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# Development of a standardized protocol for reproducible generation of matured monocyte-derived dendritic cells suitable for clinical application

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**Abstract**

There is increasing interest in the generation of dendritic cells (DC) for cancer immunotherapy. In order to utilize DC in clinical trials it is necessary to have standardized, reproducible and easy to use protocols. We describe here the process development for the generation of DC as the result of investigation of culture conditions as well as consumption rates of medium and cytokines. Our studies demonstrate that highly viable DC (93 ± 2%) can be produced from $CD14^+$ enriched monocytes via immunomagnetic beads in a high yield (31 ± 6%) with X-VIVO 15, 400 U $ml^{-1}$ GM–CSF and 2000 U $ml^{-1}$ IL-4 without serum and feeding. For the maturation of DC different cocktails (TNF-$\alpha$, IL-1$\beta$, IL-6, $PGE_2$ and TNF-$\alpha$, $PGE_2$) were compared. In both cases cells expressed typical surface molecules of mature DC and induced high proliferative responses in mixed lymphocyte reactions which led to IFN-$\gamma$ producing T-lymphocytes. The data suggest that the use of this optimized, easy to use protocol results in highly mature DC.

*Abbreviations:* DC – dendritic cells; moDC – monocytes derived dendritic cells; FACS – fluorescence activated cell sorter; FCS – fetal calf serum; GM–CSF – granulocyte macrophage–colony stimulating factor; MLR – mixed lymphocyte reaction; PBMC – peripheral blood mononuclear cell; IL – Interleukin; $PGE_2$ – Prostaglandin $E_2$; TNF – tumor necrosis factor

## Introduction

Dendritic cells (DC) are professional antigen presenting cells, inducing immune responses while stimulating naïve T-lymphocytes and controlling the activation of T-helper cells in the $T_H1$–$T_H2$ pathway (Banchereau and Steinman 1998; O'Garra and Arai 2000).

The use of these potent stimulators as vaccines for the immunotherapy of cancer is a very promising approach to overcome the tumor escape in immune surveillance (Costello et al. 1999). To date, several clinical trials have been performed utilizing DC preparations, which demonstrated anti-tumor responses (Nestle et al. 1998; Thurner et al. 1999a; Kugler et al. 2000; Fong et al. 2001; Kikuchi et al. 2001; Kobayashi et al. 2001; Schuler-Thurner et al. 2002).

Different sources can be used for the generation of DC: proliferating $CD34^+$ precursors in blood (Caux et al. 1992) after G-CSF mobilization (Mackensen et al. 2000), non-proliferating $CD14^+$ monocytes in peripheral blood after enrichment via magnetic beads (Pickl et al. 1996; Dietz et al. 2000) or adhesion (Bender et al. 1996; Romani et al. 1996; Thurner et al. 1999b; Berger et al. 2002) and enrichment of DC after cultivation of PBMC via elutriation (Bernard et al. 1998; Goxe et al. 2000). Rare circulating DC can also be isolated, but although patients can be pretreated with Flt3 ligand the yield

of DC is comparably small (Fong et al. 2001). While DC from $CD34^+$ cells require a prolonged culture and special cytokine setup in order to increase the small number of precursors, monocyte-derived DC (moDC) are easy to obtain after enrichment of monocytes by magnetic separation or adherence, followed by differentiation using granulocyte macrophage–colony stimulating factor (GM–CSF) and interleukin-4 (IL-4). This method developed by Sallusto and Lanzavecchia (1994) and Romani et al. (1994) is applied widely in experimental protocols. Due to the ease of availability of monocytes, rapidness of fully differentiation to unmature (5–6 days) or mature (6–8 days) DC using special maturation cocktails (e.g., TNF-$\alpha$, IL-1$\beta$, IL-6, $PGE_2$ (Jonuleit et al. 1997) or TNF-$\alpha$, $PGE_2$ (Kalinski et al. 1998)) and the possibility of avoiding foreign antigens (fetal calf serum (FCS)), this protocol was adapted for clinical applications (Thurner et al. 1999b). For studies where DCs are to be cultured *ex vivo* and then reintroduced to the patient, it is advisable to avoid FCS or any kind of foreign antigen due to possible infections and immunogenicity. However, the use of autologous serum or plasma may cause non-standardized cultivation conditions and so is best to avoid.

Here, we describe the development of a standardized protocol, which circumvents the disadvantages of non-uniform culture conditions caused by supplemented serum, non-defined cell densities and adherence steps. Furthermore, the protocol eliminates the need for feeding the cells, which increases the risk of contamination. In this study, we have examined the influence of several important cultivation parameters to optimize and setup the serum-free generation of DC in X-VIVO 15. Cell density, GM–CSF and IL-4 concentration, medium components like glucose, lactate and amino acids were assessed. Moreover the influence of different maturation cocktails (TNF-$\alpha$, IL-1$\beta$, IL-6, $PGE_2$ and TNF-$\alpha$, $PGE_2$) on the maturation status of DC was investigated.

## Material and methods

### *Medium, serum and cytokines*

As standard medium X-VIVO 15 (Bio Whittaker, Walkersville, MD) was used for the generation of DC. RPMI 1640 (Gibco BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated (56 °C, 30 min) FCS (Gibco BRL, Eggenstein, Germany) was used for the mixed lymphocyte reaction (MLR). The differentiation of monocytes into DC was performed using rhuGM–CSF (Leucomax$^{TM}$, Norvartis, Nuernberg, Germany) and rhuIL-4 (R&D, Wiesbaden, Germany). Factors added for maturation of DC included rhuTNF-$\alpha$, rhuIL-1$\beta$, rhuIL-6 (all from R&D) and $PGE_2$ (Sigma, Deisenhofen, Germany). For the MLR rhuIL-2 (from BHK 21 cells, kindly provided by Dr Wagner, GBF, Braunschweig, Germany) was utilized.

### *Generation of human monocyte derived dendritic cells*

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coat preparations from healthy donors (kindly provided by Dr T. Tonn, Blutspendedienst Hessen, Germany) by standard density gradient centrifugation on Biocoll (Ficoll separating solution) (Biochrom KG, Berlin, Germany). Monocytes $CD14^+$ were affinity-purified utilizing the MACS$^{TM}$ CD14 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions. Briefly, PBMC were incubated in recommended buffer with MACS$^{TM}$ CD14 MicroBeads for 15 min at 4 °C, centrifugated and resuspended in buffer. Later cells were passed through a positive selection column. This step was done twice to obtain highly purified CD14 positive cells.

After resuspension of monocytes in X-VIVO 15 they were seeded at defined cell densities described at the results' section (Standard concentration: $1.3 \times 10^6$ ml$^{-1}$) and placed in an incubator at 37 °C and 5% $CO_2$. Cytokines including rhuGM–CSF and rhuIL-4 were added at day 0 (standard concentration: rhuGM–CSF (400 U ml$^{-1}$), rhuIL-4 (2000 U ml$^{-1}$)). All comparison experiments were performed in 48 well plates (Greiner, Solingen, Germany) and with optimized parameters upscaled in 75 cm$^2$ T-flasks (Greiner). After 6 days of differentiation of monocytes into DC different maturation cocktails were added: cocktail I (TNF-$\alpha$ (1000 U ml$^{-1}$), IL-1$\beta$ (1000 U ml$^{-1}$), IL-6 (1000 U ml$^{-1}$), $PGE_2$ (1 $\mu$g ml$^{-1}$, 0.003 $\mu$M)) and cocktail II (TNF-$\alpha$ (1000 U ml$^{-1}$), $PGE_2$ (18 $\mu$g ml$^{-1}$,

0.051 $\mu$M)). After 8 days the resulting suspension-cells were harvested and analyzed as described.

*Cell counting and viability*

After harvesting of DC on day 8, counting and viability determination was performed using a hemocytometer with standard trypan blue dye exclusion and a CASY 1 particle counting system (model TT, Schaerfe System, Reutlingen, Germany).

*Phenotyping and fluorescence activated cell sorter analysis*

For analysing of PBMC and DC populations we used the following mAbs (all mAbs from Becton Dickinson, Heidelberg, Germany): CD1a (CyChrome), CD3 (CyChrome), CD4 (PE), CD8 (FITC), CD14 (FITC), CD16 (PE), CD19 (PE), CD25 (FITC), CD40 (FITC), CD54 (PE), CD56 (FITC), CD80 (FITC), CD83 (PE), CD86 (FITC), anti-HLA-A,B,C (FITC), anti-HLA-DR (CyChrome, PE). Cells ($2 \times 10^5$ per specimen) were suspended in 90 $\mu$l of ice-cold PBS and incubated with 5 $\mu$l of corresponding mAb for 30 min at 4 °C. After staining cells were washed once with ice-cold PBS and fixed in 200 $\mu$l of 1% paraformaldehyde in PBS. FACS analysis was performed using a FACSCalibur (Becton Dickinson) and CellQuest 3.1 software (Becton Dickinson).

*Cytokine enzyme-linked immunosorbent assay (ELISA) kits*

Cytokine ELISA kits for rhuGM–CSF (detection limit: 4.7 pg ml$^{-1}$), rhuIL-4 (detection limit: 7.8 pg ml$^{-1}$) and rhuIL-12p70 (detection limit: 7.8 pg ml$^{-1}$) were purchased from Becton Dickinson (OptEIA human ELISA Set, BD PharMingen, Heidelberg, Germany) and were used following the manufacturer's instructions. The readout of the ELISA-plates was performed using a photometer (Wallac Victor$^2$, PerkinElmer Life Science, Bad Wildbad, Germany) reader at 450 nm with a correction at 570 nm.

*Metabolic analysis*

Osmolality was measured using a freezing-point osmometer Osmomat 030 (Gonotec, Berlin, Germany). Glucose (Ebio compact, Eppendorf, Hamburg, Germany), lactate (YSI 1500L, Yellow Springs Instruments, Yellow Springs, USA), glutamine and glutamate (YSI 2700 select, Yellow Springs Instruments, Yellow Springs, USA) were quantified enzymatically using the indicated automatic analyzer according to the manufacturers instructions. Amino acids analysis was realized using HPLC (Amino Quant 1090 AX, Hewlett Packard, Waldbronn, Germany).

*The MLR*

DC were added to $5 \times 10^5$ allogeneic PBMC at a ratio of 1 : 10 in 6 well plates and co-incubated for 4 days in RPMI 1640 supplemented with 10% FCS. After 4 and 7 days 50% of the culture medium were replaced and 100 U ml$^{-1}$ rhuIL-2 were added. After 8 days the proliferating T-cells were counted, phenotyped and an Interferon-$\gamma$ secretion assay was performed.

*Interferon-$\gamma$ secretion assay*

For determination of the portion of IFN-$\gamma$ producing T-cells an IFN-$\gamma$ Cytokine Secretion Assay (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instructions (Manz et al. 1995).

## Results

*Cell enrichment and starting population of monocytes*

There are different methods of monocyte enrichment currently discussed in literature. Due to the availability of GMP (Good Manufacturing Practice) quality immunomagnetic beads from Miltenyi (CliniMACS$^{TM}$) to isolate CD14$^+$ monocytes (Dzionek et al. 2002), this method was used in a laboratory setup with Mini- and Midi-MACS$^{TM}$ magnets and appropriate columns. This magnetic cell sorting approach was highly effective in isolating CD14$^+$ monocytes from PBMC with a yield of $12.5 \pm 4.8\%$ ($n = 6$) and a purity of $\geq 98\%$ (data not shown).

*Influence of different cell densities on yield and maturation of moDC*

To setup and optimize a protocol for the generation of moDC we first investigated the influence of different cell densities on the consumption and accumulation of medium components such as glucose, lactate, glutamine, glutamate and supplemented cytokines. In different experiments $3.3 \times 10^5$, $6.6 \times 10^5$, $1.3 \times 10^6$ and $2.6 \times 10^6$ monocytes $ml^{-1}$ were inoculated in X-VIVO15 supplemented with 800 U $ml^{-1}$ GM–CSF and 500 U $ml^{-1}$ IL-4 and incubated for 6 days without feeding. A maturation cocktail consisting of TNF-$\alpha$ (1000 U $ml^{-1}$), IL-1$\beta$ (1000 U $ml^{-1}$), IL-6 (1000 U $ml^{-1}$) and $PGE_2$ (1 $\mu$g $ml^{-1}$) was then added and the cells incubated for further 2 days.

After 6 days of cultivation partly non-adherent DC could be observed which did not express CD83 a marker of mature DC (Zhou and Tedder 1995). The maturation cocktail added for two additional days induced the expression of CD83 and an increase in CD80 and CD86 as well as HLA-DR. These non-adherent DC with many motile veils showed the typical pattern of mature moDC. The size of the cells increased from an average of 10 $\mu$m (monocytes) to 16 $\mu$m (moDC), which was determined by a CASY 1 particle counter.

The yield (as defined by size of cells, morphology and surface antigen expression) of matured moDC was similar for the cultures initially inoculated with $3.3 \times 10^5$, $6.6 \times 10^5$, $1.3 \times 10^6$ monocytes $ml^{-1}$ and about 25% lower for the highest inoculated cell density (Figure 1). All cell densities except for the $2.6 \times 10^6$ $ml^{-1}$ showed the typical mature DC phenotype with high expression of HLA-A, B, C (data not shown), HLA-DR, CD80, CD83 and CD86 (Figure 2). The DCs differentiated and matured from $2.6 \times 10^6$ $ml^{-1}$ expressed reduced HLA-DR, CD80, CD83 and CD86 antigens. The HLA-DR/CD80 and HLA-DR/CD83 dot plots for $6.6 \times 10^5$ and $1.3 \times 10^6$ $ml^{-1}$ showed two distinct populations of DC: a population with higher HLA-DR/CD80 and HLA-DR/CD83 expression and a lower one, which may be caused by a different maturation status. Especially for the HLA-DR/CD80 dot plot for the cell density of $1.3 \times 10^6$ $ml^{-1}$ this observation was significant. Lower HLA-DR expression seems to correlate with a decrease in CD80 surface antigen expression. The mean

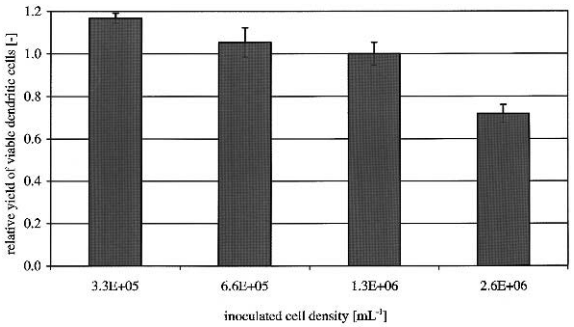


*Figure 1.* Influence of different cell densities on yield after generation of moDC. Monocytes were enriched via immunomagnetic beads, inoculated at specified cell densities and differentiated with 800 U $ml^{-1}$ GM–CSF and 500 U $ml^{-1}$ IL-4. For maturation a cytokine cocktail consisting of TNF-$\alpha$, IL-1$\beta$, IL-6 and $PGE_2$ was used. The data represent the mean (± SD) of triplicates from a single donor. Similar data were obtained with two other donors.

fluorescent intensity as an indicator for the level of expression of CD40 decreased about 50% with the highest cell density (data not shown). We could not observe any differences in expression of CD1a (data not shown).

We also analyzed medium components and found an increase in lactate concentration at the highest cell density at $\geq$25 mmol $l^{-1}$ (Figure 3), which caused a decreased culture pH and might be responsible for the lower yield. Accumulation of metabolic products like lactate produces an acidic environment and therefore inhibits proliferation (Bohnenkamp and Noll 2002; Patel et al. 2000).

The glucose concentration in all experiments remains above limiting levels. Amino acid analysis demonstrated no limitation for glutamine and serine. The highest glutamate concentration was 1.4 mmol $l^{-1}$.

An ELISA for GM–CSF and IL-4 showed no limitation for GM–CSF (GM–CSF residual content: $3.3 \times 10^5$ $ml^{-1}$: 780 U $ml^{-1}$, $6.6 \times 10^5$ $ml^{-1}$: 720 U $ml^{-1}$, $1.3 \times 10^6$ $ml^{-1}$: 450 U $ml^{-1}$ and $2.6 \times 10^6$ $ml^{-1}$: 500 U $ml^{-1}$, respectively) but IL-4 was limiting (detection limit: 7.8 pg $ml^{-1}$) (data not shown), which might explain the non-homogenous DC population especially for the $6.6 \times 10^5$ and $1.3 \times 10^6$ $ml^{-1}$ inoculated monocytes.

Due to a cost-effective utilization of the cultivation system in terms of highest yield of matured moDC per volume, all further experiments were

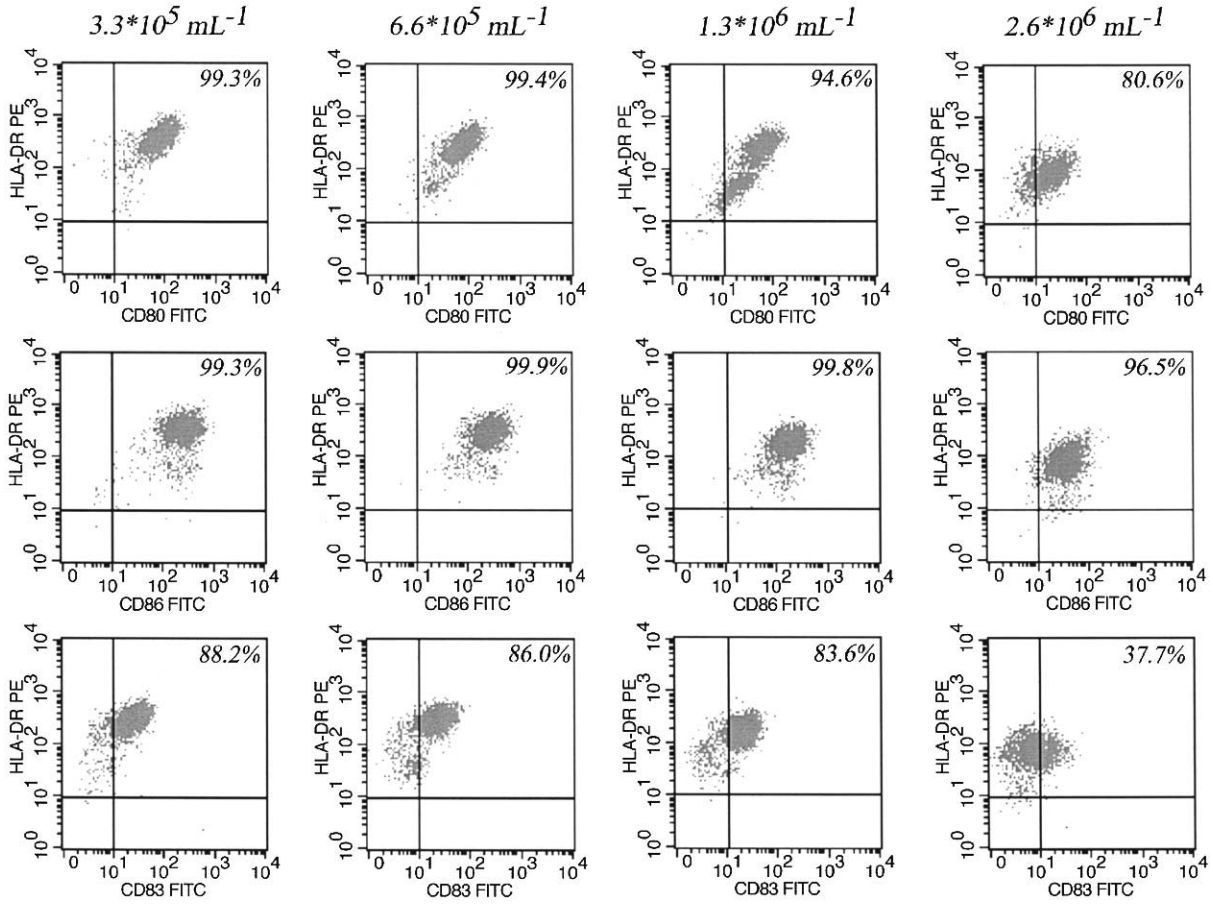


*Figure 2.* Phenotype of moDC generated with in figure 1 specified cultivation parameters. HLA-DR/CD80, HLA-DR/CD86 and HLA-DR/CD83 dot plots are shown for different cell densities ($3.3 \times 10^5$, $6.6 \times 10^5$, $1.3 \times 10^6$ and $2.6 \times 10^6$ ml$^{-1}$ respectively). Decreased CD80, CD83 and CD86 was expressed for the highest cell density, HLA-DR/CD80 and HLA-DR/CD83 dot plots for $6.6 \times 10^5$ and $1.3 \times 10^6$ ml$^{-1}$ featured a higher and a lower expressing population. The data shown are from one representative experiment out of three performed.

performed at a cell density of $1.3 \times 10^6$ ml$^{-1}$ inoculated monocyte.

### *Optimization of GM–CSF and IL-4 concentrations*

Based on the finding that no substrate feeding is necessary for the generation of matured moDC at a cell density of $1.3 \times 10^6$ ml$^{-1}$ inoculated monocytes, we investigated the influence of different concentrations of GM–CSF and IL-4 on yield and phenotype of the cells in order to get a homogenous moDC population. Monocytes differentiate in the presence of GM–CSF and IL-4 to immature DC but both cytokines are also necessary for maturation, therefore suggesting that both cytokines are not exhausted after 8 days of culture.

We did several experiments to test the stability, half-life and consumption of the mentioned cytokines. The stability and half-life test was established utilizing triplicates of T-flasks we also used for the generation of DC. We inoculated 800 U ml$^{-1}$ GM–CSF and 500 U ml$^{-1}$ IL-4 respectively in X-VIVO 15 and incubated at 37 °C and 5% $CO_2$ for 8 days. Samples were taken every [2$^{nd}$] day, frozen and after collection thawed for quantification of GM–CSF and IL-4. The GM–CSF used (Leucomax$^{TM}$) showed no decrease of concentration after 8 days (data not shown). For the IL-4

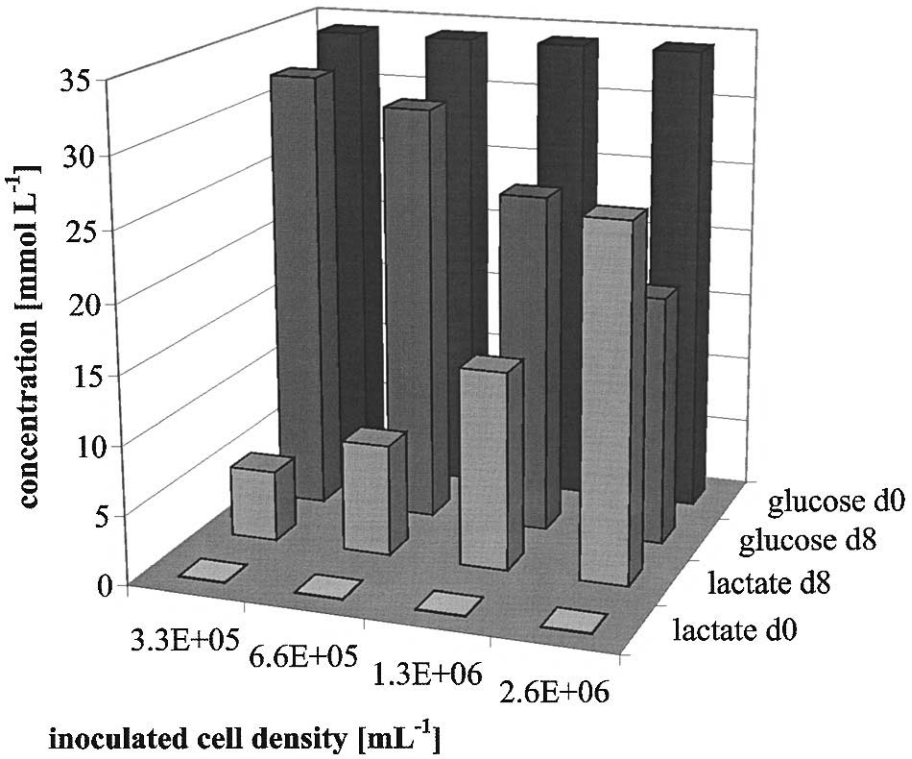


*Figure 3.* Glucose and lactate analysis on day 0 (start concentration) and on day 8 (after generation of moDC). Shown is one representative experiment out of three.

(R&D) we could demonstrate that 80% of the cytokine adhered to the surface of the flask after 2 h. The remaining IL-4 concentration did not show any further decay (data not shown). Therefore, the monocytes were inoculated first and then the cytokines were added to ensure that enough IL-4 remained in solution (data not shown).

The next step was to investigate the consumption of GM–CSF and IL-4. Therefore we started with 200, 400 and 800 U $ml^{-1}$ GM–CSF while IL-4 was inoculated at 500 U $ml^{-1}$. The 200 U $ml^{-1}$ were exhausted completely while in the other two experiments about 300 U $ml^{-1}$ were consumed (296 U $ml^{-1}$ and 320 U $ml^{-1}$, respectively) (Figure 4). The yield and phenotype was comparable to previous experiments, see Figure 2, 1.3 $\times$ $10^6$ $ml^{-1}$ inoculated monocytes. The 200 U $ml^{-1}$ GM–CSF concentration resulted in the same number of DCs but in a lower expression of CD80, CD83 and CD86 (data not shown).

We then determined which concentration of IL-4 resulted in a homogenous populations of matured

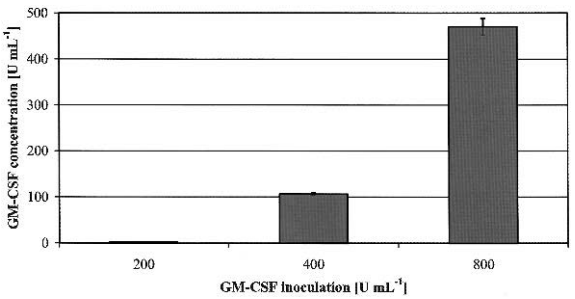


*Figure 4.* GM–CSF consumption of cultivated cells after 8 days. Data shown are the mean (± SD) of triplicate cultures from one representative experiment of three performed.

moDC. We added different IL-4 concentrations (500, 1000 and 2000 U $ml^{-1}$ respectively) with a constant concentration of 800 U $ml^{-1}$ GM–CSF to alter only one parameter. The IL-4 ELISA indicated that only at an initial concentration of 2000 U $ml^{-1}$ IL-4 was the not limiting, as we could still measure 25 U $ml^{-1}$ after 8 days of cultivation (data not shown) and phenotypic analysis showed

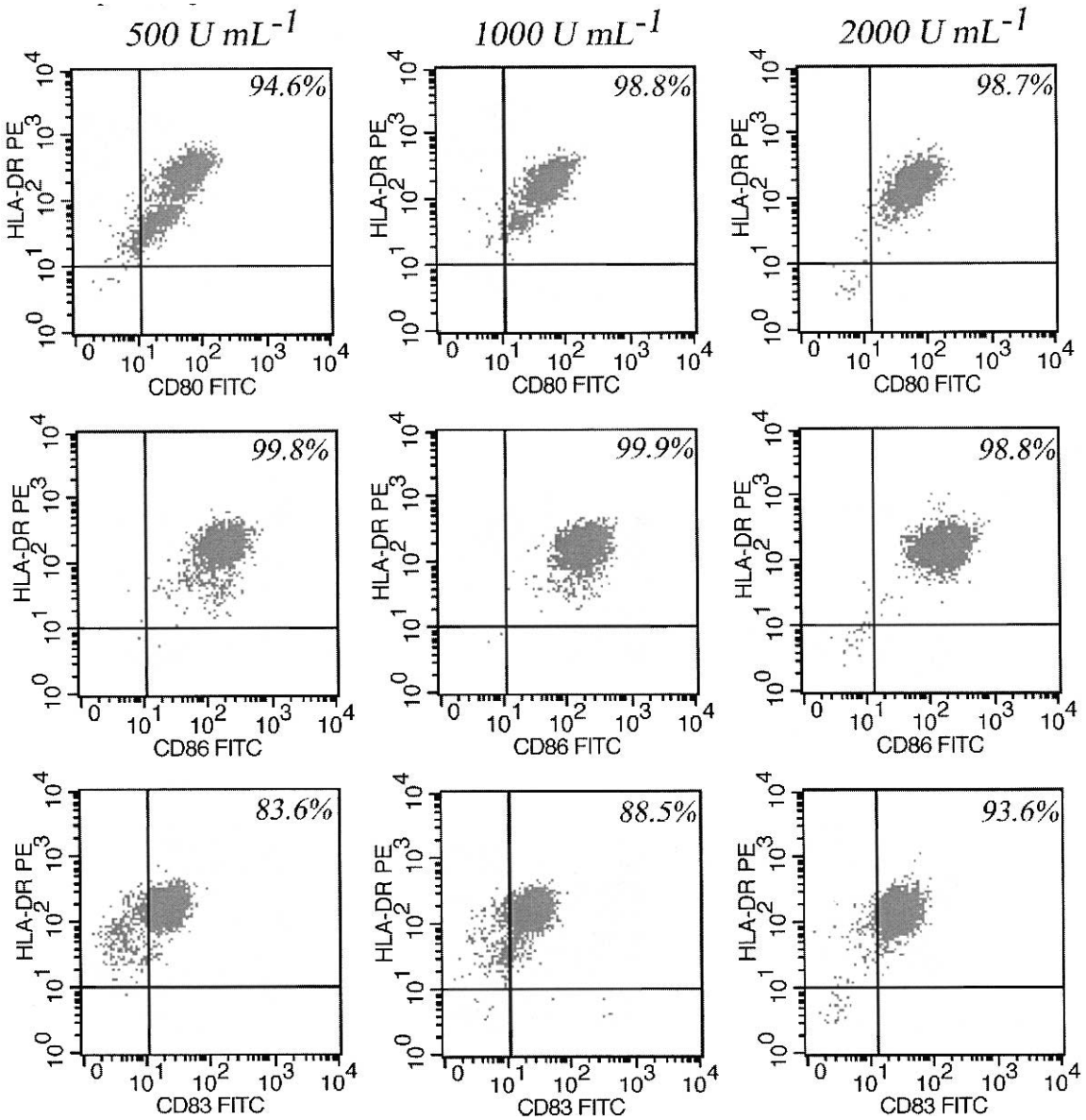


*Figure 5.* Phenotype of moDC generated with different IL-4 concentrations (500, 1000 and 2000 U ml$^{-1}$). Only moDC generated with 2000 U ml$^{-1}$ demonstrated a homogenous population of fully matured dendritic cells. The data shown are from one representative experiment of three performed.

a homogenous population of matured moDC only for the highest IL-4 concentration (Figure 5). However, the number of DCs was similar for all cytokine concentrations.

The data demonstrated that 800 U ml$^{-1}$ GM–CSF and 2000 U ml$^{-1}$ IL-4 resulted in homogenously matured moDC. We also could show that 400 U ml$^{-1}$ GM-CSF is sufficient for the generation of mature moDC without feeding.

*Influence of different maturation cocktails*

In previous experiments for maturation of moDC a cocktail consisting of TNF-$\alpha$, IL-1$\beta$, IL-6 and

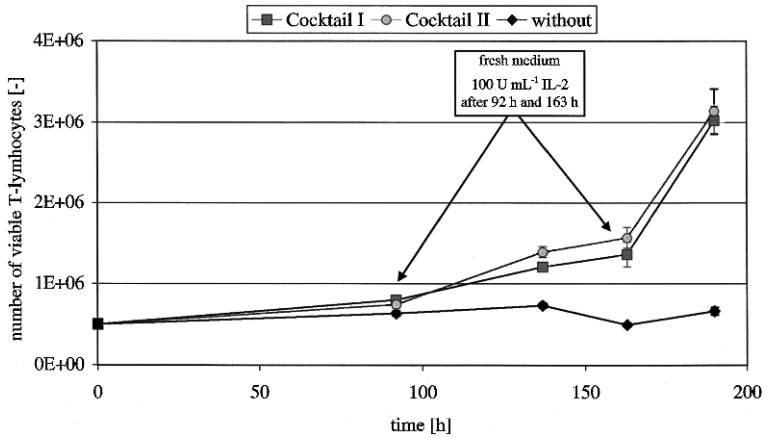


*Figure 6.* Allostimulatory capacity for PBMC from healthy donors. MoDC matured with different maturation cocktails (cocktail I: TNF-$\alpha$, IL-1$\beta$, IL-6 and $PGE_2$; cocktail II: TNF-$\alpha$, $PGE_2$) induced a similar stimulatory capacity in the allogeneic MLR. Shown are mean values (± SD) from three experiments in triplicates.

$PGE_2$ was used. In the following experiments, we examined the feasibility to simplify this maturation cocktail while obtaining the same yield, viability, phenotype and distinct functional capacity of DC. We used 400 U $ml^{-1}$ GM–CSF, 2000 U $ml^{-1}$ IL-4 and inoculated a cell density of $1.3 \times 10^6$ $ml^{-1}$ to differentiate monocytes to dendritic cells.

Therefore, we compared two maturation cocktails: Cocktail I composed of 1000 U $ml^{-1}$ TNF-$\alpha$, 1000 U $ml^{-1}$ IL-1$\beta$, 1000 U $ml^{-1}$ IL-6 and 1-$\mu$g $ml^{-1}$ $PGE_2$ and Cocktail II consisted of 1000 U $ml^{-1}$ TNF-$\alpha$ and 18 $\mu$g $ml^{-1}$ $PGE_2$. We found in 12 experiments similar results in yield, viability and phenotype for both cocktails (data not shown).

A consequence of maturation is usually the secretion of inflammatory cytokines by moDC (Sallusto and Lanzavecchia 1999). However, it has been demonstrated that $PGE_2$ induces the final maturation of IL-12p70 deficient DC (Kalinski et al. 1998). To investigate the amount of bioreactive IL-12p70, we analyzed by ELISA the cell culture supernatant of moDC after 2 days of stimulation with the respective cytokine cocktail. With both stimuli no detectable level of IL-12p70 was produced at any time point (data not shown).

*Mixed lymphocyte reaction*

The allostimulatory capacity of matured moDC was tested using a DC T-cell ratio of 1 : 10. After an induction phase cells were fed twice at 92 h and 163 h by replacement of 50% of the medium and adding of 100 U $ml^{-1}$ IL-2. Figure 6 illustrates the potent stimulatory capacity produced by moDC samples.

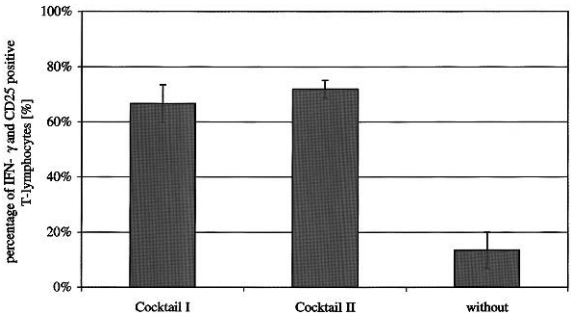


*Figure 7.* Phenotype of PBMC after MLR. Either cocktail I and II induced IFN-$\gamma$ producing T-cells which were also CD25 (IL-2 $\alpha$-chain) positive. Results are expressed as mean (± SD) from three experiments in triplicates.

Testing of the supernatant of the MLR after 4 days for levels of IL-12p70 showed no detectable amount of cytokine. Since $PGE_2$ suppressed IL-12p70 production in matured moDC, we investigated whether a typical $T_H1$ or $T_H2$ cytokine pattern was being induced. After 190 h of cultivation and proliferation of T-lymphocytes the supernatant was tested for secreted IFN-$\gamma$. Either T-cells stimulated by moDC matured with cocktail II or I were highly IFN-$\gamma$ and CD25 positive (Figure 7). ELISA for IL-4 showed no level of cytokine (data not shown). These data suggest that T-lymphocytes stimulated by moDC matured by either cytokine cocktails were polarized towards $T_H1$-type.

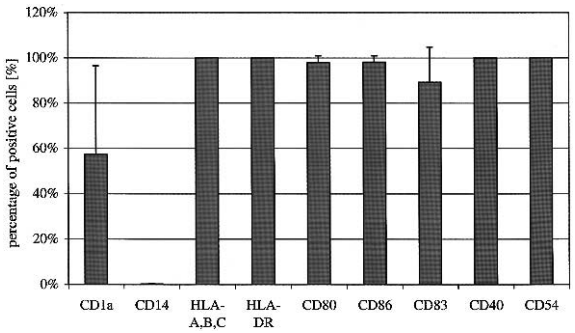


*Figure 8.* Phenotypic analysis of mature moDC generated with the standardized protocol. Shown are results of surface antigen expression as indicated as mean (± SD) from four independent experiments.

*Generation of moDC with optimized parameters*

Based on the optimized parameters, generation of moDC was carried out in 75 $cm^2$ T-flasks. Monocytes were inoculated at a cell density of $1.3 \times 10^6$ $ml^{-1}$ and differentiated with 400 U $ml^{-1}$ GM–CSF and 2000 U $ml^{-1}$ IL-4. On day 6 1000 U $ml^{-1}$ TNF-$\alpha$ and 18 $\mu$g $ml^{-1}$ $PGE_2$ were added to mature the cells and on day 8 the cells were harvested.

No difference was observed regardless of using 48 well plates or 75 $cm^2$ T-flasks. The mean yield as calculated from inoculated CD14+ monocytes was 31 ± 6% with a mean viability of 93 ± 2% ($n = 4$). Figure 8 illustrates the mean percentage of positive DC for important surface antigens like HLA-DR, HLA-A,B,C, costimulatory molecules like CD40, CD80 and CD86, the intercellular adhesion molecule (ICAM-1) CD54 and the standard maturation marker CD83.

## Discussion

The aim of this study was to determine optimal conditions for the generation of moDC by developing a standardized, easy to use and reproducible protocol. We accomplished within this study a non-feeding protocol in serum-free conditions that is suitable for clinical use. Culture of enriched monocytes using serum-free conditions for 6 days resulted in immature moDC with low levels of CD83. Final maturation was induced for additional 2 days by pro-inflammatory cytokines TNF-$\alpha$, IL-1$\beta$ and IL-6 supported by $PGE_2$ (Jonuleit et al. 1997) or TNF-$\alpha$ and $PGE_2$ (Kalinski et al. 1998) alone, both of which resulted in high levels of HLA-DR, CD80, CD83, CD86, CD40 and CD54.

Using this protocol we could obtain moDC with a yield of 31 ± 6% and a viability of 93 ± 2%. We demonstrated that feeding the cells is not necessary and that it is sufficient to only add 400 U $ml^{-1}$ GM–CSF and 2000 U $ml^{-1}$ IL-4 at the beginning of the cultivation. Dietz et al. (2000), who generated moDC using MACS Micro-beads enriched $CD14^+$ monocytes in X-VIVO 15, 1% human AB serum, 800 U $ml^{-1}$ GM–CSF and 1000 U $ml^{-1}$ IL-4 fed the cells every 3rd day by replacing of one-third with fresh medium containing 1600 U $ml^{-1}$ GM–CSF and 1000 U $ml^{-1}$ IL-4 obtained a yield between 11.4% and 31.2%. Berger et al. (2002) reported a moDC yield of 19.9 ± 9.6% with a viability of 94.0% ± 12.0%, using the adherent fraction of PBMC: the cells were cultivated in RPMI 1640 supplemented with 1% autologous, heat-inactivated human plasma, 800 U $ml^{-1}$ GM–CSF and 500 U $ml^{-1}$ IL-4 and fed twice on day 3 and 5 with additional medium, 800 U $ml^{-1}$ GM–CSF and 500 U $ml^{-1}$ IL-4.

Our data suggest that under serum-free conditions the cytokine-cocktails consisting of either TNF-$\alpha$, IL-1$\beta$, IL-6 and $PGE_2$ or TNF-$\alpha$ and $PGE_2$, a simplified cocktail with just two components, are sufficient to induce the final maturation of immature moDC into mature, homogenous immunostimulatory DC. Stimulation of allogeneic naïve T-cells in MLR led to high proliferation with either cytokine cocktail. Production of IFN-$\gamma$ was significantly induced, while no effect on the production of IL-4 or IL-12p70 was seen. These findings are in agreement with Jonuleit et al. (1997), who demonstrated that addition of $PGE_2$ to a cocktail of TNF-$\alpha$, IL-1$\beta$ and IL-6 led to higher IFN-$\gamma$ production in allogeneic primary stimulation. They also observed, that neither $CD4^+$ nor $CD8^+$ T-cells produced IL-4 or IL-10, indicating that these moDC could not support the development of type-2 T-cells. Two studies showed recently that $PGE_2$ regulates the migratory capacity of moDC (Luft et al. 2002; Scandella et al. 2002) and that these migratory-type DC produce lower level of cytokines (including IL-12p70) and induce IFN-$\gamma$ production of T-cells in MLR. These

conclusions were confirmed by several clinical investigations using moDC matured with the cytokine-cocktails utilized in this study (TNF-$\alpha$, IL-1$\beta$, IL-6, $PGE_2$), which induced more potent T-cell immune responses in undergoing immunotherapy patients (Schuler-Thurner et al. 2002; Dhodapkar et al. 2001). These findings were in contrast to Kalinski et al. (1998) who reported that DC matured in the additional presence of $PGE_2$ bias naïve Th cell development toward the Th2. However, this may be caused by different cultivation parameters including FCS in the cultivation setup.

In conclusion, we have shown that we could further simplify the generation of fully mature moDC while maintaining their high stimulatory capacity. Although for the clinical application of DCs the culture will need to take place in a fully enclosed system, for instance cell bags (Guyre et al. 2002). Because of the simplicity of the protocol described here the transfer to such a system should be easily achieved.

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