DC was evaluated in a ^{51}Cr-release assay with peptide-loaded Croft cells (HLA-A2+) as targets. We found specific lysis of relevantly loaded target cells at higher effector to target ratios. There was only background reactivity to Croft cells loaded with the control peptide of human immunodeficiency virus (HIV).

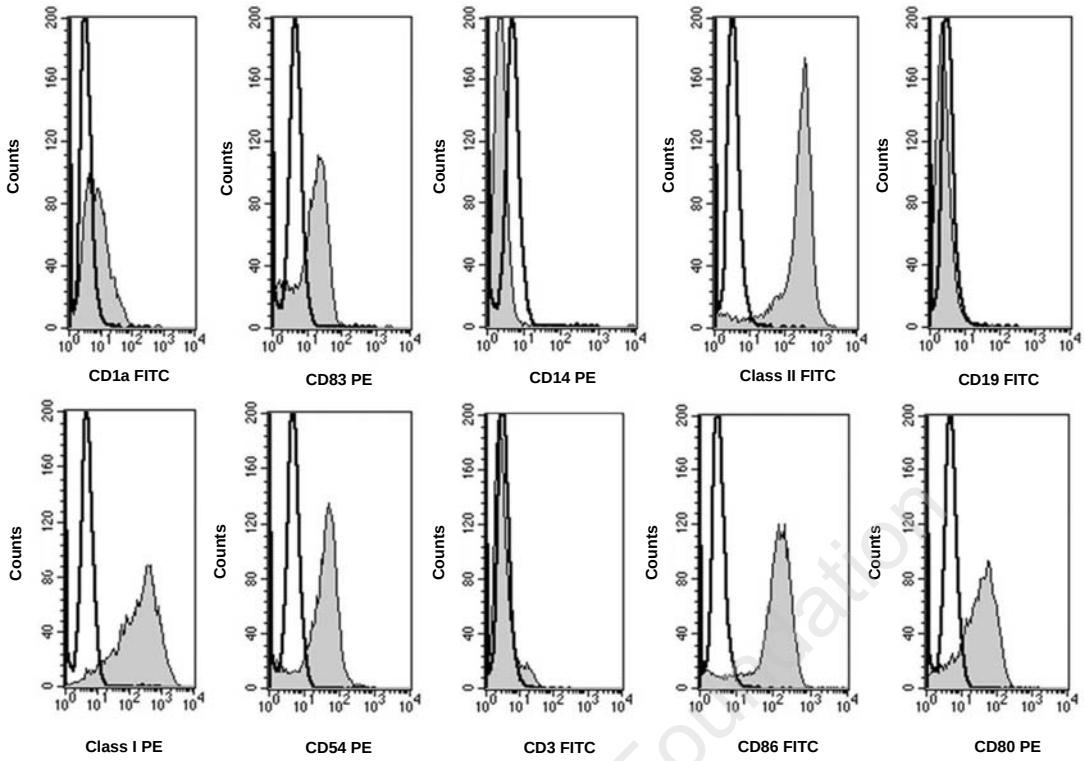

Figure 4. Immunophenotype of serum-free-generated DC of patient MM09. DC were generated in X-VIVO 15 as outlined in the material and methods and pulsed with individual Id on day 5. On day 10 all cells were harvested and predominantly veiled large cells of *typical* DC morphology by light microscopy (*not shown*) were found. An aliquot was analyzed by FACS with double-staining and no gate was set. Bold lines depict the control isotype antibody staining whereas specific antibody staining is shown by the filled curve.

Serum-free-generated DC for the phase I trial

All DC used in the clinical phase I trial were generated starting from freshly drawn whole blood of MM patients with the optimized methodology outlined above. All 24 preparations (2 Id/DC vaccines for each patient) were grown without antibiotics and tested for microbiological contamination prior to i.v. administration. The yield of DC starting from 100 mL of fresh blood varied among patients, mostly due to the absolute monocyte counts 3 to 6 months after HDT and PBSCT. On average, 4.5×10^6 DC were harvested after the 10 days *in vitro* culture. We reliably found veiled DC morphology using serum-free culture conditions without the addition of either autologous or AB serum (*not shown*). Trypan blue staining of harvested DC proved that there was >95% viability of the cells used for the cellular vaccine. FACS analyses revealed a DC phenotype with almost all cells expressing high amounts of class II molecules, high expression of adhesion and co-stimulatory molecules and the lack of expression of monocytic, B- and T-cell markers (Figure 4). As depicted in Figure 5, the majority of DC generated from all trial patients showed expression of mature DC markers as determined by high levels of CD80 and CD86 as well as CD83 expression.

Toxicity

For all patients enrolled we were able to isolate and purify 10 to 20 mg of individual Id protein (11 IgG and 1 IgA) with standard procedures. The quality was assessed by SDS-PAGE and isotype-specific ELISA and revealed a purity of greater than 95%. Id preparations and Id/KLH conjugates were negative for microbiological contamination prior to use as vaccine. One Id/DC vaccine tested positive for *Bacillus sp.* contamination without subsequent signs of infection. All 12 patients received two Id/DC vaccinations starting 3 to 6 months after single or double HDT with PBSCT. No toxicity was seen when autologous Id-pulsed DC were given intravenously as a bolus injection. Ten of 12 patients received all scheduled s.c Id/KLH booster injections with co-injection of GM-CSF as adjuvant. With increasing number of Id/KLH booster injections we found a moderate to advanced local reaction with erythema, local soreness at the s.c. injection site and swelling. All patients developed some degree of

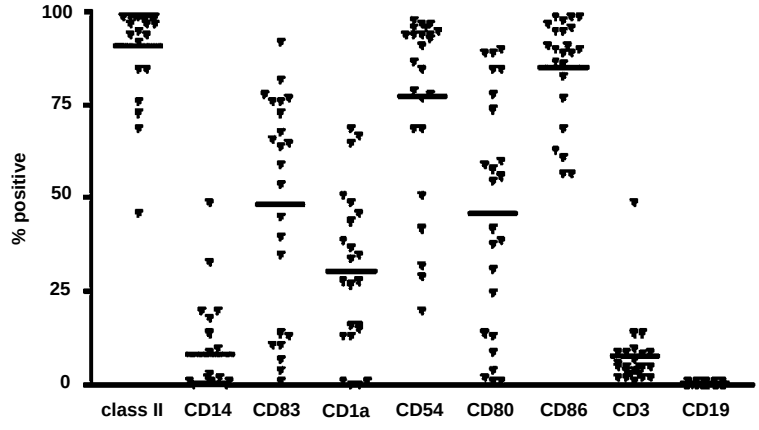

Figure 5. Phenotype of Id-pulsed monocyte-derived DC generated in X-VIVO 15 from 12 MM patients. Three to 6 months following HDT and autologous PBSCT monocyte-derived DC were generated in serum-free X-VIVO 15 medium, pulsed with individually purified Id protein and used as patient-specific Id/DC vaccine. The summarized FACS data of all 24 DC preparations which were used as vaccines are shown. The vast majority of harvested cells were uniformly large and of high granularity as determined by FACS forward and sideward scatter. Gates were set on this population before measuring the depicted phenotypes. The horizontal bar represents the mean of 23 or 24 individual data points.

local reaction, some as early as after the second Id/KLH boost, others not until after the fourth Id/KLH vaccination (two patients). With subsequent booster injections we then reduced the GM-CSF dose to 40% and omitted the day+1 to day+3 GM-CSF injections to avoid local hyperreactivity. All local reactions were easily controlled by oral acetaminophen and resolved within 48 to 72 hours.

Idiotype-specific immune responses

One heavily pretreated patient (MM06) developed a weak (SI 1,7) but specific Id response in a standard T-cell proliferation assay already after the first Id/DC vaccination that disappeared thereafter (Figure 6A). No cytotoxic Id-specific reactivity was found in this patient. Patient MM09 developed a not pre-existing Id-specific T-cell proliferative response after administration of two Id pulsed DC vaccines (Figure 6B). This reactivity was accompanied by Id-specific TNFα secretion which was repeatedly found in patient MM09 (Figures 7A and 7B). After *in vitro* re-stimulation we also found Id-specific cytotoxic T-cell reactivity at a low level in patient MM09 (Figure 8). None of the other patients mounted a measurable Id-specific T-cell response in our assay systems. Since 11 of 12 patients studied had residual circulating Id protein during the whole course of vaccination we did not analyze the induction of anti-idiotypic antibodies.

KLH-specific immune responses

All patients who received at least three Id/KLH booster injections with GM-CSF as adjuvant mounted strong KLH-specific T-cell responses, as found in our earlier trial[6] (Figure 9). These KLH responses were not found prior to vaccination and first became measurable in T-cell proliferation assays after the first Id/KLH vaccine (4 patients), after 2 Id/KLH vaccines (6 patients) and in one

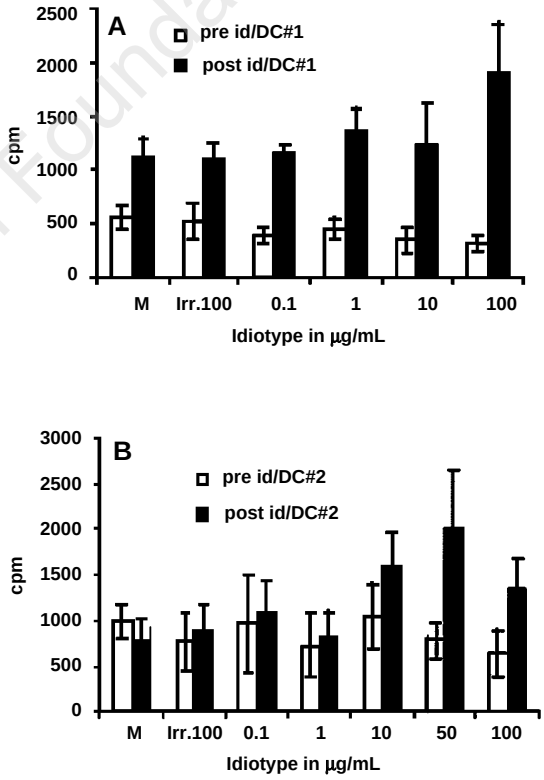

Figure 6. Idiotype-specific T-cell proliferative responses after Id/DC vaccination. Two patients (MM06 and MM09) developed transient Id-specific T-cell proliferative responses after one (6A, MM06) or two (6B, MM09) Id/DC vaccinations. For patient MM06 a maximal stimulation index (SI) of 1.7 and for patient MM09 a SI of 2.6 was calculated. Neither patient showed pre-existing Id-specific proliferation but both were found to develop dose-dependent Id-specific T-cell reactivity after a 6-day restimulation period as described in the material and methods. The background proliferation at 100 µg/mL irrelevant isotype matched Id (Irr. 100) is shown.

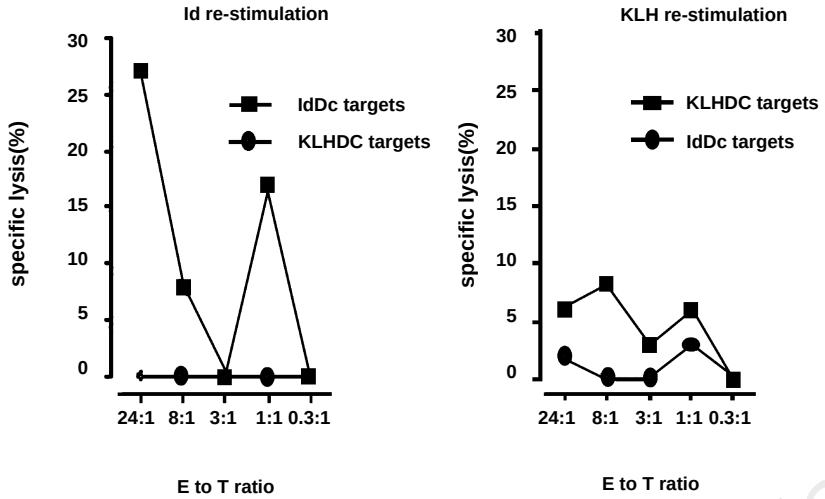

Figure 8. Idiotype-specific CTL response in patient MM09 after two Id/DC vaccines. PBMNC of patient MM09 were collected after two Id/DC vaccinations and re-stimulated in vitro twice (see Design and Methods). Id-specific cytotoxicity was seen at higher effector to target ratios after Id re-stimulation when using autologous Id pulsed DC as target cells in ^{51}Cr- release assays. The control target cells, comprising KLH-pulsed autologous DC, were not killed. *In vitro* re-stimulation with KLH did not yield significant KLH-specific cytotoxic T-cell reactivity.

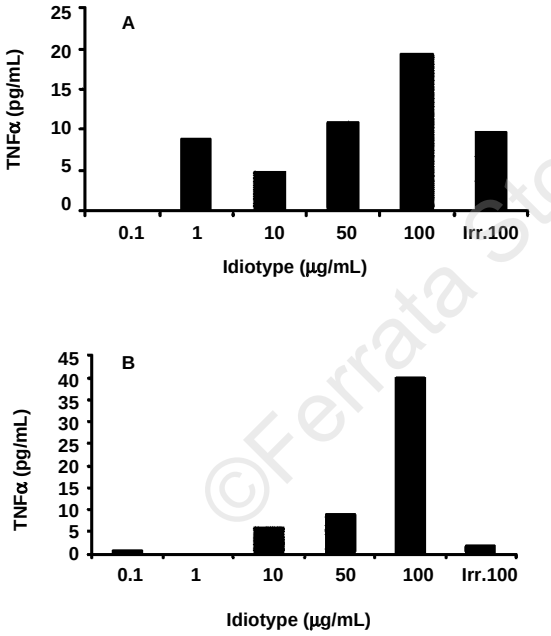

Figure 7. Id-specific TNFα secretion in patient MM09. At different time points after Id/DC and Id/KLH booster immunizations, Id-specific TNFα secretion was measured in the pooled supernatant of re-stimulation PBMNC of patient MM09. 7A: TH1 cytokine secretion after the second Id/DC vaccination; 7B: TNFα secretion after two Id/DC vaccinations and four Id/KLH booster immunizations. The TNFα secretion into medium without Id was 0 pg/mL in 7A and 2 pg/mL in 7B and is subtracted from the appropriate values. TNFα secretion at 100 μg/mL irrelevant idiotype (Irr. 100) stimulation is depicted next to the individual Id at 100 μg/mL concentration.

patient after four Id/KLH booster immunizations (MM06). All T-cell proliferation specific for KLH was accompanied by KLH-specific and concentration-dependent TNFα secretion (*data not shown*). We did not analyze the induction of KLH-specific cytotoxic T cells in our patients.

We found delayed KLH-specific antibody reponses in all of 7 patients studied (*data not shown*). Specifically, KLH antibodies were detected for the first time after two Id/KLH immunizations in 3 patients and in 4 patients after three immunizations.

Clinical course of study patients

Two patients (MM05 and MM08) relapsed during the course of the vaccination and were withdrawn from the experimental protocol in order to receive immediate salvage chemotherapy. Despite aggressive therapy no lasting remission could be induced and both patients succumbed to disease within 3 and 9 months after initiation of salvage therapy. Eight of ten patients who received all scheduled vaccinations progressed. Four of these patients died due to progressive disease or infectious complications, whereas the other four are alive and well with salvage therapy (MM01, MM06, MM07 and MM12). Two patients who were in PR at the start of the vaccine protocol remain in a clinically stable condition without any further treatment after completion of the vaccination series with a follow-up of 29 months (MM09), and 25 months (MM11) after starting the trial (Table 2).

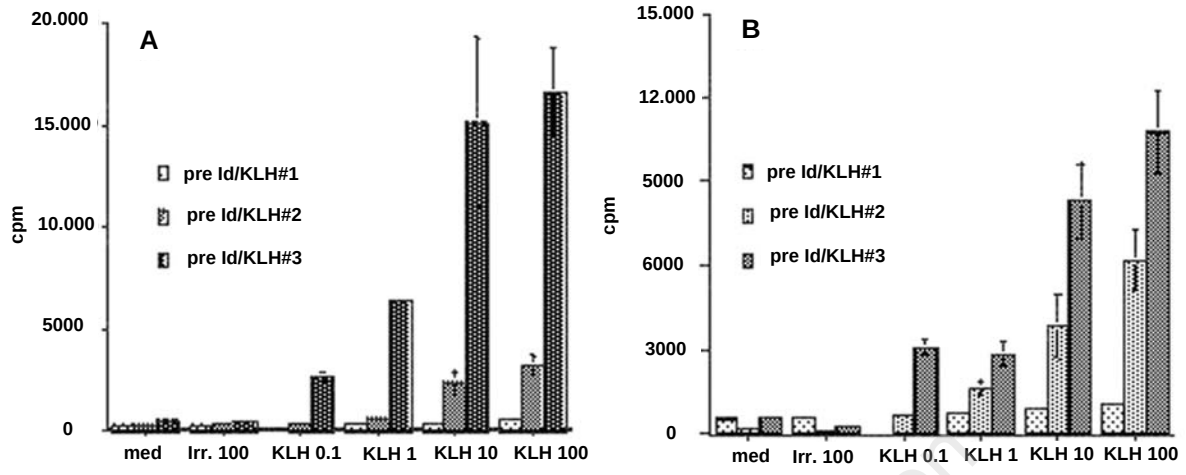

Figure 9. KLH-specific T-cell proliferation in patients MM01 and MM02. KLH-specific immune responses of patients MM01 (9A) and MM02 (9B) are depicted as representative of KLH-specific T-cell responses in all patients. The KLH-specific T-cell responses before, during and after the first two Id/KLH booster vaccinations are shown. PBMNC were collected and stimulated with titrated concentrations of KLH *in vitro*. The incorporation of ^{3}H-thymidine was measured after 6 days of culture. Results for medium (med.) and irrelevant isotype control at 100 μg/mL (irr.100) are also shown.

Table 2. MM Id vaccine. Patients' clinical and immunological outcome.

Patient	*Status pre Vac*	*Status post Vac*	*Time to PD from start of Vac*	*Immune response*		*Status as of 08/02 (f/u)*
				KLH	*Id*	
MM01	PR	PR	20 mo	++	–	a/w (50 mo)
MM02	PR	PR	14 mo	++	–	dec
MM03	PR	PR	11 mo	++	–	dec
MM04	SD	SD	7mo	++	–	dec
MM05	CR	PDa	3 mo	++	–	dec
MM06	PR	PR	6 mo	++	+	a/w (37 mo)
MM07	PR	PR	17 mo	++	–	a/w (32 mo)
MM08	PR	PR[a]	5 mo	++	–	dec
MM09	PR	PR	+29 mo	++	++	a/w (29 mo)
MM10	PR	PD	4 mo	++	–	dec
MM11	PR	PR	+25 mo	++	–	a/w (25mo)
MM12	PR	PR	7 mo	++	–	a/w (24 mo)

[a]patients MM05 and MM08 did not receive all scheduled vaccines due to progressive disease during the vaccination protocol. Vac: vaccination; f/u: follow-up; a/w: alive and well; dec: deceased; mo: months.

Discussion

We report here on the development of a system to generate a reliable source of DC using serum-free culture conditions for the production of a specific vaccine for MM patients after HDT when pulsed with individual Id protein. The DC yield was comparable to that in our previous trial[6] but the purity of the DC preparations was significantly higher. The serum-free monocyte-derived DC were morphologically and functionally mature DC. In line with other reports[14] these DC were potent stimulators of allogeneic MLR and induced primary CTL responses *in vitro*. In contrast to the results of the thorough investigation of Tarte and co-workers, we found a high (>95%) viability of DC under our serum-free culture conditions without the addition of human serum or albumin.[14,15] This might be due to the fresh starting material in our trial as compared to leukapheresis products used by Tarte's group.

It remains unknown whether the DC dose is important for the immunological or even clinical outcome in the human system. The dose we applied was easily achievable with blood samples of 100 mL, whereas higher doses would require leukapheresis for DC generation. A recent paper by Yi and co-workers proposed the use of higher numbers of DC.[16] Due to the limited number of patients in this trial it remains to be seen whether these positive results are based on higher DC numbers or potentially on the route of DC administration, as assumed by the authors. Different investigators have shown

that subcutaneously administered DC of mature phenotype migrate to draining lymph nodes[17] and can induce immunological and clinical responses.[7] Despite the notion of many investigators that intravenously administered DC are trapped in the pulmonary capillaries, there is good immunological and clinical evidence that DC given intravenously are functional.[1,2,6] There is only preliminary evidence from one group of investigators that CTL precursor frequency decreases when changing from s.c to i.v. DC vaccine administration.[4]

We found measurable Id-specific immune responses in two of 12 MM patients, which is in line with our previous results using Id pulsed DC precursors from peripheral blood.[6] It remains unclear whether this low rate of specific immune responses is due to a DC defect in MM, as proposed by various groups,[18,19] or is a reflection of poor immune reconstitution early after HDT. In contrast, we found early, strong and lasting T-cell and antibody responses to the neoantigen KLH in our group of patients, which is in line with the findings of Massaia and colleagues.[20] We vaccinated MM patients who were in clinical PR; single patients were in SD or CR. Based on investigations by Bogen and coworkers in their murine model of MM, it should be emphasized that the lowest tumor mass possible would allow better immunological and potentially better clinical outcome. Therefore, the immunization of patients in clinical CR after HDT and PBSCT seems most promising and will be investigated in our future trials. Whether Id vaccination schedules which target MM patients earlier in the course of their disease (i.e. in stage I) or which offer Id vaccination before and after HDT will translate into higher rates of immune responses or will yield better clinical responses remains to be seen. So far, Id vaccination in MM has not achieved the remarkable immune and subsequent clinical responses already found in NHL.[1,2]

With regard to the tumor antigen, there is a large body of evidence that Id-specific CTL responses can be induced not only *in vitro*[21-23] but also *in vivo*.[1,6] There is growing interest in other tumor-associated antigens, which will make the identification or purification of individual antigens such as Id unnecessary. So far there is no clinical data available on the vaccination of MM patients with MAGE or MUC1 proteins or peptides. RNA array technology will certainly allow better characterization of individual MM clones in order to define risk profiles based on the vast amount of individualized data, but will also allow promising targets of immunotherapy to be identified.[24,25]

The clinical outcome of our study population is limited but probably realistic whereas others have postulated clinical effectiveness of Id vaccination based on preliminary data.[26] Whether the induction of myeloma-specific immune responses in clinical trials will be achieved by techniques such as hybrid cell vaccination, which has yielded promising results in murine models of MM,[27] or by means of RNA transfection, as found by our group in myeloma cell lines,[28] remains to be seen.

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Pre-publication Report & Outcomes of Peer Review

Contributions

VLR and his technicians planned, performed and analyzed the experiments reported. He recruited the participating patients and conducted the clinical trial. He wrote and revised the manuscript. CM performed experiments forming the basis of several data presented in the publication. She critically revised the manuscript and approved the submitted manuscript. WB was significantly involved in the conception of the *in vitro* studies as well as in the induction of the clinical study in myeloma patients. He revised the manuscript critically and approved the submitted manuscript. HE was involved in the conception and design of the clinical trial of Id/DC vaccination of myeloma patients. He critically revised the manuscript's content and approved the version to be published. LK was substantially involved in the conception and design of the clinical trial of Id/DC vaccination of myeloma patients. He critically revised the manuscript's content and approved the version to be published. PB was significantly involved in the conception of the *in vitro* studies and substantially contributed to the development of the methodology outlined in the manuscript. He actively participated in the induction of the clinical study in myeloma patients. He revised the manuscript critically and approved the submitted manuscript.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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In the following paragraphs, Dr. Biragyn summarizes the peer-review process and its outcomes.

What is already known on this topic

Idiotype (Id), immunoglobulin expressed by clonally expanded B cells, is the only widely accepted tumor marker and is a promising therapeutic target for immunotherapy of B-cell malignancies. Although Id vaccination of patients with follicular lymphoma (FL) has been proven to be effective, the vaccine efficacy for multiple myeloma (MM) is, so far, not encouraging.

What this study adds

In this issue of Haematologica, Reichardt *et al.* report data from a clinical phase I trial of twelve MM patients vaccinated shortly after HDT and PBSCT with two doses of Id-pulsed DC followed by 5 doses of a protein-based formulation. Idiotype was used as a tumor-specific antigen and GM-CSF as an adjuvant. They established a simpler approach to produce autologous monocyte-derived DC under serum-free culture conditions, which circumvents repetitive stimulation with xenogenic antigens of fetal calf serum.