mRNA-transfected dendritic cell vaccine in combination with metronomic cyclophosphamide as treatment for patients with advanced malignant melanoma

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\textbf{ABSTRACT}

Introduction: Vaccination with dendritic cells (DCs) has generally not fulfilled its promise in cancer immunotherapy due to ineffective translation of immune responses into clinical responses. A proposed reason for this is intrinsic immune regulatory mechanisms, such as regulatory T cells (Tregs). A metronomic regimen of cyclophosphamide (mCy) has been shown to selectively deplete Tregs. To test this in a clinical setting, we conducted a phase I trial to evaluate the feasibility and safety of vaccination with DCs transfected with mRNA in combination with mCy in patients with metastatic malignant melanoma (MM). In addition, clinical and immunological effect of the treatment was evaluated.

Experimental design: Twenty-two patients were enrolled and treated with six cycles of cyclophosphamide 50 mg orally bi-daily for a week every second week (day 1–7). During the six cycles patients received at least $5 \times 10^6$ autologous DCs administered by intradermal (i.d.) injection in the week without chemotherapy. Patients were evaluated 12 and 27 weeks and every 3rd mo thereafter with CT scans according to RECIST 1.0. Blood samples for immune monitoring were collected at baseline, at the time of 4th and 6th vaccines. Immune monitoring consisted of IFN-γ ELISpot assay, proliferation assay, and flow cytometry for enumeration of immune cell subsets.

Results: Toxicity was manageable. Eighteen patients were evaluable after six cycles. Of these, nine patients had progressive disease as best response and nine patients achieved stable disease. In three patients minor tumor regression was observed. By IFN-γ ELISpot and proliferation assay immune responses were seen in 6/17 and 4/17 patients, respectively; however, no correlation with clinical response was found. The percentage of Tregs was unchanged during treatment.

Conclusion: Treatment with autologous DCs transfected with mRNA in combination with mCy was feasible and safe. Importantly, mCy did not alter the percentage of Tregs in our patient cohort. There was an indication of clinical benefit; however, more knowledge is needed in order for DCs to be exploited as a therapeutic option.

\textbf{Abbreviations:} ALC, absolute leucocyte count; ANC, absolute neutrophil count; BID, bidaily; BTLA, B- and T-lymphocyte attenuator; CBR, clinical benefit rate; CM, central memory; CTCAE, Common Terminology Criteria for Adverse Events; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DC, dendritic cell(s); DTH, delayed-type hypersensitivity; EM, effector memory; EMRA, effector memory RA positive; FACS, flow cytometry; i.d., intraderma; IFN, interferon; LAG-3, lymphocyte-activation gene 3; mCy, metronomic regimen of cyclophosphamide; MDSC, myeloid-derived suppressor cells; MM, metastatic malignant melanoma; ON, overnight; OS, overall survival; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cells; PD, progressive disease; PD-1, programmed cell death-1 protein; PFS, progression-free survival; RECIST, Response Evaluation Criteria In Solid Tumors; SD, stable disease; TIM-3, T-cell immunoglobulin and mucin domain containing 3; Tregs, regulatory T cells

\textbf{Introduction}

During the past decade, huge advances have been achieved in the treatment of metastatic melanoma (MM). Especially, the immune checkpoint inhibitors, such as anti-CTLA-4 and anti-PD-1 antibodies, have altered cancer treatment essentially, as these drugs target the immune system, instead of targeting the tumor. Immune checkpoint inhibitors release the brake on the immune system and re-activate already existing anticancer immune responses. Patients benefitting from checkpoint blockade often have durable responses, and for Ipilimumab, pooled analysis shows a plateau in overall survival (OS) at about 20% with follow-up time for up to 10 years in some patients.\textsuperscript{1} Anti-PD-1 antibodies hold the promise of further improvement with improved response rates compared to Ipilimumab;\textsuperscript{2} however, long-term benefit from these therapies is not yet determined.

Even with these advances, only a fraction of patients respond to immunotherapy and other strategies are needed. It has been shown that patients with tumors infiltrated by...
activated immune cells, the so-called immunogenic tumors, have a greater chance of responding to immunotherapy as compared to patients with non-immunogenic tumors.\(^3\)\(^4\) One proposed way of altering the immune balance is by inducing increased tumor-reactive T-cell frequencies by therapeutic vaccines as reviewed by Spranger and Gajewski.\(^5\) A much tried vaccine approach is loading autologous dendritic cells (DCs) with antigens in vitro and injecting the DCs back into the patients taking advantage of their capacity to initiate immune responses. In previous studies, we have loaded DCs with antigens through peptide pulsing; however, this approach is limited by the HLA-restricted nature of the peptides, which necessitate patient selection based on HLA expression. To overcome this limitation, we tested electroporation of DCs with tumor-associated antigen-encoding mRNA. Indeed, we found that we were able to electroporate DCs with a high transfection function. As reviewed by Galuzzi et al.,\(^9\) chemotherapy is known to affect the immune system either by direct effects on different immune cell subsets or by the modulation of the tumor microenvironment. In line with this, Ghiringhelli et al. published that a metronomic (low-dose) regimen of the DNA alkylating agent cyclophosphamide (mCy) selectively decreased the number of Tregs and impair their function.\(^10\) An enticing notion as a low-dose regimen of chemotherapy expectantly would induce less side effects than standard doses.

In general, clinical studies evaluating the effect of DC vaccines have been disappointing with only few objective clinical responses and no clear correlation between clinical and immunological response. A possible explanation for this discrepancy is that intrinsic mechanisms in the immune system inhibit the vaccine-induced immune responses. Regulatory T cells (Tregs) have been thought to play a central role in inhibiting immune responses and consequently a lot of effort has been put into finding ways to regulate their number and function. As reviewed by Galuzzi et al.,\(^9\) chemotherapy is known to affect the immune system either by direct effects on different immune cell subsets or by the modulation of the tumor microenvironment. In line with this, Ghiringhelli et al. published that a metronomic (low-dose) regimen of the DNA alkylating agent cyclophosphamide (mCy) selectively decreased the number of Tregs and impair their function.\(^10\) An enticing notion as a low-dose regimen of chemotherapy expectantly would induce less side effects than standard doses.

Thus, this phase I clinical trial was performed to evaluate the feasibility and safety of therapeutic vaccination with DCs transfected with p53, survivin, and hTERT encoding mRNA in combination with mCy in patients with progressive MM. In addition, clinical and immunological effect of the treatment was evaluated.

**Results**

**Patient characteristics**

In total, 26 patients were enrolled in the trial between November 2009 and May 2012. All patients had progressive disease (PD) upon study entry. Four patients were excluded before first vaccination due to an allergic reaction to leukapheresis, brain metastases, atrial fibrillation, and patients were own wish, see patient flowchart, Fig. S1. Four patients were excluded before first evaluation due to rapidly progressing disease, however, are included in the description of PFS and OS. Eighteen patients were available for formal evaluation after receiving six cycles of mCy and six vaccines. Nine patients achieved stable disease as best overall response and nine patients had PD at first evaluation, which translates to a clinical benefit rate (CBR) of 41% (9/22). Four patients had prolonged SD (> 6 mo) and three of these achieved a minor response, however, not enough to fulfill the criteria for a partial response.

Patient characteristics are shown in Table 1. Patient’s mean age was 68 (range 41–77) with slightly more females (12/22). The majority of patients were PS 0 (15/22), seven had elevated LDH (>205 U/L) and six patients had stage M1a disease, three had M1b, and thirteen had M1c. All but two patients had received prior treatment for their disease; however, one patient had only had regional treatment.

Median PFS for the 22 patients was 3.1 mo (95% CI 2.8–3.4) and median OS was 10.4 mo (95% CI 1.5–19.3), see Fig. 1A and B. If patients were grouped according to response, patients achieving SD had a significantly better median OS compared to the PD group (24 mo vs. 6.4 mo, respectively, \(p = 0.04\)), see Fig S2. Patients in the SD and PD group were similar when comparing the prognostic parameters LDH, age, PS, and tumor burden (\(p = 0.52\), \(p = 0.56\), \(p = 0.58\), and \(p = 0.73\), respectively).

DTH response was evaluated at baseline in 11 patients, see Table S1. At first evaluation (6th vaccine), DTH responses from seven patients were evaluated, as four patients were excluded due to fast PD. Of these, a positive DTH response, as defined by a red induration > 2 mm 48 h after i.d. injection, was observed in five patients. In four patients, the responses were only present after treatment initiation. However, all of the responses were positive for both DCs with or without antigen and are thus not antigen-specific. Three of the four patients with induced DTH responses achieved stable disease.

**Toxicity**

Generally, the treatment was well tolerated. Three CTC grade III events were reported. Following leukapheresis, one patient had a lung embolus, which was associated with the catheterization. Anticoagulant treatment was applied and the patient recovered completely. Another patient had a lung embolus and a grade III pleural effusion due to his malignant disease. Apart from these events, only CTC grade I/II events were reported, see Table S2 for most frequent and important events. Also, one patient developed an allergic reaction during leukapheresis and was consequently excluded.

**Vaccine characterization**

DCs from 17 patients were available for phenotypic surface marker analysis. The markers were found to be expressed in a pattern suggestive of a mature DC phenotype,\(^11\) i.e., high expression of costimulatory molecules CD80, CD86, CD40, and maturation markers CD83 and HLA-DR, however, with a lower expression of the homing receptor CCR7 than we have seen in previous trials,\(^6,12\) see Table S3. There was no evident difference between DC characteristics in the PD and SD patient group when comparing percentages of marker expressing DCs, see Fig. S3, or MFI of markers (data not shown).

**Indirect IFN\(\gamma\) ELISPOT**

To evaluate if the DCs transfected with p53, survivin, and hTERT encoding mRNA were capable of stimulating the
secretion of the proinflammatory cytokine IFNγ, we performed indirect ELISpots in 17 patients with paired samples at baseline, 4th and 6th vaccines. In total, a significant difference in IFNγ secretion from PBLs stimulated with mRNA-transfected DCs compared to mock-electroporated DCs was seen in six out of 17 patients. In Fig. 2A, response patterns are depicted as number of spots pr. 1 × 10⁵ PBLs. Responses were tested statistically using actual spot counts. In one patient (patient 4), the spots developed rapidly in test wells and were too numerous to count and was arbitrarily set to 500 for wells at baseline and time of 4th vaccine. Response pattern were unique in each patient; however, in three patients, a baseline response was observed (pts. 4, 5, and 13). In one, response subsequently disappeared (pt. 13). In another (pt. 5), response was still present at the time of 4th vaccine, however, no longer significant due to high background. In the last (pt. 4), response was sustained.

Table 1. Patient characteristics.

| Patient no. | Age | Sex | PS | No. of sites | Baseline LDH > ULN | Stage | Prior therapy | Vacc (no.) | Best response | Best change in tumor size (%) | PFS (mo) | OS (mo) | Subsequent therapy |
|-------------|-----|-----|----|-------------|-------------------|-------|---------------|------------|--------------|--------------------------|----------|---------|---------------------|
| 1           | 68  | M   | 1  | 3           | No M1c            | IL-2  | 5 PD (f)      | NA         | 2.1          | 2.8                       | None     |
| 3           | 73  | F   | 0  | 2           | ND F            | M1b   | 6 PD          | 23         | 2.9          | 38.9                     | Ipi, Ipi-r, V, TIL, T+1f |
| 4           | 50  | F   | 0  | 1           | No M1a            | IL-2  | 6 SD          | −21        | 7.0          | 38.4                     | Ipi, Ipi, V, TIL, T+1f |
| 5           | 50  | M   | 0  | 4           | Yes M1c          | IL-2  | 6 PD          | 21         | 3.1          | 10.4                     | Ipi       |
| 6           | 73  | M   | 0  | 4           | No M1c            | Vaccine | 6 PD          | 30         | 2.6          | 5.6                       | None     |
| 7           | 72  | M   | 0  | 3           | No M1c            | RT, IL-2 | 6 PD          | 25         | 3.0          | 6.4                      | Ipi       |
| 8           | 50  | F   | 0  | 2           | No M1b            | IFNγ, HRP | 16 SD         | −25        | 12.4         | 30.4                     | HR, surgery |
| 9           | 71  | M   | 0  | 1           | No M1a            | RT, IL-2 | 10 SD         | 16         | 5.6          | 23.9                     | Ipi, T    |
| 10          | 66  | F   | 0  | 1           | Yes M1c           | IL-2  | 8 SD          | 0          | 4.6          | 5.9                      | None     |
| 11          | 51  | M   | 0  | 2           | No M1c            | IL-2  | 6 PD          | 24         | 2.6          | 5.9                      | None     |
| 12          | 77  | F   | 1  | 2           | No M1c            | T     | 6 PD          | 11         | 3.3          | 12.5                     | None     |
| 13          | 71  | M   | 1  | 1           | No M1a            | None   | 10 SD         | −2         | 6.1          | 19.0                     | T         |
| 14          | 63  | F   | 1  | 3           | Yes M1c           | IFN, IL-2, Ipi | 3 PD (f)  | NA         | 1.1          | 1.4                      | None     |
| 15          | 41  | M   | 0  | 1           | No M1a            | IL-2, Ipi | 9 SD         | 0          | 5.4          | 43.8                     | TIL       |
| 16          | 72  | F   | 0  | 1           | No M1c            | None   | 10 SD         | 18         | 5.8          | 24.0                     | T         |
| 17          | 43  | F   | 0  | 2           | No M1b            | IL-2  | 6 PD          | 12         | 3.0          | 8.4                      | Ipi, T    |
| 18          | 72  | F   | 0  | 1           | No M1a            | HRP   | 6 PD          | 76         | 2.8          | 9.0                      | T         |
| 19          | 68  | F   | 1  | 3           | No M1c            | IL-2, RT, Ipi | 6 PD          | 38         | 3.1          | 36.8                     | V, T      |
| 20          | 63  | F   | 0  | 1           | Yes M1c           | IL-2, Ipi | 2 PD (f)  | NA         | 1.3          | 3.1                      | None     |
| 21          | 73  | F   | 0  | 2           | Yes M1c           | Ipi, HRP | 9 SD         | 0          | 5.7          | 16.1                     | T         |
| 22          | 77  | M   | 1  | 2           | Yes M1c           | Ipi, T, HRP | 3 PD (f)  | NA         | 1.4          | 2.3                      | None     |
| 23          | 66  | M   | 0  | 1           | Yes M1c           | IL-2, Ipi | 10 SD         | 0          | 7.0          | 26.7                     | V, T      |

n = 22

Median 68 3.1 10.4

*aLDH upper limit of normal 205 U/L.
*bBest response according to RECIST.
*cInterleukin-2.
*dFast progressive disease (PD).
*eNot applicable.
*fNot done.
*gIpilimumab.
*hIpilimumab re-induction.
*iTemozolomide.
+jHyperthermic regional perfusion.
+kYemurafenib.
*lAdaptive T cell transfer using tumor infiltrating lymphocytes (TIL).
+mTemozolomide + IDO vaccine.
+nGanglioside vaccine.
+oLocal radiotherapy.
+pAdjuvant interferon-α.

Figure 1. Clinical outcome. Kaplan–Meier plot showing progression-free survival (PFS) for all treated patients (A). Overall survival is shown in (B). mo, months.
during treatment. Interestingly, this patient had tumor shrinkage on scans and prolonged SD > 6 mo. In the three other patients (pts. 3, 6, and 9), a response developed after treatment initiation, indicating a treatment-induced effect. However, for two of these patients, the response was transient and disappeared at the next time point (pts. 6 and 9).

Pooled analysis of ELISpot data show no general change in IFN-g secretion during treatment ($p = 0.81$), see Fig. 2B, no difference between DC vacc or DC mock and no difference between PD and SD patients (data not shown). Clinical and immunological responses were not correlated (data not shown).

**Proliferation assay**

In addition to investigating IFN-g secretion, we also performed proliferation assays to evaluate if mRNA-transfected DCs were able to stimulate T-cell proliferation in both the CD4+ and CD8+ T-cell subsets. Paired samples from baseline, 4th and 6th vaccines from 17 patients were analyzed.

In total, proliferation responses against mRNA-transfected DCs were seen in 4/17 patients, see Fig. 3A (see Materials and Methods section for response definition). In patients 3 and 6, responses were seen in both CD4+ and CD8+ T-cell subsets, whereas responses in patients 4 and 7 were found only in the CD8+ compartment. In patient 3, 4, and 6, responses were only seen at the time of 4th or 6th vaccine, indicating responses could be treatment-induced. In patient 7, a response was seen at baseline, indicating either treatment-induced tolerance or response disappeared due to the progression of disease. Proliferation responses were not associated with clinical response to treatment.

Pooled analysis of CD4+ T cells showed an increase in proliferation from baseline to the time of 4th and 6th vaccines ($p < 0.01$, adjusted $p = 0.18$), see Fig. 3B. However, there is no apparent difference in proliferation between PBLs stimulated with mRNA-transfected DCs or mock-electroporated DCs. A similar analysis of CD8+ T cells shows no change in proliferation during treatment ($p = 0.47$), see Fig. S4.

Patients 3, 4, and 6 had responses in both ELISpot and proliferation assays. Only patient 4 achieved SD.

**White blood cell counts, Tregs, and MDSC**

Baseline absolute leucocyte (ALC) and neutrophil counts (ANC) were registered on study entry and data are available for 22 patients. At baseline, there was no difference between leucocyte and neutrophil counts in the SD group as compared to the PD group (data not shown). Baseline data for absolute lymphocyte count were available for 17 patients and also showed no difference between the two groups (data not shown). Full data sets of values from baseline, 4th and 6th
vaccines were available for 15 patients for ALC and ANC and 14 patients for absolute lymphocyte count. Both ALC and ANC were unchanged during treatment (data not shown), whereas a significant decrease was seen for absolute lymphocyte count (adjusted $p = 0.03$), see Fig. S5.

mCy have been reported to selectively deplete Tregs; thus, we performed FACS analysis on both whole blood and cryopreserved PBMCs in order to evaluate the effect of mCy on our patient cohort. In paired samples from baseline, 4th and 6th vaccines from eight patients, we enumerated absolute Treg levels in whole blood and found a borderline significant decline ($p < 0.09$, adjusted $p = 1.95$), see Fig. 4A. However, the absolute number of CD4$^+$ T cells also declined ($p < 0.01$, adjusted $p = 0.21$), thus leaving the percentage of Tregs of the CD4$^+$ T cells unchanged ($p = 0.88$), see Fig. 4B and C, respectively. The absolute number of CD8$^+$ T cells was also reduced slightly, see Fig. S6.

In cryopreserved and thawed PBMCs, we analyzed paired samples from 17 patients and found no difference in the percentage of Tregs as a percentage of CD4$^+$ T cells through the treatment ($p = 0.59$), see Fig. 5A. In five patients, we had paired samples also including 10th vaccine and even after discontinuation of mCy, there was no increase in Treg levels (data not shown). We also looked at difference in Treg levels in patients with PD or SD at baseline and during treatment, but found no difference between the two groups (data not shown).

MDSCs are another subset of inhibitory immune cells proposed to be affected by cyclophosphamide. Since we analyzed cryopreserved samples, we only looked at monocytic MDSCs. Paired samples from 16 patients were analyzed. The percentage of MDSCs of live PBMCs did not change throughout treatment ($p = 0.83$), see Fig. 5B. However, patients obtaining SD had significantly lower levels of MDSCs than patients with PD already at baseline ($p = 0.05$), see Fig. 5C, and this difference was sustained during treatment (data not shown). MDSC levels were not associated with previous treatment or the number of previous treatments; however, the number of patients was not large enough to formally test this (data not shown).

**T-cell phenotyping**

In the pursuit of gaining more knowledge on the immunological impact of the treatment, we investigated T-cell phenotype at baseline and during treatment. We performed these analyses on 16 paired samples at three time points. Overall, there was a statistically significant decrease in the median percentage of naïve CD4$^+$ T cells from baseline, to 4th and 6th vaccines (46.78%, 42.62% and 40.21% respectively, adjusted $p < 0.01$) with a corresponding increase in EM CD4$^+$ T cells (27.86%, 33.03% and 33.84% respectively, $p = 0.02$, adjusted $p = 0.46$), see Fig. 6A, whereas CD4$^+$ CM and EMRA T-cell percentages were unchanged. Also, a significant difference was observed between the median percentage of naïve CD4$^+$ T cells in the SD versus the PD group at baseline ($p = 0.03$), 54.54% vs. 35.86%, respectively), see Fig. 7A. Again, the main corresponding difference was in the EM subset. Thus, there was a distinct difference in the changes of CD4$^+$ T-cell subsets between the two groups, most clearly envisioned by the stable percentage of EM CD4$^+$ T cells in the PD group during treatment, whereas there was an increase in the SD group, see Fig. 7C.

In the CD8$^+$ subset, the percentages of different phenotypes were almost unchanged, see Fig. 6B; however, as seen in the CD4$^+$ subset, there was significantly higher median percentage

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**Figure 4.** Absolute number of regulatory T cells (Tregs) and CD4$^+$ T cells and percentage of Tregs of the CD4$^+$ T cells in whole blood in paired samples from eight patients. Each symbol represents a value for one patient. Horizontal lines represent median values.

**Figure 5.** Levels of regulatory T cells (Tregs) (A) and myeloid-derived suppressor cells (MDSCs) (B) in peripheral blood from cryopreserved samples. Paired samples from 17 and 16 patients were available for Treg and MDSC analysis, respectively. In (C) baseline MDSC levels for patient in the progressive disease or stable disease groups are shown. Each symbol represents a value for one patient. Horizontal lines represent median values.
of naive CD8⁺ T cells in the SD group versus the PD group at baseline (p = 0.03, 28.52% vs. 7.31%, respectively), see Fig. 7B. In the CD8⁺ subset, this difference was balanced out by a lower level of EMRA cells in the SD group compared to the PD group (data not shown).

The differences in the percentage of naive T cells of both subsets were not attributable to differences in age, previous treatment, and the number of previous systemic treatments (data not shown). The CD4⁺/CD8⁺ T-cell ratio remained unchanged throughout treatment (data not shown).

Activation and exhaustion markers on T cells
A possible explanation for the failure of vaccine trials is the intrinsic inhibitory mechanisms of the immune system. Already inhibitory cell types have been mentioned, but immune regulatory molecules such as CTLA-4, PD-1, LAG-3, TIM-3, and BTLA might also play a role. Analogous to the phenotype and MDSC enumeration, we performed these analyses on paired samples including three time points for 16 patients.

In the CD4⁺ T-cell subset, there was a statistically significant increase in the percentage of PD-1⁺ cells (adjusted p = 0.02), TIM-3⁺ cells (p = 0.05), and BTLA⁺ cells (p = 0.03) during treatment; however, the latter two were non-significant after adjustment (adjusted p = 0.99 and p = 0.59, respectively), see Fig. 8A–C. For PD-1 and TIM-3, the increase was seen primarily in PD patients, whereas for BTLA the increase was primarily in the SD group, see Fig. 8D–F. For LAG-3 and CTLA-4, there were no changes during treatment (data not shown).

In the CD8⁺ T-cell subset, only TIM-3 exhibited a borderline significant increase (p < 0.01, adjusted p = 0.13), which was attributable to increases in both SD and PD patients. LAG-3, PD-1, BTLA, and CTLA-4 expression in the CD8⁺ T-cell subset did not change during treatment (data not shown).

Discussion
In this single-center phase I trial, the primary endpoint was to test safety and feasibility of the treatment with autologous mRNA-transfected DCs and concomitant mCy. The treatment was well tolerated and only mild and manageable side effects were observed. This is in line with what we have seen in previous vaccine studies treating patients with autologous DCs.⁶,¹²-¹⁴

At the time of inclusion all patients had PD as verified by CT imaging. The CBR was 41%, which is lower than seen in our previous trial using autologous DCs and mCy in combination with IL-2 and Celecoxib.⁶ Median OS was 10.4 mo, with a significantly longer survival of patients achieving disease stabilization; however, survival data could be affected by the fact that a large proportion of patients had either ipilimumab or vemurafenib as later therapies. Two of the patients received adoptive T-cell transfer with TILs in another clinical trial,¹⁵ whereof one (patient 20) achieved complete response and is to date still without evidence of disease. In this patient, TILs showed high reactivity against DCs from the vaccine used in the trial, indicating that vaccine-induced T cells were able to home to the tumor site, however, were unable to induce a clinical response.

We performed immune monitoring using indirect IFNγ ELISPot and proliferation assay and detected responses in 6/17 and 4/17, respectively. Some of the responses were only present after initiation of treatment indicating a treatment induced effect; however, this could also be due to stochastic variation. Spontaneous responses not attributable to the treatment were
also detected in some patients. Findings were not consistent
between the two assays and response in neither of them could
be correlated with clinical benefit. The lack of correlation
between immune response and clinical response has also been
described by other groups. A possible explanation is that
immune monitoring was performed on immune cells from
peripheral blood due to the accessibility; however, conditions
of the tumor microenvironment are not necessarily re
lected in
peripheral blood and inducing an immune response in itself
does not automatically translate into an effective antitumor
response, if tumors do not express the antigens in the vaccine. One way of addressing this issue is by inducing a peripheral
tissue immune reaction by i.d. injection of antigen, collect
DTH biopsies and analyze antigen-speci
cific skin in
filtrating
lymphocytes as done by others.
Frequency of in vitro detectable immune responses was
lower than expected from other DC vaccination trials; however, several factors could be attributable to this. First off,
due to using full length mRNA-transfected DCs, the sensitivity
of our functional assays was probably not as good as it would
have been using peptides alone, as DCs in themselves are stim-
ulatory and thus produce high levels of background. On the
other hand, using mRNA encoding full-length protein increases
the chance of detecting a response due to the increase in the
number of potential immunogenic epitopes. Higher reactivity
against mRNA-transfected DCs compared to peptide-pulsed
DCs has been reported previously. Secondly, patients were
treated with chemotherapy and immune cells were in poor
shape when looking in the microscope (personal observation).
Third, it is possible that adding three mRNA encoding full-
length proteins results in competition between immunogenic
epitopes and hampers induction of robust T-cell responses.
Others have shown that transfection of DCs with three different
mRNAs simultaneously does not lead to inferior antigen
expression or CTL reactivity compared to single mRNA
transfection; however, transfection with six mRNAs reduced
CTL reactivity drastically. Fourth, we selected our TAAs on
the basis of reported frequent spontaneous or induced CTL
responses in peripheral blood in cancer patients; however,
it is possible that responses against the vaccine antigens are
rare in our patient cohort.
Due to the proposed effect of mCy to selectively deplete
Tregs, we treated patients with mCy 50 mg BID biweekly. We
did find slightly lower absolute numbers of Tregs; however, this
was accompanied with a decrease in absolute lymphocyte count,
absolute CD4+ and absolute CD8+ numbers, and so in contrast
to Ghiringhelli et al., we neither found a selective decrease in
Tregs in whole blood nor in cryopreserved samples, and the
percentage of Treg of CD4+ T cells remained unchanged during
treatment in our patient cohort. This is in line with previous
findings at our center and a randomized phase II trial treating
prostate cancer patients when looking at mean frequencies of
Tregs. In a mouse model, mCy preferentially depleted CD8−
cells (including Tregs), and had synergistic antitumor effects
in combination with a vaccine. However, timing of mCy
treatment was found to be of great importance, as mCy admin-
istration at the same time as or within a week of immunization
was detrimental to proliferation of cytotoxic T lymphocytes
(CTL). mCy administration 3 mo after immunization did not
affect CTLs and data suggested that cells with restimulatory
capacity (memory cells) were spared, whereas proliferating
CTLs (effector cells) were affected. This finding makes sense
due to Cy being a DNA alkylating agent and is supported by
findings in another mouse model, where it was demonstrated
that Tregs were affected due to higher cell turnover compared
to non-regulatory T cells. This mechanism could explain the
relative decrease in naïve T cells and corresponding relative
increase in CD4+ EM subset seen in our study. It could also

Figure 8. Expression of PD-1 (A), TIM-3 (B), and BTLA (C) on CD4+ T cells. Paired samples from 16 patients were available for activation marker analysis. In (D–E–F),
expression of PD-1, TIM-3, and BTLA, respectively, is divided between patients achieving disease stabilization (SD; n = 9) or not (PD; n = 7). Each symbol represents a
value for one patient. Horizontal lines represent median values.
be speculated that naïve cells differentiated upon antigen recognition when interacting with the mRNA-transfected DCs. In the studies by Lutsiak et al. and Ghiringelli et al., inhibition of Treg functionality was also demonstrated. In the present study, we did not find a selective decrease in Tregs, but we did observe an antigen-independent tendency toward an increased proliferative potential in the CD4+ T-cell compartment in the proliferation assay, indicating attenuated intrinsic immune inhibitory mechanisms. Expression of PD-1 on CD4+ T cells was increased significantly during treatment and could indicate increased immune activation. Others have shown that increased PD-1 expression in malignant disease is a result of skewing of the T-cell phenotype, as EM T cells have higher PD-1 expression (and other immune regulatory receptors) compared to naïve T cells. Overall this corresponds with our findings, however, does not explain the observation that PD-1 expression primarily was increased in patients not achieving stable disease, whereas the increase in the CD4+ EM subset was more pronounced in the patients achieving stable disease.

In both animal models and in the randomized phase II study treating prostate cancer patients, MDSCs were demonstrated to be increased by mCy. In contrast, MDSC levels in our study were not changed by treatment. In a previous DC and mCy trial, we found an initial decrease in MDSC levels followed by an increase returning to baseline levels. In both trials, MDSC levels were found to be significantly lower in patients with clinical benefit.

In the field of DC research, there has been some debate as to which kind of maturation would provide the most potent DCs. In this study, we used the standard maturation cocktail, which is known to yield DCs lacking production of IL-12 important for T cell priming, whereas others propose to use IL-12 producing DCs which in turn have reduced migratory capacity.

The migratory capacity of DCs is important when injected i.d., however, could be circumvented using intranodal (i.n.) or intratumoral injection; however, i.d. has been shown to be superior to i.n. in terms of immunogenicity and clinical feasibility. Furthermore, in addition to antigen loading mRNA, electroporation can be used to induce expression of immune stimulatory molecules such as CD70, CD40L and toll-like receptor 4 or others, to optimize DC function in vivo.

Even though DC vaccination has generally not fulfilled its promise, there are indications of clinical benefit and induced immune responses. In a parallel study soon to be published (Ahmad et al., submitted), we show that the addition of PD-L1 long peptides to PBMC and transfected DC co-cultures significantly increased reactivity against DCs after two re-stimulations, indicating that self-reactive T cells against PD-L1 epitopes could be activated. It has already been shown that self-reactive T cells specific for PD-L1 are present in the circulation of cancer patients. Thus, it can be hypothesized that vaccination with PD-L1 peptides could boost such a response, lead to killing of PD-L1 expressing cells in the tumor microenvironment and by inhibiting the immune suppression, allow an efficient anticancer response.47,48

Also, with the emergence of checkpoint inhibitors, combination with DC vaccines is an obvious approach to fuel the immune cascade leading to tumor control. Indeed, Ribas et al. reported that the combination of a CTLA-4 inhibitor (tremelimumab) and DC vaccine induced objective responses at higher ranges than expected with either agent alone. Even more promising is the combination of Iplimumab and TriMix-DC, which produced objective responses in 38% of treated patients with both complete and durable responses.

In conclusion, treatment with autologous DCs transfected with p53, survivin, and hTERT encoding mRNA and concomitant mCy regimen was feasible and well tolerated. Sign of clinical benefit was seen, as 9 of 22 (41%) treated patients achieved SD and 3 of 18 evaluable patients experienced tumor shrinkage. In our patient cohort, mCy did not selectively decrease Treg levels. Even with this indication of clinical benefit in some patients, more knowledge on the complex immune interplay is needed in order for DC vaccinations to enter clinical daily life.

Materials and methods

Study design

The study was designed as an open-labeled, non-randomized phase I trial. The primary aim of the study was to evaluate feasibility and safety of the treatment. The secondary aim was to show whether vaccination could induce a measurable immune response and to evaluate clinical effect (objective response rate/CR, progression-free survival (PFS), and OS).

The protocol was approved by the Scientific Ethics Committee for The Capital Region of Denmark (H-A-2009-013), the Danish Medicines Agency (2612–4030), and the Danish Data Protection Agency and conducted in accordance with the provisions of the Declaration of Helsinki. Written informed consent from the patients was obtained before study entry. ClinicalTrials.gov identifier: NCT00978913.

Patients, treatment and evaluation

Patients with histologically confirmed MM in progression, performance status ≤ 1 (ECOG scale), age > 18 years, life expectancy of > 3 mo, at least one measurable lesion according to response evaluation criteria in solid tumors (RECIST) version 1.0, and absence of brain metastasis were eligible if there were no other treatment options, the patient had no history of other malignancies within 5 years, the patient had no received chemotherapy within 4 weeks, and were not treated with immunosuppressive drugs.

Patients were treated with six cycles of cyclophosphamide 50 mg orally BID for a week every 2nd week (day 1–7). During the six cycles patients received intradermal (i.d.) injection with at least 5 × 10^6 autologous DCs administered on the proximal thigh during the week without chemotherapy (day 9). If patients continued on treatment after first evaluation, they received DC vaccination alone every 4 weeks, see treatment overview, Fig. S6.

Evaluation with CT scan was performed before treatment (baseline), after 6 (12 weeks), and 10 (27 weeks) vaccines and every 3 months thereafter until progression. Radiologic evaluation was performed according to RECIST version 1.0. Adverse events were graded according to Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

A skin test for delayed type hypersensitivity (DTH) was performed as an i.d. injection of 5 × 10^6 mRNA-transfected DCs on the palmar side of the forearm at baseline, at the time of 4th
and 6th vaccines. Negative controls were mock-electroporated DCs and media alone. A red indurated area of >2 mm 48 h after injection was defined as a positive DTH skin test reaction.

**Vaccine preparation**

DCs were generated as previously described in a facility meeting Good Manufacturing Practice standards and approved by the Danish Medicines Agency. Briefly, patients underwent leukapheresis in order to isolate peripheral blood mononuclear cells (PBMC). PBMCs were incubated in a humidified incubator for 1 h at 37°C to allow plastic adherence. The adherent cell fraction was used for DC culture by incubation for 5 d in X-VIVO15 medium (Lonza, Basel, Switzerland) supplemented with 1% autologous heat-inactivated plasma, 1,000-U/mL GM-CSF (Leukine) (Genzyme, Cambridge MA, USA) and 250-U/mL IL-4 (CellGenix, Freiburg, Germany). Maturation of DCs were performed on days 5–7 with 1,000-U/mL TNF-α, 1,000-U/mL IL-1β, 1,000-U/mL IL-6 (CellGenix), and 1-μg/mL PGE2 (ProstinE2, Pfizer Freiburg, Germany). Cells were harvested on day 7 and transfected with mRNA. The transfection of mature DCs by electroporation was slightly modified compared to previously described. Briefly, mature DCs were washed twice, suspended in Opti-MEM medium (Invitrogen, Paisley, UK) and adjusted to a final cell density of 6.25 × 10^6 cells/mL. The cell suspension (800 μL) was preincubated in a 4-mm gap electroporation cuvette for 5 min on ice. 20 μg of mRNA encoding p53, survivin, or hTERT were transferred to the cuvette and DCs were pulsed using a BTX 830 square-wave electroporator (Harvard Apparatus, Holliston MA, USA). Electroporation settings were adjusted to a single pulse of 500 V and 2 ms. After electroporation, DCs were rested overnight (ON) in 24-well plates in X-VIVO 15 before frozen in aliquots of 1 × 10^7 DCs in 85% autologous serum, 10% DMSO (Wak-Chemie Medical GMBH, Steinbach, Germany), and 5% Glucosteril 40% (Fresenius, Albertslund, Danmark) using automated cryopreservation (Planer freezing unit, Planer, UK). For each patient, a concurrent transfection with green fluorescent protein (pSP73/pGEM-sig-survivin-DClamp plasmid (kindly provided by Kris Thielemans, Medical School of the Vrije Universiteit Brussel, Brussels, Belgium) using restriction site Brussel, Brussels, Belgium) using restriction site

Plasmid constructs and in vitro transcription of mRNA

The generation of p53, survivin, and hTERT mRNA was slightly modified compared to previously described. Briefly, prior to serving as DNA templates for in vitro transcription, the plasmids pSP73/p53/A64 and pSP73/survivin/A64 were linearized with SpeI, whereas the plasmid pCI/hTERT/A102 was linearized with MfeI, and all linearized plasmids were subsequently purified using Wizard DNA Clean-Up System (Promega, Madison WI, USA). The in vitro transcription was performed with mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin TX, USA) and mRNA was purified with MEGAclear kit (Ambion) according to manufacturer’s instructions. The mRNA length, concentration, and purity were evaluated with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto CA, USA), using RNA 6000 Nano LabChip Kit (Agilent Technologies) according to manufacturer’s instructions. Data analysis was performed with 2100 Bioanalyzer software (Agilent Technologies).

**Blood samples**

Heparinized blood samples for immune monitoring were collected at baseline and during treatment at the time of 4th and 6th vaccinations. Samples during treatment were taken at the same day as vaccination, which is 2 d after the last dose of cyclophosphamide. Samples were kept at room temperature for a maximum of 4 h until handling according to standard operating procedure. Shortly, PBMC were separated by centrifugation on a Lymphoprep (Takeda, Roskilde, Denmark) density gradient. Aliquots of PBMC were cryopreserved in RPMI medium with 10% AB serum and 10% DMSO using controlled-rate freezing (CoolCell, Biocision) in a ~80°C freezer and the next day moved to and stored in liquid nitrogen until analysis. When used for cell culture assays, cells were counted manually using trypan blue exclusion. When thawed for analysis, median live cell yield was 72.1% (data not shown). Data are available upon request.

**Indirect IFNγ ELISpot assay**

The ELISpot assay was performed according to the guidelines provided by CIP (http://cimt.eu/cimt/files/dl/cip_guidelines.pdf) and a thorough description of the assay has been reported elsewhere. Briefly, cryopreserved PBMCs were thawed and rested overnight (ON) in 24-well plates in X-VIVO 15 in a humidified 5% CO₂ 37°C incubator. The day after, a vial of DCs in excess from vaccine production was thawed, rested for 1 h, washed, resuspended in Opti-MEM and electroporated with p53, survivin, and hTERT encoding mRNA (DC vacc). After electroporation, DCs were transferred directly to 37°C X-VIVO 15 and rested for 30 min. Non-adherent PBMCs (PBLs) were harvested and co-cultured with DCs in a DC to PBL ratio.
of 1:10 in 2 mL X-VIVO 15 in 24 well plates with a concentration of 2–3 × 10⁵ PBLs/mL for 7 d. On day 1, 40 U/mL IL-2 were added. On day 7, another vial of DC was thawed and handled as described above however also electroporating DCs without mRNA added as control (DC mock). PBLs were harvested and co-cultured with DC vacc in triplicates in a DC:PBL ratio 1:10 aiming at using 3 × 10⁵ PBLs pr. well ON in a nitrocellulose-bottomed 96-well plate (MultiScreen MSIPN4W50, Merck Millipore, Billerica MA, USA) precoated with anti-IFNγ antibody (mAb 1-D1K, Mabtech, Sweden) and blocked with X-VIVO 15 before addition of cells. The day after medium was discarded and wells washed prior to addition of biotinylated secondary antibody (mAb 7-B6-1-Biotin, Mabtech). Plates were incubated at room temperature (RT) for 2 h, washed and streptavidin-ALP (Mabtech) was added. Plates were incubated for 1 h at RT before washing and addition of the enzyme substrate BCIP/NBT (Mabtech). Upon appearance of purple spots, the reaction was terminated by washing with tap water. Spots were counted using “Grand” ImmunoSpot S6 Ultimative UV Image Analyzer and analysis software ImmunoSpot 5.0 Analyzer. Positive controls were either PBLs stimulated with the super antigen Staphylococcal enterotoxin B or autologous DCs transfected with mRNA encoding Cytomegalovirus, Epstein-Barr Virus, or Flu virus epitopes. Raw data are available upon request.

Responses are determined using a statistical DFR(2×) method in test wells with >six spots pr. 1 × 10⁵ PBLs.

**Proliferation assay**

A thorough description of the assay has been reported elsewhere. In short, cryopreserved PBMCs were co-cultured for 7 d in the presence of 40-U/mL IL-2 from day 1 as described above. On day 7, PBLs were harvested, stained with PKH26 (Sigma, St. Louis MO, USA) dye according to manufacturer’s protocol. Subsequently, DCs and PBLs were co-cultured in triplicates in a 96-well round-bottom plate in a ratio 1:10 aiming at using 3 × 10⁵ PBLs pr. well for 3 d. FACS analysis was performed to make sure that PBLs were adequately and uniformly stained. After 3 d, DCs were harvested, relevant triplicates pooled, stained with anti-CD3 APC, anti-CD4 HV500, anti-CD8 BV421, and NIR for flow cytometric analysis. To establish background proliferation, a gate was set on PBLs without added DCs and the percentage of proliferation subtracted the percentage of proliferated cells in wells added mRNA-transfected or mock-electroporated DCs. A response was defined post hoc as the percentage of proliferated DCs in DC vacc stimulated PBLs being equal to or more than twice the proliferation in DC mock-stimulated PBLs (stimulation index ≥ 2) and more than 5% higher compared to wells with PBLs alone. Staphylococcal enterotoxin B, DCs transfected with mRNA encoding virus epitopes from CMV, EBV, and flu or anti-CD3 antibodies were used for positive controls. A representative example and gating strategy is shown in Fig. S9. Raw data are available upon request.

**Flow cytometry on PBMC**

Multicolor flow cytometry (FACS) was performed on whole blood to enumerate the Treg subset and on cryopreserved PBMCs to enumerate Treg and myeloid-derived suppressor cell (MDSC) subsets, to evaluate phenotype and the expression of activation or exhaustion markers. Analysis was performed using a FACScanto II flowcytometer (BD) and FACSDiva software (BD).

Monoclonal antibodies used for PBMC FACS analysis were anti-CD3 APC, anti-CD4 HV500, anti-FoxP3 PE, anti-IgG2a PE, anti-CTLA-4 PE, anti-BTLA PE, anti-PD-1 PE-Cy7, anti-CD33 FITC, anti-HLA-DR PerCP, anti-CD3 PE-Cy7, anti-CD19 PE-Cy7, anti-CD56 PE-Cy7, anti-CD11b APC, anti-CD15 HV500 (BD), anti-CD127 FITC, anti-CD45RA FITC (eBioscience), anti-CD25 BV421, anti-CD27 PerCP, anti-CCR7 PE-Cy7, anti-CD8 BV421, anti-CD14 BV421 (Biolegend), anti-LAG-3 FITC (Life-Span Bioscience), anti-TIM-3 PerCP (R&D Systems), and life/dead marker NIR APC-Cy7 (Invitrogen). Information on clone is available upon upon request.

Tregs are defined as being live cells in the lymphocyte gate in the forward side scatter plot, CD3⁺, CD4⁺, CD25⁺, CD127⁺, and FoxP3⁺. In analysis performed on whole blood, FoxP3 was not included. MDSCs are defined as being live cells in the PBMC gate in the forward side scatter plot and are lineage⁻ (CD3⁻CD19⁻CD56⁻), HLA-DR⁻flow, CD14⁺, CD11b⁺, and CD33⁺. T cells of both CD4⁺ and CD8⁺ subsets were further characterized according to phenotype. Naïve T cells being CCR7⁺, CD45RA⁻; central memory (CM) T cells being CCR7⁺,CD45RA⁻; effector memory (EM) T cells being CCR7⁻,CD45RA⁺; and effector memory RA positive (EMRA) being CCR7⁻, CD45RA⁺. Expression of activation or exhaustion markers programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), B- and T-lymphocyte attenuator (BTLA), lymphocyte-activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin domain containing-3 (TIM-3). Representative examples of the gating strategies are shown in Figs. S10A–C and S11.

**Statistics**

Survival was described using the Kaplan–Meier method and was defined as the time interval from first vaccination until death, last date of follow-up or date of data cut-off (18th August 2014). Progression-free survival was defined as the time interval from first vaccination until radiologic or clinical progression.

Responses in the ELISpot assay was assessed in tests with >six spots pr. 1 × 10⁵ PBLs using the DFR(2×) method as recommended in Ref. 46. A one-way p value ≤ 0.05 was considered significant.

The Wilcoxon Mann–Whitney test was used to explore differences marker values at baseline between responder (SD) and non-responder (PD) subgroups. Median and/or interquartile range was used for descriptive statistics. As this exploratory analysis was done post hoc and was descriptive in nature, no formal multiple testing corrections were performed. p values ≤ 0.05 were considered significant.

Marker values for paired data at different time points were tested with the Friedman test. p values were adjusted using the Holm–Bonferroni correction, taking into account the multiplicity of the number of time points. Adjusted p values ≤ 0.05 was considered significant.
Tests were two-sided except otherwise mentioned and were performed in R version 3.2.2.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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