Chapter 3

Is Anticancer Vaccine Possible: Experimental Application of Produced mRNA Transfected Dendritic Cells Derived from Enriched CD34+ Blood Progenitor Cells

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Additional information is available at the end of the chapter

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1. Introduction

The dendritic cells (DCs) are the most powerful antigen-presenting cells (APC) specialized to induce and regulate immune responses (1,2). The clinical use as cellular adjuvants in vaccination strategies has been aided by the development of methodologies to generate large production of these cells in culture. DCs can be grown ex-vivo from blood monocytes (3,4,5) or enriched CD34+ progenitors (6,7), using combinations of several cytokines/growth factors. Since our laboratory in Oslo routinely uses enriched CD34+ stem cells as stem cell support following high dose radio- and chemotherapy, it was of interest to test if such cells also could be applied for vaccine purposes (8,9), with a long term strategy of combining the two forms of therapy.

There are some publications indicating that CD34+ derived DC may work more efficiently as APC than those derived from monocytes (10), and recent data confirm that vaccine programs using CD34+ cell derived DCs lead to improved clinical results (11).

However, most in vitro culture systems for production of DCs include serum (9,11,12).

Since DCs are able to take up and process serum–derived antigens that are present in the cell cultures, such DC can when injected create unwanted reactions in the patients, in particular when fetal calf serum (FCS) is used. Thus, serum-free culturing condition is preferable, but in most previous culture experiments these conditions resulted in a lower yield of DCs (13,14).

Recently we reported a protocol for producing DCs from monocytes by use of gas-permeable Teflon bags and serum-free medium (15). We have used in the present study this

experience and have developed a similar serum-free culture system for CD34+ cell derived DC and investigated the optimal immunological properties of these cells.

2. Materials and methods

2.1. Growth factors, recombinant human cytokines and medium

Flt-3 ligand (Flt-3L), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), tumor necrosis factor-alpha (TNF-α) and stem cell factor (SCF) were purchased from CellGenix, Freiburg, Germany. Interferon-alpha (INF- α) was from Roche, Basel, Switzerland. Serum free medium CellGro/SCGM and CellGro/DC medium (CellGenix, Freiburg, Germany) were employed during the culture. To compare the serum free growth conditions with serum containing medium, CellGro/SCGM with 25% human albumin or Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS were used. Penicillin and Streptomycin was added to all mediums.

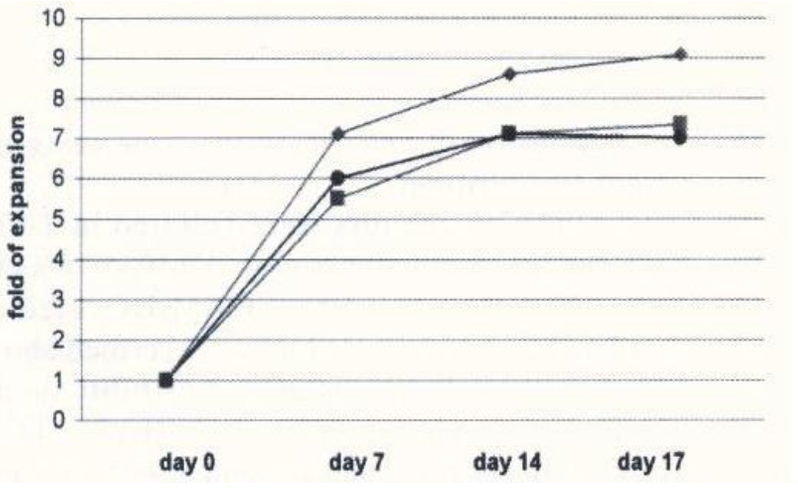

Figure 1. CD34+ cells (three different samples) cultured in serum-free CellGro SCGM/DC medium with added cytokines (as shown in materials and methods). The graphs indicate the fold expansion over time

2.2. CD34+ cell isolation

Leukapheresis-harvested samples were obtained from cancer patients undergoing peripheral blood (PB) stem cell mobilization after informed consent, using a CS 3000 Fenwall Cell Separator (Baxter, Deerfield, IL, USA). The isolation of CD34+cells was carried out using an Isolex 300i magnetic cell selector (Nexell, Irvine, CA, version 2.5CE/2.5CE+) as described earlier (16). The CD34+ samples were frozen in liquid nitrogen using PBS with 10% DMSO and 40% human serum albumin. The purity and viability of thawed CD34+ cells used for DC production was >98% and >95% respectively.

2.3. Generation of CD34+-derived DCs

CD34+ cells were rapidly thawed in a 37ºC water bath and washed once with culture medium. Then cells (0.5-1x10^5/ml) were transferred into VueLife™ FEP Teflon bags

(CellGenix, Freiburg, Germany) with serum containing DMEM/10% FCS medium, serum-free CellGro/SCGM medium or CellGro/SCGM/25% human albumin respectively. The following cytokine cocktail was added: GM-CSF 1000u/ml, IL-4 500u/ml, TNF-α 50ng/ml, Flt-3L 150ng/ml and SCF 50ng/ml. The bags were cultured for 14 days at 37ºC/5% CO_2. To keep a cell concentration of 10^5cells per ml through the entire culture period, re-feeding of the cells with culture medium employing equal concentration of cytokines was performed at weekly intervals. The serum-free medium cultures were from day 7, supplemented with CellGro DC medium instead of CellGro SCGM medium.

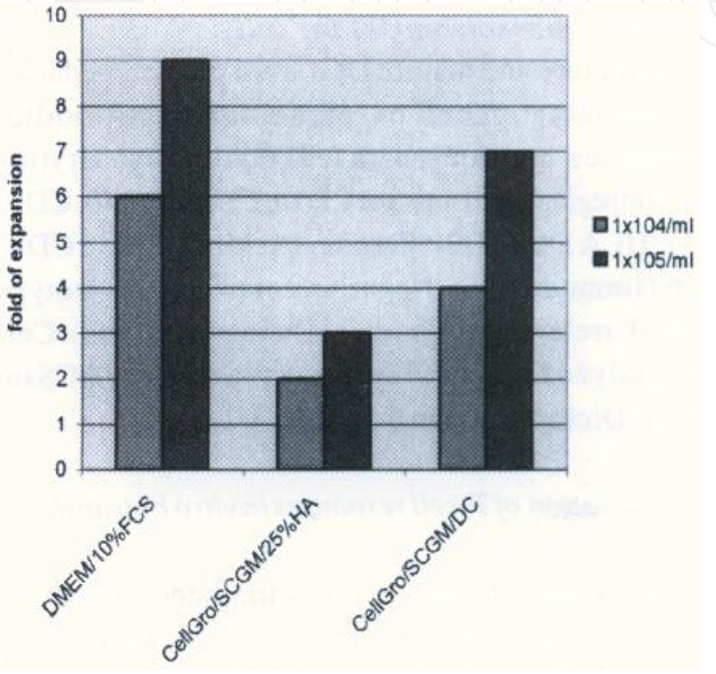

Figure 2. Expansion fold on Day 14. Different initial concentrations of CD34+ cells incubated in different culture medium. Cell concentration 10^5/ml are expanded better than 10^4/ml especially in serum free medium

2.4. Isolation of mRNA from cell line of human prostate cancer origin

Prostate cancer cell line DU 145 obtained from American Type Culture Collection (ATCC), was cultured in RPMI 1640 supplemented with 10% FCS. The method employed for isolation of mRNA from the tumor cell line has been described earlier (15). Briefly, $5x10^7$ cells were washed with cold PBS and transferred to a 1.5 ml microtube. Five hundred microliter ice-cold 2% IGEPAL (polyoxyethylene 9 nonylphenyl ether) (Sigma-Aldrich) was added to lyse the cells. The supernatant containing the cytosol fraction was obtained after centrifugation (10000xG for 1 minute at 4°C) and transferred to a 1.5 ml tube placed on a cooling block (4°C). To the supernatant 80 µl 10% LiDS (Lithium Lauryl Sulfate) (Sigma-Aldrich), 80 ul 5M LiCl (Lithium Chloride) (Sigma-Aldrich) and 0.5 ml Lysis & Binding Solution (Geno Vision) was added. Samples were frozen and stored at –80ºC until use. Isolation and purification of mRNA from the frozen or fresh samples were prepared in a GenoM™-48 Robotic Workstation (Genovision AS, Oslo, Norway) following the procedure

as described by the manufacturer (GenoM™-48, Automated mRNA Isolation Handbook, http:// www.qiagen.com /genovision/technical.htm). Denaturing agarose/formaldehyde gel electrophoresis was used to evaluate the quality of mRNA. The prepared mRNA was either used fresh or stored at -80°C until use.

2.5. Transfection of tumor mRNA into immature DCs

Teflon bags containing immature DCs (day14) were concentrated by centrifugation (600xG, 5 min., 4°C), the supernatant was removed using a plasma extractor (FENWAL Laboratories, USA) and the cell pellet was transferred by a syringe to a 50 ml tube. After one additional wash by centrifugation, the DCs were resuspended in cold culture medium to give a final volume of 0.6-0.8 ml and placed in a 4°C cooling block until use. mRNA transfection was performed as described earlier (17) using a BTX ECM 830 square-wave electroporator (Genetronics Inc.,San Diego,CA). Electroporation settings were adjusted to single pulse, 500 volt and 2 ms. The BTX-4mm electroporation cuvette (Genetronics Inc.) was washed twice with sterile DC culture medium. Then mRNA extracted from $5x10^7$ cells (40 µl) was added to the prepared immature DCs and transferred to the electroporation cuvette . After eletroporation, DCs were transferred back into the tube and stored on the cooling block for 1 min. before further incubation and maturation. All mock-mRNA transfected DCs used as control underwent electroporation following the same procedure as described above. The cell processing and electroporation procedure took place in a sterile laminar hood inside the GMP (good manufacturing practice) laboratory facility. In order to assess the transfection efficacy, immature DCs were also electroporated with enhanced green fluorescence protein (EGFP) mRNA as a reporter gene instead of mRNA from tumor. The experimental conditions used and the flow cytometry measurement has been described previously (17).

2.6. Maturation of DCs in sterile VueLife™ FEP Teflon bags

The two cells, mRNA-transfected and mock-transfected DCs, were removed from the tube by a syringe and injected through a sterile sampling site coupler into VueLife™ FEP Teflon bags. In order to mature the DCs, serum-free or serum-containing medium was supplemented with a mixture of the cytokines: 50 ng/ml TNF-α (CELLGenix, Freiburg) and 1000u/ml INF-α (Sigma-Aldrich). The final cell concentration during the incubation at 37°C with 5% CO_2 for 72 hours was $5x10^5$cells per ml.

2.7. Cryopreservation of mature DCs

The bag containing matured DC was centrifuged at 600xG for 10 minutes at room temperature. By the use of plasma extractor the supernatant was removed and the remaining DCs were transferred to a 50 ml tube. Following cell enumeration and sterility testing, cells were transferred into Nunc vials. The cryoprotectant solution was CellGro DC medium with 50% human albumin and 10% DMSO. The final cell concentration was $1x10^7$ cells/ml. Total volume in each Nunc vial was 500 µl. Freezing was performed in a control

rate freezer giving a rate of cooling of 1°C/min to -40°C with compensation for heat of fusion, then 1-2°C/min to -90°C. The prepared samples were thereafter transferred to liquid nitrogen and stored until use. The quality control of the frozen DCs consisted of sterility tests, phenotyping and viability testing by trypan blue staining before freezing and after thawing.

2.8. Immunophenotyping of the cells

Immature and mature DCs were phenotyped using the following panel of monoclonal antibodies: fluorescence isothiocyanate (FITC)- or phycoerythrin (PE)- conjugated anti-human CD1a, CD14, CD40, CD33, CD34, HLA DR, CD80 (Becton Dickinson), and CD83, CD86 (Immunotech). Negative controls were isotype-matched irrelevant antibodies (Dakocytomation). Cells were analyzed by flow cytometry using a FACSsort (Becton Dickinson, San Jose, Ca, USA).

2.9. Generation of T-cell responses in vitro by transfected DCs

Autologous-T cells were stimulated four times with weekly intervals in vitro by transfected DCs as described before (15). Briefly, $CD34^-$ PBMC from the same patient were thawed and plated in 6-well plates to get rid of adherent cells. Non-adherent cells containing high numbers of T lymphocytes were collected and used as responder cells. Thawed mRNA-transfected DCs used as stimulator cells were washed and irradiated with 3000 cGy. They were co-incubated in 24-well plates at a ratio 10:1 in serum-free CellGroDC-medium with 20 ng/ml of IL-7 and 100 pg/ml of IL-12. After 7 days incubation at 37°C in 5% CO_2, 1 ml of the suspension from each well was replaced with 1 ml of fresh DC medium containing 20 ng/ml of IL-7. On day 12, 19 and 26 the responder cells were restimulated by new batches of thawed and irradiated transfected DCs as on day 0. On day 14, 21 and 28 the cell cultured were given 1 ml of DC medium containing 20 IU/ml of IL-2. Finally on day 33 the cells were harvested and tested using ELISPOT assay as described below.

2.10. ELISPOT assay

The conditions for the ELISPOT assay have been described previously (15). A 96 well plate (Millipore-MAIP N45) were coated with 75 μl antibodies against human IFN-γ (Mabtech 1-D1K, 1 mg/ml diluted with PBS to a final concentration of 2 μg/ml) and incubated overnight at 4°C. The plate was left at room temperature (RT) for 1 hour and washed six times with PBS, 200 μl/well. Then RPMI-1640 + 1% human albumin was added 100 μl/well and incubated for 1-2 hours at 37°C to block unspecific binding of the antibody. The responder cells and stimulator cells were transferred to the precoated wells in different cell concentrations. As control mock-transfected DC, responder cells alone or medium alone was used. After incubation over night at 37°C, the plates were washed six times with PBS/0.05% Tween. To each well 75 μl of a stock solution of 0.75 μg/ml Biotinylated antibody against human IFN- γ (Mabtech, 7-B6-1-biotin, 1 mg/ml) was added and incubated for 2 hours at RT. Following six repeated washings the plate was incubated for one hour with 75μl per well of Streptavidin-ALP (Mabtech, 3310-8) from a stock solution (diluted 1:1000 in PBS+1%BSA). The plate was again washed 5 times with

PBS/0.05% Tween and one additional time with PBS alone. Then, after adding 75 µl of substrate BCIP/NBT (Sigma B911) to each well the plate was incubated for 4-5 minutes. When spots appeared, water was added to stop the reaction. The number of spots per well was counted under a stereomicroscope and the frequency of reactive T cells was calculated.

3. Results

3.1. Expansion of cells in serum-containing and serum-free medium

CD34+ progenitors were cultured in Teflon bags using serum containing DMEM/10%FCS and serum-free CellGro SCGM/DC medium supplemented with cytokines as described above. Table 1 shows cell expansion and viability on day14. The total cell expansion is 7.9±0.8 fold for CellGro/SCGM/DC medium and 8.3±0.6 fold for DMEM/FCS medium. Both conditions gave high cell viability, but when human albumin was added to the serum-free medium, lower cell expansion and viability was observed.

CD34+ cells proliferate and differentiate very rapidly during the first week, while in the remaining culture period only a minor expansion took place. When different cell concentrations of CD34+ cells were seeded and cultured for 14 days, a cell concentration of $1x10^5$ cells per ml kept through the culture period gave optimal growth conditions. Lower initial cell concentrations ($1x10^4$/ml) in the cultures gave no growth advantages (data not shown).

3.2. Yield and phenotypes of immature and mature DCs

Cultured cells lost their CD34 marker rapidly and no CD34 positive cells could be detected after 7 days of culturing. At day 14, DC purity was 35.9 ± 7.7 % as assessed by expression of CD86 and HLA DR antigens. Figure 2 (A and B) shows the phenotype of DCs in serum free medium on day 14 and 17. The CD86, CD83 and CD80 were up regulated greatly during maturation and the phenotypic profile obtained was comparable to DCs cultured in serum containing medium.

Different transfection parameters with regard to voltages and time of exposure were tested and the most suitable protocol was found to be 500V at 2ms. By the use of these parameters a transfection efficacy of >95% could be achieved and the mean fluorescence levels using EGFP (Enhanced green fluorescent protein) were increased to about 100-fold above background (figure 3). The percentages of surviving DC following mRNA tranfection using propidium iodide staining were 76%. A similar survival was obtained in the mock-transfected DC.

3.3. T-cell responses to thawed transfected DCs

To assess the function of matured DCs we use transfected DCs to stimulate autologous T cells four times weekly. Thereafter the T cells were tested in the ELISPOT assay, which give information of both transfection efficacy, processing and antigen stimulation capacity of transfected DCs. As shown in figure 4, after four times stimulation by thawed transfected DCs in vitro, a significant and specific T-cell response to transfected DCs as compared to the control experiment employing mock-transfected DCs was achieved.

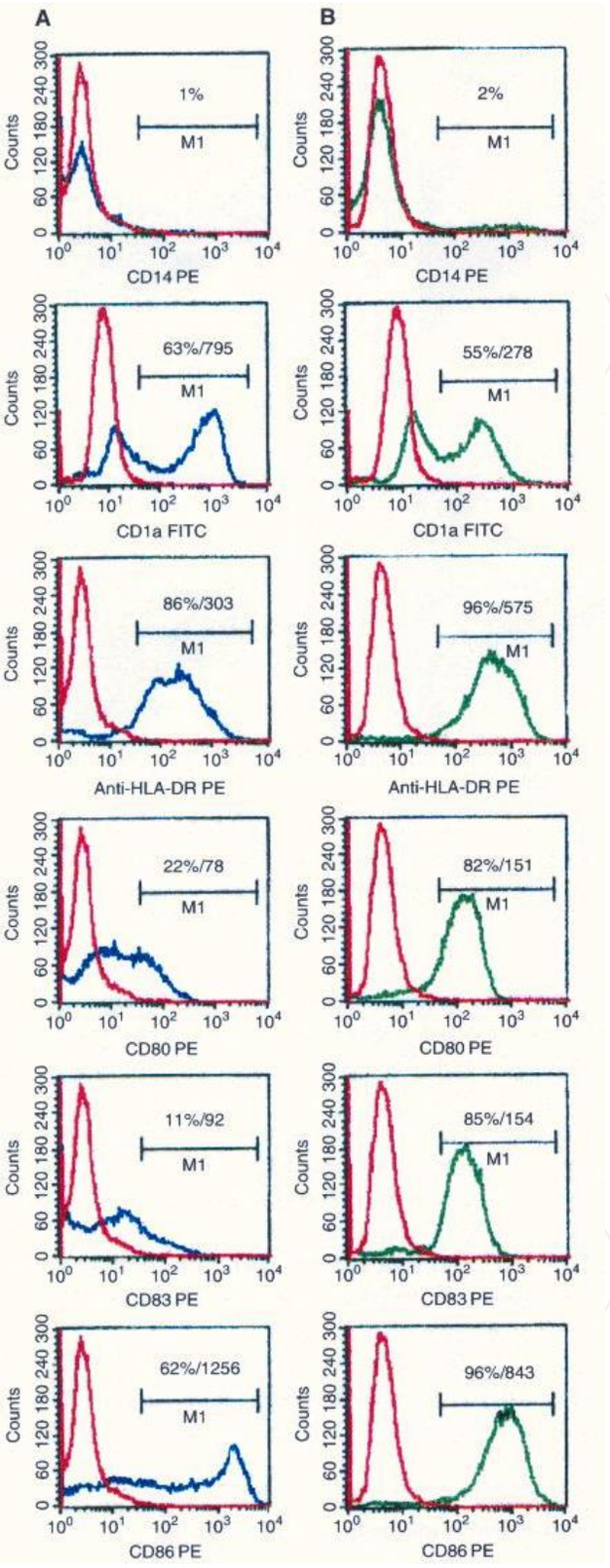

Figure 3. Immunophenotyping profile of: A) Immature DC; B) Mature DC generated from enriched monocytes. Overlay histograms show the expression of relevant antigens of immature (blue) and mature DC (green) versus isotype-matched control (red). The percentage of positive cells and mean fluorescence intensity value is shown too.

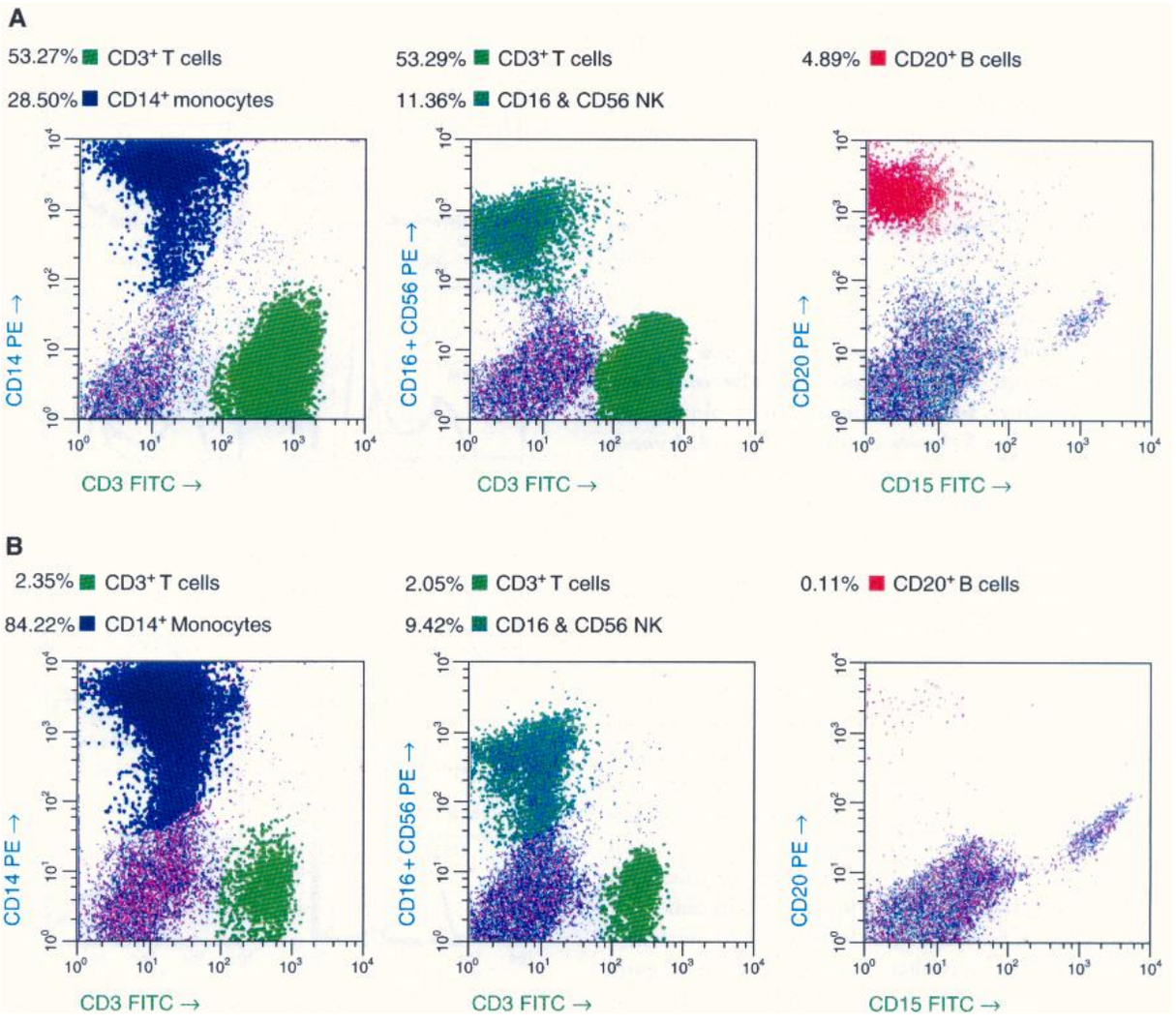

Figure 4. Transfection efficacy of mRNA demonstrated by utilized EGFP mRNA (lower part) compared to non-transfected DC (upper part). Left panels are density plots indicating large cell population gated. Middle panels are density plots showing the viability of cells (non-transfected and EGFP mRNA transfected by application of propidium iodide PI stating FL3). Right panels are histogram plots showing GFP signal in living cells. The green fluorescence intensity is increased about 100-fold in transfected cells than in non-transfected cells.

4. Discussion

In our hospital, enriched CD34+ stem cells are routinely being prepared and used as progenitor stem-cell support to patients receiving high dose therapy (16). In the cases that such patients also are candidates for DC-based vaccine treatment, spared frozen CD34+ cells would be available as a source for DC production thereby avoiding new expensive procedures for production of monocyte-derived DCs (18).

The present study describes the establishment of a clinical ex- vivo culture system for expansion of mature DCs derived from CD34+ cells employing VueLife™ FEP Teflon bags and serum free CellGro/SCGM/CellGro. Most methods applied for production of DC include FCS or pooled human serum. As a foreign protein, FCS is highly unwanted not only

because of the danger of disease transmission but also because immune responses against FCS might result in high background responses obscuring the specific T cell immunity. Other studies have indicated that human serum may inhibit DC differentiation and therefore seems not to be a good alternative to replace FCS (13). Serum free conditions have been tested previously (13,14). In these studies cell expansion and DC yield were very low though the phenotype of the DCs were considered not to be affected. In this study we have demonstrated that there is no difference between serum-free and serum-containing medium with regard to ex-vivo expansion of both the total number of cells and the estimated content of matured DCs in the cell products. The addition of serum albumin to our cultures did not result in any growth advantages.

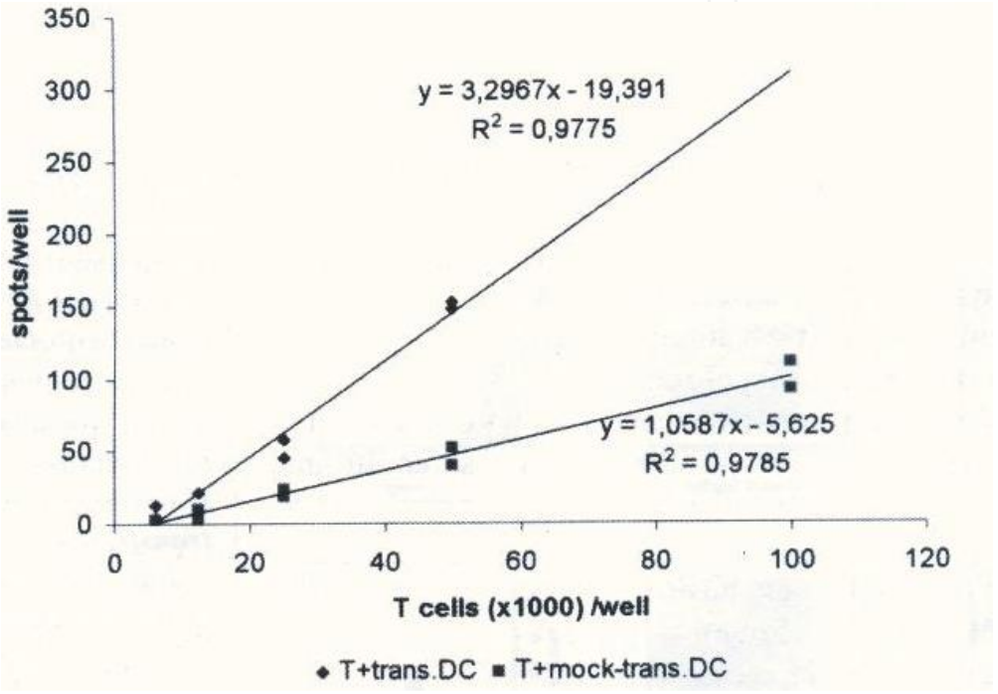

Figure 5. Autologous T cells stimulated with four times ex vivo irradiated transfected DC. ELISPOT assay indicates that stimulated T cells are able to recognize transfected DC specifically by use of mock-transfected DC as conrol

GM-CSF, TNF-α and IL-4 are cytokines that play an import role in DC differentiation (19,20) when serum-containing medium is being employed. However, our experiences and that of others indicate that this cytokine combination alone is not sufficient for ex vivo expansion of DCs from CD34+ cells. As described by others, ex-vivo culturing of CD34+ cells in the presence of SCF and Flt-3 (21) give an efficient expansion of total cell numbers without interfering with DC development. Since these early acting factors does not affect DC differentiation, but sustained the long-term expansion of CFU-DC, we chose to add them to our cultures. In contrast to monocyte- derived DCs, ex-vivo expansion of CD34 derived DCs usually occurred asynchronously over a 2 to 3 weeks period. Since INF-α can efficiently accelerate the course of maturation (22) we also included this cytokine in the cocktail the last 3 days of culture. This resulted in an up regulation of the maturation antigens CD86, CD83 and CD80.

The use of gas permeable bags for ex-vivo production has several advantages when compared to production in culture flasks. The bag system is closed and reduces the risk of contamination. DCs produced in Teflon bags do not attach to the surface and can easily be concentrated by centrifugation without any extra steps. It also facilitates large-scale production, which can be divided into aliquots containing cells with identical properties. We have shown that DCs can efficiently be produced in suspension using gas permeable Teflon bags. When CD34+ progenitors are cultured in flasks, usually the cell concentration is 10^4/ml. In our system we have shown that optimal cell concentration is 10^5/ml, which give a 10-fold reduction in the amount of medium and cytokines used.

DCs have been loaded with several antigens, such as tumor lysate, peptides, proteins, DNA and mRNA. As a source of antigens, the major limitation of using lysate, proteins or peptides isolated from patients' tumor cells is the amount of tumor tissue or the purity of the tumor specimens. The use of nucleic acids, either DNA or RNA, would overcome this practical limitation. As mRNA is a safer alternative due to its limited ability to cause permanent genetic alterations in the host, it appears to be more attractive to be used than DNA transfection (23). For this purpose, a vector-free transfection system based on square-wave electroporation to transfer mRNA into DCs has been developed (17). This method is currently successfully used in the clinic to produce mRNA-transfected monocyte-derived DCs (15). We here demonstrate that this method also resulted in efficient transfection of mRNA into immature CD34+ cell derived DCs without significantly affecting the survival of the cells.

Generally the antigen-stimulating capacity of DCs has been evaluated employing allo-reactive T cells or responses against recall antigens. We have used priming of autologous T cell against antigens encoded by a prostate tumor cell line in order to evaluate the immuno-stimulatory role of the transfected DCs. The ELISPOT assay was used to detect and quantify of single T lymphocyte forming cytokine spots after antigen contact in vitro. The ELISPOT assay is a more stringent system for testing both the efficacy of transfection and the processing and antigen-stimulating capacity of transfected DCs. Our results show that CD34+ cells derived DCs, grown in serum free conditions in clinical scale productions reproducibly are capable of inducing a tumor specific immune response. These results are similar to what was seen in our previous study using monocyte-derived DCs

The finding and results from the present study allows us to proceed with a clinical protocol for application of CD34+ derived DCs for cancer vaccine. Possible attractive candidates for such an approach are relapsed Hodgkin's patients (16) and other patients that have previously been treated with auto transplantation and with spared frozen samples of CD34+ cells.

Experiment	Cell Gro/SCGM&25% HA	DMEM / 10% FCS	Cell Gro / SCGM / DC
	fold / viability	fold / viability	fold / viability
1	1.6 / 61%	9.0 / 97%	8.6 / 94%
2	1.2 / 67%	7.8 / 98%	7.1 / 93%
3	1.8 / 73%	8.8 / 97%	7.1 / 84%

Table 1. Expansion fold and viability on Day 14

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