VRIJE UNIVERSITEIT AMSTERDAM

VU Research Portal

The contribution of ABC transporters to dendritic cell development and function

van de Ven, R.

2009

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)
van de Ven, R. (2009). *The contribution of ABC transporters to dendritic cell development and function*. [PhD-Thesis - Research and graduation internal, S.l.]. s.n.

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:
vuresearchportal.ub@vu.nl

Download date: 21. Apr. 2025

Chapter 4

Exposure of CD34$^+$ precursors to cytostatic anthraquinone-derivatives induces rapid Dendritic Cell differentiation

Submitted

Rieneke van de Ven

Anneke W. Reurs

Pepijn G.J.T.B. Wijnands

Sandra van Wetering

Ada M. Kruisbeek

Erik Hooijberg

George L. Scheffer

Rik J. Scheper

Tanja D. de Gruijl

Abstract

Appropriate activation of Dendritic Cells (DC) is essential for successful active vaccination and induction of cell-mediated immunity. The scarcity of precursor cells, as well as long culture methods, has hampered wide-scale application of DC vaccines derived from $CD34^+$ precursors, despite their suggested superior efficacy over the more commonly applied monocyte-derived DC (MoDC). Here, employing the $CD34^+/CD14^+$ AML-derived human DC cell line MUTZ3, we show that cytostatic anthraquinone-derivatives (i.e. the anthracenedione mitoxantrone and the related anthracyclin doxorubicin) induce rapid differentiation of $CD34^+$ DC precursors into functional antigen presenting cells (APC) in a three-day protocol. The drugs were found to act specifically on $CD34^+$, and not on $CD14^+$ DC precursors. Importantly, these observations were confirmed for primary $CD34^+$ and $CD14^+$ DC precursors from peripheral blood. Mitoxantrone-generated DC were fully differentiated within three days and after an additional twenty-four hours of maturation, were as capable as control 9-day differentiated and matured DC to migrate towards the lymph node-homing chemokines CCL19 and CCL21, to induce primary allogeneic T cell proliferation, and to prime functional MART1-specific $CD8^+$ T lymphocytes. Anthraquinone-derivatives like mitoxantrone may thus be employed either as a differentiation-inducing agent for rapid in vitro generation of DC, or might even be exploited to mature DC precursors in vivo in support of DC-based therapies.

Introduction

Antigen presenting cells are key players in the initiation of an effective immune response.[1] Dendritic cells (DC), which reside in peripheral tissues, are professional APC. DC take up antigens and present derived epitopes to naïve T cells in the context of MHC class I or class II molecules. If proper danger signals are present at the site of antigen uptake, DC will mature and migrate from the tissue to draining lymph nodes (LN), where they encounter, and subsequently activate, antigen-specific naïve T cells. Unfortunately, these processes are often hampered in cancer patients due to prevailing tumor-induced immune suppression, which interferes with the generation of an effective anti-tumor response.[2,3]

Although chemotherapeutic agents at high systemic levels are invariably lethal to immune effector cells, we previously reported that they can actually activate DC when applied locally and might thus act as an adjuvant in vaccination settings.[4] A similar observation was made by Yu et al. who combined DC vaccination with paclitaxel treatment resulting in increased anti-tumor responses.[5] Aiming to develop new immunotherapeutic regimens for cancer treatment, the direct effect of cytostatic anthraquinone-derivatives, i.e. mitoxantrone and doxorubicin, on DC precursor cells was studied. Both mitoxantrone and doxorubicin are used in the clinic to treat various types of cancer [6-12], while mitoxantrone is also used to treat multiple sclerosis. [13] As a source of DC precursor cells we made use of blood-derived $CD14^+$ monocytes and $CD34^+$ precursors as well as of the acute myeloid leukemia cell line MUTZ3. MUTZ3 consists of a $CD34^+$ proliferating fraction, which passes through a $CD34^-CD14^-$ (double negative; DN) 'intermediate' state, to eventually differentiate into a $CD14^+$ fraction with direct DC differentiating potential.[14,15] These MUTZ3 progenitor cells can be differentiated into interstitial DC (MUTZ3-IDC) in a 7-9 day culture protocol with granulocyte macrophage-colony-stimulating factor (GM-CSF), interleukin 4 (IL4) and tumor necrosis factor α (TNFα) or Langerhans Cells (MUTZ3-LC) in a 10-12 day culture protocol with GM-CSF, transforming growth factor β (TGFβ) and TNFα, as previously described.[14,15] In extensive studies MUTZ3-IDC and -LC were shown to accurately reflect their *in vivo* primary skin counterparts –both in terms of phenotype and of function.[16-18] Here we show that *in vitro* exposure of $CD34^+$ precursors to mitoxantrone in the presence of appropriate cytokine cocktails results in accelerated DC/LC differentiation. These DC were fully

functional with respect to migration and T cell stimulation and priming. These data suggest that short-term exposure to anthraquinone-derivatives like mitoxantrone accelerates DC differentiation from $CD34^+$ precursor cells and may be applied as a fast differentiation stimulus in DC cultures for the efficient production of clinical DC vaccines *in vitro*. This is of particular interest for the use of $CD34^+$ precursors, since their general application has been hampered due to the relatively long culture periods required, despite their suggested superiority over monocyte-derived DC in terms of vaccination efficacy.[19]

Materials and methods

Chemicals:

Unless otherwise stated, all chemicals and drugs were obtained from Sigma Chemical Co. (St. Louis, MO) except for Ko-143 which was kindly provided by Dr. Allen (Netherlands Cancer Institute, Amsterdam, The Netherlands) and has been described before.[20]

Cell culture

The AML-derived $CD34^+$ MUTZ3 cell line was cultured as previously described.[14] In brief, MUTZ3 progenitors were cultured in MUTZ3 routine maintenance medium, consisting of MEM-α (Minimum essential medium, Lonza, Verviers, Belgium) containing 20% fetal calf serum (FCS) (Hyclone, Perbio Science, Etten-Leur, The Netherlands), 100 IU/ml sodium-penicillin (pen), 100μg/ml streptomycin (strep), 2 mM L-glutamine (glut), 50μM β-mercaptoethanol (2ME) and 10% conditioned medium (CM) from cultures of the 5637 renal cell carcinoma cell line, in 12-well plates (Co-star) at a concentration of 0.2 million cells/ml and were passaged twice weekly. For mitoxantrone and doxorubicin effects on progenitor cell cultures, 0.1-0.2 million cells/ml of unseparated MUTZ3 cells or $CD34^+$, $CD14^+$ and double negative (DN) magnetic bead sorted (MACS) MUTZ3 subpopulations (Miltenyi Biotec, Bergisch Gladbach, Germany) were seeded in 12-well plates in MUTZ3 routine medium in the absence or presence of 1-16.7 nM mitoxantrone (IC30-IC70) or 16,7-100 nM doxorubicin (IC30-IC70) and were cultured for 72 hours before quantification of viable cells by trypan blue exclusion and phenotypic characterization by flow cytometry. In case of ABC transporter inhibition, 200nM Ko-143 was added on day 0 to block BCRP activity.

MUTZ3-derived Langerhans cells (MUTZ3-LC) were cultured in MEM-α containing 20% FCS, pen/strep/glut, 2ME supplemented with 10ng/ml TGF-β1 (Biovision, Mountain View, CA), 1000 IU/ml rhGM-CSF (Sagramostim, Berlex) and 120 IU/ml TNFα (Strathmann Biotec) for 10 days in 12-well plates at a concentration of 0.1 million cells/ml, adding fresh cytokines on day 4 and 7. Interstitial DC derived from MUTZ3 (MUTZ3-IDC) were cultured in MEM-α containing 20% FCS, pen/strep/glut, 2ME supplemented with 20ng/ml IL-4 (R&D systems Europe, Abingdon, United Kingdom), 1000 IU/ml rhGM-CSF and 120 IU/ml TNFα (MUTZ3-IDC medium) in 12 well plates for 6 days, adding fresh cytokines on day 3. Mitox-DC were generated by culturing MUTZ3 progenitor cells into MUTZ3-IDC with the addition of 2.1 nM mitoxantrone for 3 days. Mitox –LC received 2.1-nM-16.7 nM mitoxantrone for 4 days before phenotypic analysis. Immature cells were matured by adding 2400 IU/ml TNFα, 100ng/ml IL-6 (Strathmann Biotec), 25ng/ml IL-1β (Strathmann Biotec) and 1μg/ml prostaglandin E2 (PGE2) (Sigma Aldrich) for 24 hours. Monocyte-derived DC (MoDC) were generated from monocytes isolated from healthy donor buffy-coats (Sanquin, Amsterdam, The Netherlands) in IMDM supplemented with 10% FCS, pen/strep/glut, 2ME, 1000 IU/ml GM-CSF and 10ng/ml IL4 in the absence or presence of 16.7nM mitoxantrone for 4-5 days.

.

$CD34^+$ haematopoietic progenitor cells

$CD34^+$ haematopoietic progenitor cells were isolated from blood of healthy donors and expanded for 2-5 weeks with 25ng/ml *fms*-like tyrosine kinase-3 ligand (Flt3-L) and 10ng/ml stem cell factor (SCF) as described previously.[21] To study the effect of mitoxantrone on these cells, thawed expanded $CD34^+$ progenitor cells were cultured with or without 16.7nM mitoxantrone for 72 hours in the presence of 10ng/ml Flt3-L and SCF. To study effects on LC differentiation, $CD34^+$ progenitors were cultured with or without 16.7nM mitoxantrone for 72 hours in the presence of 1000 IU/ml rhGM-CSF, 10ng/ml TGFβ and 120 IU/ml TNFα. After 72 hours, phenotypic analysis was performed by flow cytometry.

Flow cytometric immunophenotypical analyses

Cells were immunophenotyped using the following FITC- and /or PE-conjugated Mabs reactive against: CD1a (1:25), CD54 (1:25), CD80 (1:25), CD86 (1:25), CD40 (1:10) (PharMingen, San Diego, CA), CD14 (1:25), HLA-DR (1:25), DC-SIGN (1:10) (BD Biosciences, San Jose, CA), CD83 (1:10), CD34 (1:10), Langerin (1:10) (Immunotech, Marseille, France). In short, 2.5 to $5 \cdot 10^4$ cells were washed in PBS supplemented with 0.1% BSA and 0.02% NaN_3 and incubated with specific or corresponding control Mabs for 30 minutes at 4°C. Cells were washed and analyzed on a FACS-Calibur flow cytometer (Becton and Dickinson, San Jose, CA) equipped with CellQuest analysis software. Results were expressed as mean or median fluorescence intensity or the percentage of positive cells, as indicated.

Allogeneic Mixed leukocyte reaction (alloMLR)

$1 \cdot 10^2$ - $3 \cdot 10^4$ DC were co-cultured with $1 \cdot 10^5$ peripheral blood lymphocytes (PBL) for 4 days in 96-wells plates in IMDM containing 10% human pooled serum (Sanquin, Amsterdam, The Netherlands), pen/strep/glut and 2ME. At day 4, 2.5 μCi/ml [^{3}H]-thymidine (6.7Ci/mmol, MP Biomedicals, Irvine, CA) was added per well for 16 hours. Plates were harvested onto glass fiber filtermats (Packard Instruments, Groningen, The Netherlands) using a Skatron cell harvester (Skatron Instruments, Norway), and [^{3}H]-thymidine incorporation was quantified using a Topcount NXT Microbetacounter (Packard, Meriden, CT).

Trans-well migration towards CCL19 and CCL21

For in vitro trans-well migration assays, 10^5 mature d7 MUTZ3-IDC or d4 mitox-DC were seeded in the upper compartment of Costar 24-well trans-wells with a pore-size of 6 μm. The lower compartment contained 600μl serum free MEM-α supplemented with pen/strep/glut, and 250 ng/ml CCL19 (Peprotech, Huissen, The Netherlands) or CCL21 (Invitrogen, Carlsbad, CA). Cells were allowed to migrate for 4 hours at 37°C. After migration, 500 μl medium was harvested from the lower compartment and migrated cells were quantified with flow-count fluorospheres (Beckman Coulter, Fullerton, CA) by flow cytometry.

In vitro CTL-priming

The *in vitro* priming of MART1 specific CTL was performed as described previously.[22] In short, mature d7 MUTZ3-DC and d4 mitox-DC, at a concentration of 1.0 million cells/ml, were loaded with 1μg/ml MART1 $_{26\text{-}35L}$ peptide in serum free IMDM for 3-4 hours in the presence of 3μg/ml β2-microglobulin (β2M). After loading, cells were irradiated at 5000 rad, washed and seeded at 0.2 million cells/ml in Yssels medium[23] supplemented with 2% hAB serum (ICN Biochemicals), pen/strep/glut, 2ME, 10ng/ml IL6 and 10ng/ml IL12 in 24-well plates. 0.1million loaded DC were co-cultured with 1.0 million CD8β^+ T cells, isolated from an HLA-A2$^+$ donor by magnetic-bead sorting and 0.75-1.0 million, irradiated (5000 rad) CD8β^- cells from the same donor, both diluted in Yssels medium. For each DC condition, 6 priming wells were started and the experiment was performed with 3 different HLA-A2$^+$ donors. On day 10 and 19, CTL were re-stimulated with 10ng/ml MART1 $_{26\text{-}35L}$ loaded mature d7 MUTZ3-DC or d4 mitox-DC in the presence of 10ng/ml IL-7. On day 12 and 21, 10 IU/ml IL-2 was added per well. MART1 tetramer (Tm) analysis was performed on CD8$^+$ T cells on day 10 (1st restimulation) and 24 (2nd restimulation) using PE- and APC-labeled MART1 $_{26\text{-}35L}$ Tm.

Intracellular IFNγ assay

The determine whether the primed MART1 $_{26\text{-}35L}$ specific CTL could recognize and respond to target cells, an intracellular IFNγ staining was performed as described previously. [22] As target cells, JY cells were pulsed with either irrelevant peptide (BCRabl $_{926\text{-}935}$) or with the MART1 $_{26\text{-}35L}$ peptide (1μg/ml) in the presence of 3μg/ml β2M. CTL were cultured with the JY cells in a 2:1 E:T ratio (effector:target cell) for 4 hours. 0.5μl Golgiplug (BD Biosciences) was added to each well after 1 hour of stimulation. After 4 hours, cells were harvested, washed and stained with APC-labeled MART1 Tm and PE-labeled anti-CD8 antibodies. After fixation with cytofix/cytoperm (BD Biosciences) and permeabilization with 1x BD perm/wash solution (BD Biosciences), cells were stained with FITC-labeled anti-IFNγ. Stained cells were analyzed by flow cytometry. The percentage of cells responding to the irrelevant peptide was subtracted from the percentage of cells responding to the relevant peptide.

Statistical analysis

Statistical analysis of the data was performed using the paired two-tailed student's T-test. Differences were considered statistically significant when $p<0.05$.

Results

Mitoxantrone induces differentiation of MUTZ3 progenitors

MUTZ3 progenitor cells were analyzed for their sensitivity to mitoxantrone, in a ninety-six hour toxicity assay, MUTZ3 progenitor cells were found to be sensitive to mitoxantrone with an IC50 value of 1.5 ± 0.5 nM (n=3).

In studying the cytotoxic effects of mitoxantrone on MUTZ3 progenitors, morphological changes reflecting DC differentiation became apparent at concentrations exceeding 5.6nM (IC60). MUTZ3 progenitors were therefore cultured for seventy-two hours in the presence of 5.6nM or 16.7nM (IC70) mitoxantrone and subsequently analyzed for expansion [Figure 1A] and DC marker expression [Figure 1B]. These analyses revealed that at these dose levels, mitoxantrone dramatically compromised cell division and drove the surviving MUTZ3 progenitors to DC differentiation, reflected by an altered $CD34^+/CD14^+$ ratio in favor of the direct $CD14^+$ DC precursor subset and low-level expression of the LC markers CD1a and Langerin and the co-stimulatory molecules CD86 and CD80 [Figure 1B]. Similar experiments were carried out with the related drug doxorubicin. Like mitoxantrone, the IC70 concentration of doxorubicin (~100nM) induced LC differentiation of MUTZ3 progenitors [Figure 2], whereas no such effects were observed at lower concentrations of mitoxantrone or doxorubicin (data not shown).

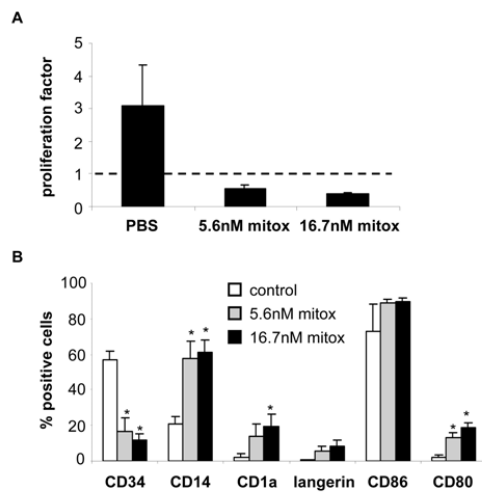

Figure 1. Mitoxantrone induces LC differentiation of MUTZ3 precursor cells.
A) MUTZ-3 precursor cell proliferation was determined in the presence of PBS, 5.6nM or 16.6nM mitoxantrone. Shown is the fold expansion over 3-4 days of culture. **B)** MUTZ-3 precursor cells cultured in the presence of PBS, 5.6nM or 16.7nM mitoxantrone were phenotyped by flow cytometry for typical DC/LC markers (n=3). P<0.05 compared to the PBS cultures.

Table 1: percentage of viable cells after drug-treatment (IC70), relative to PBS control.

	Percentage viable cells compared to PBS control	p-value
$CD14^+$		
mitoxantrone	120 ± 27	p > 0.05
doxorubicin	100 ± 71	p > 0.05
$CD34^+$		
mitoxantrone	4 ± 3	p < 0.01
doxorubicin	6 ± 6	p < 0.01
double negative (DN)		
mitoxantrone	20 ± 4	p = 0.02
doxorubicin	27 ± 7	p = 0.04

CD34⁺, but not CD14⁺, MUTZ3 cells undergo drug-induced differentiation

Isolated $CD34^+$ and $CD14^+$ MUTZ3 cells were incubated with 16.7nM mitoxantrone or 100nM doxorubicin (both IC70) for seventy-two hours to analyze which population was drug-responsive, i.e. was induced to differentiate. Table I shows the percentage
of viable cells after seventy-two hours relative to the amount of viable cells present
in the control cultures, as determined by trypan blue exclusion. $CD14^+$ cells were not reduced in viable cell numbers upon mitoxantrone or doxorubicin exposure, whereas $CD34^+$ cells were. Further analysis revealed that the phenotypic effects of mitoxantrone and doxorubicin on the MUTZ3 progenitor cells were entirely attributable to effects on the $CD34^+$ population [Figure 2]. $CD14^+$ cells were not affected by incubation with these anthraquinone-derivatives [Figure 2A and C]. In contrast, higher frequencies of $CD14^+$ cells, as well as *de novo* arising fully differentiated $CD1a^+Langerin^+$ DC, were found in cultures of $CD34^+$ sorted cells upon treatment with both drugs [Figure 2B and C]. This differentiation effect could also be visualized by alterations in the forward/side scatter (FSC/SSC) as a clear shift in SSC in the drug-treated $CD34^+$ cells, which was not present in drug-treated $CD14^+$ cells [figure 2A and B]. The graphs in figure 2c show the average of induced CD1a and Langerin expression rates within the two isolated subsets after seventy-two hours of PBS, mitoxantrone, or doxorubicin treatment (n=3; p= 0.02 for CD1a and p= 0.03 for Langerin, comparing the $CD34^+$ mitoxantrone and PBS treated cells). Similarly to mitoxantrone, doxorubicin induced CD1a and Langerin expression on $CD34^+$ MUTZ3 cells.

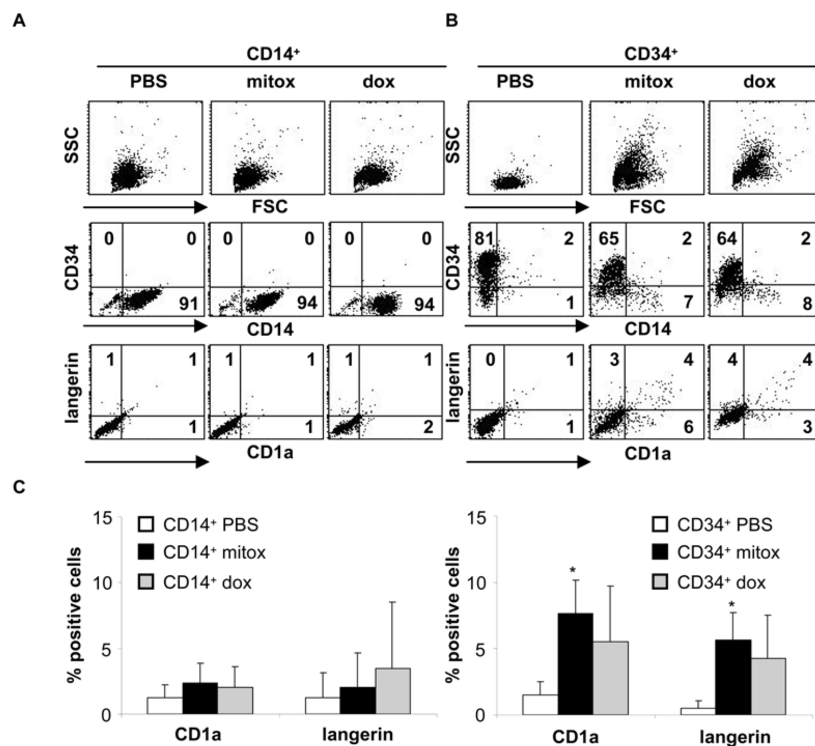

Figure 2. Cytostatic anthraquinone-derivatives induce differentiation in $CD34^+$, but not $CD14^+$, precursors.
A) $CD14^+$ and $CD34^+$ MUTZ-3 precursor cells were isolated and cultured in the presence of 16.7nM mitoxantrone or 100nM doxorubicin for 72 hours. Shown are the FSC/SSC plots, CD14, CD34, CD1a and Langerin expression on the PBS- and drug-treated precursor cells. **B)** The percentages of CD1a and Langerin expressing cells in $CD14^+$ and $CD34^+$ MUTZ-3 precursor cells after 72 hours in the presence of PBS, 16.7nM mitoxantrone or 100nM doxorubicin are shown (n=3). P<0.05 compared to the PBS cultures.

Cytostatic anthraquinone-derivatives accelerate LC differentiation

Next, we tested whether the addition of the anthraquinone-derivatives mitoxantrone or doxorubicin at the start of MUTZ3-LC differentiation cultures (i.e. in the presence of GM-CSF, TNFα, and TGFβ) could boost differentiation. Addition of a single dose of 16.7nM mitoxantrone at day-0 of MUTZ3-LC differentiation, resulted in fully differentiated cells with high expression levels of specific LC markers on day 4, whereas control cultures usually take 8-10 days to induce fully fledged LC with all the typical phenotypic hallmarks. In figure 3A FSC/SSC and CD1a/Langerin plots of day-4 LC cultures with PBS or mitoxantrone are shown. Clearly, the mitoxantrone-treated cells were more differentiated as they displayed a more typical dendritic morphology in the FSC/SSC plot (i.e. high SSC levels) and three-fold higher CD1a and Langerin expression rates as compared to the control culture. Mitoxantrone-treated cells also showed enhanced expression of the co-stimulatory molecules CD80 and CD86 and of HLA-DR and CD54 [Figure 3B]. Figure 3C shows combined CD1a, Langerin and CD83 expression data from 3 experiments. Beside an increase in the percentage of CD1a- and Langerin-expressing cells (p= 0.02 and p= 0.01 respectively), the percentages of $CD14^+$ and $CD34^+$ cells were decreased (p= 0.04 and p= 0.02, respectively; data not shown) and the amount of cells expressing the maturation marker CD83 was increased upon mitoxantrone treatment (p= 0.03). Comparable results were obtained for doxorubicin (data not shown).

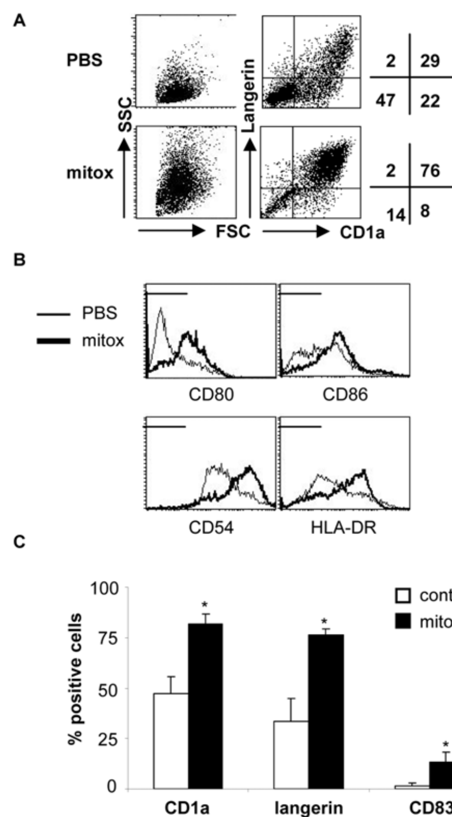

Figure 3. Langerhans cell (LC) differentiation is accelerated by mitoxantrone.
A) MUTZ3-LC differentiation was performed in the presence of PBS or 16.7nM mitoxantrone. At day 4, flow cytometric analysis was performed for DC markers. FSC/SSC and CD1a/Langerin dotplots are shown, revealing enhanced differentiation in the mitoxantrone-treated sample. **B)** Histogram plots for the markers CD80, CD86, CD54 and HLA-DR are shown. PBS control expression levels are indicated with the normal lines, whereas expression levels on mitoxantrone-treated LC are indicated with the bold lines. Markers indicate isotype fluorescence range. (a and b, experiment representative of three). **C)** Average percentages of CD1a, Langerin and CD83 positive cells within PBS control (white bars) and mitoxantrone-treated cultures (black bars) are shown (n=3). P<0.05 compared to the PBS cultures.

CD34$^+$ haematopoietic progenitors, but not CD14$^+$ monocytes, respond to mitoxantrone

To establish whether primary human CD34$^+$ precursors from blood responded in a similar manner to anthraquinone-derivatives as the CD34$^+$ MUTZ3 cells, CD34$^+$ haematopoietic precursors were isolated from human blood, expanded over a period of 1-4 weeks with Flt3Ligand (Flt3L), trombopoietin and stem cell factor (SCF), to obtain sufficient numbers while maintaining their DC differentiation capacity,[21] and treated with mitoxantrone.

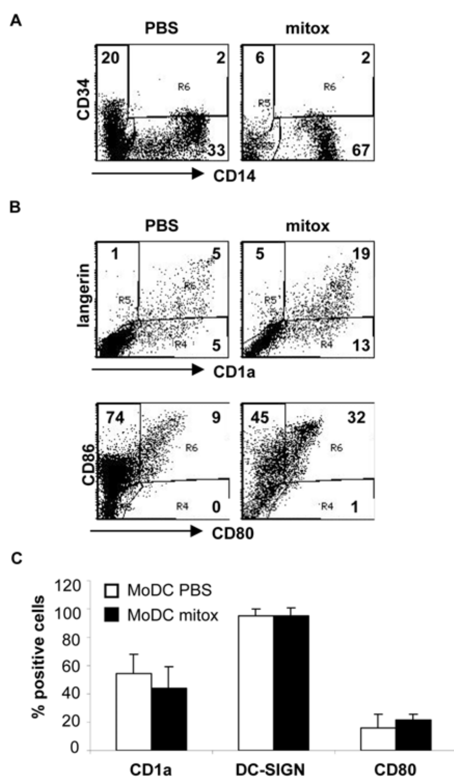

Figure 4. CD34$^+$, and not CD14$^+$, blood precursors respond to mitoxantrone.
A) Expanded CD34$^+$ blood precursor cells were cultured with PBS or 16.7nM mitoxantrone and were analyzed for marker expression after 72 hours (representative of 2 experiments). **B)** Expanded CD34$^+$ blood precursor cells were cultured in the presence of LC-differentiating cytokines supplemented with PBS or 16.7nM mitoxantrone and were analyzed for LC marker expression after 72 hours. Shown are CD1a/Langerin and CD80/CD86 dot plots (representative of 2 experiments). **C)** CD14$^+$ monocytes were differentiated into MoDC in the presence of PBS or 16.7nM mitoxantrone for 96 hours. Shown are the average percentages for CD1a, DC-SIGN and CD80 of PBS- (white bars) or mitoxantrone-treated (black bars) MoDC (n=4).

Mitoxantrone treatment of the expanded precursors resulted in a loss of CD34 expression and a gain of CD14 expression [Figure 4A] but not in a significant increase in CD1a or Langerin expression (data not shown). This is consistent with an incomplete DC differentiation induction. However, in the presence of LC differentiation inducing cytokines (i.e. GM-CSF, TGFβ and TNFα), the addition of 16.7nM mitoxantrone did result in an increased percentage of CD1a$^+$ Langerin$^+$ cells and in the induced expression of the co-stimulatory markers CD80 and CD86 within seventy-two hours [Figure 4B] (n=2), indicative of accelerated complete DC differentiation. In contrast, when similar experiments were performed with CD14$^+$ monocytes isolated from blood, in analogy with the CD14$^+$ MUTZ3 data, there was no effect on cell viability (data not shown), nor was any accelerated differentiation observed as illustrated by the percentages of cells positive for CD1a, DC-SIGN and CD80 [Figure 4C; n=4].

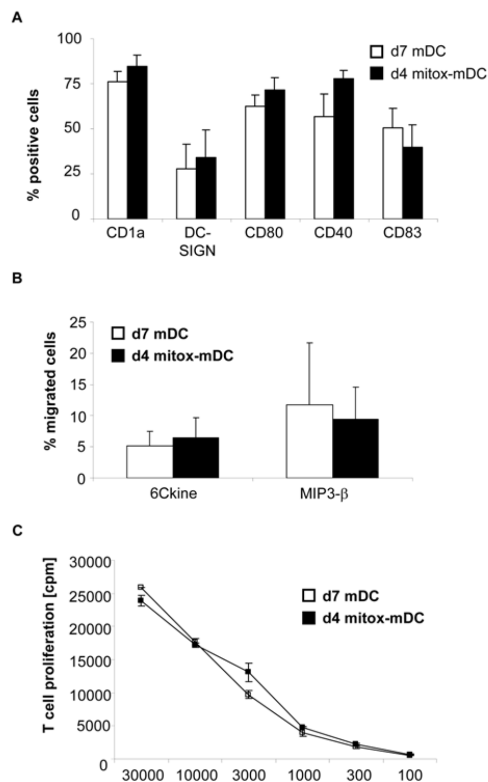

Figure 5. Day 4 mitox-DC are equally functional as day 7 control DC.A) Mitox-DC differentiated for 3 days in the presence of 2.1nM mitoxantrone and matured for 24 hours with a maturation cocktail were phenotyped by flow cytometry and compared to conventional day 7 MUTZ3-mDC (n=5). B) D4 mitox-mDC were compared to d7 MUTZ3-mDC for their migratory capacity towards CCL19 and CCL21 in a trans-well migration assay (n=3). C) D4 mitox-mDC were compared to d7 MUTZ3-mDC for their T cell stimulatory capacity in an alloMLR (representative experiment of 3).

High yields of rapidly differentiated and fully functional DC upon exposure to low-dose mitoxantrone

Their differentiation-accelerating capacity, might make anthraquinone-derivatives attractive supplements to culture media formulated for the *in vitro* differentiation of DC from CD34$^+$ progenitors for immunotherapeutic purposes. Hence, we analyzed whether the concentration of mitoxantrone could be reduced in order to increase the yield of viable cells without losing the rapid differentiation advantage. A concentration range of mitoxantrone (1-16.7nM) was tested to identify the least toxic concentration with maintained stimulatory effects on DC differentiation in the presence of DC-differentiating cytokines. The phenotype of the cells was analyzed for DC characteristics after seventy-two hours. A robust accelerating effect on MUTZ3-IDC [Figure 5A] and –LC (data not shown) differentiation was still observed when 2 nM mitoxantrone was added (n=5), with eighty percent viability of the cells in both the control and the mitoxantrone-exposed cultures. The percentages of CD1a, DC-SIGN, CD80, CD40 and CD83 expressing cells were determined among matured MUTZ3-IDC from 7-day conventional cultures (i.e. d7 mDC) and from 4 day cultures containing 2 nM mitoxantrone (i.e. d4 mitox-mDC) [Figure 5A]. The short culture protocol in the presence of 2 nM mitoxantrone resulted in a comparable phenotype to conventional 7 day cultures [Figure 5A], without the need for further addition of fresh cytokines during culture –as is the case for the conventional IDC cultures. If mitox-DC are to be used for future vaccination strategies, they need to be functionally active in at least a comparable fashion to conventional DC. Indeed, d4 mitox-mDC were as able as conventional d7 MUTZ3-mDC to migrate towards the chemokines CCL19 and CCL21 in a trans-well assay [Figure 5B] and to stimulate allogeneic T cell proliferation in a Mixed Leukocyte Reaction (MLR) [Figure 5C].

Efficient priming of MART-1 specific T cells by mitox-DC

A crucial function of DC in anti-tumor vaccination strategies is the ability of the cells to prime tumor antigen-specific $CD8^+$ effector T cells. Hence, d4 mitox-mDC and d7 MUTZ3-mDC were compared in their capacity to *in vitro* prime $CD8^+$ T cells against the immunodominant HLA-A2-restricted aa26-35L epitope of the well characterized melanoma antigen MART-1. Upon peptide loading, both DC types displayed a similar priming efficiency, as revealed by the frequencies of MART1-specific tetramer (Tm)-positive cells among $CD8^+$ T cell bulk cultures from three separate HLA-A2-matched donors. Figure 6 shows the percentage of Tm+ CTL for 6 bulk cultures from two HLA-$A2^+$ donor after the first re-stimulation and the second re-stimulation [Figure 6A] with peptide-loaded DC. Unloaded (d7) DC were taken along as a negative control. From the total of 24 bulk cultures (n=6 per donor) in which CTL-priming was initiated, MART1 Tm+ CTL could be detected in 22/24 cultures primed with d4 mitox-DC, compared to 23/24 cultures primed with d7 control MUTZ3-mDC. Figure 6B shows the capacity of the primed CTL (determined for 6 bulk cultures) to secrete IFNγ upon recognition of the MART-$1_{25\text{-}36L}$ epitope. JY cells loaded with control peptide were included as a negative control. MART-1 Tm-positive CTL primed with d7-mDC or d4 mitox-mDC produced IFNγ upon stimulation with MART-1 peptide-loaded JY cells (47.3±4.6% for d7 mDC-primed CTL and 52±2.7% for d4 mitox-mDC-primed CTL), showing that rapidly differentiated mitox-DC are fully functional and equivalent to their conventionally differentiated counterparts with respect to their capacity to prime functional antigen-specific $CD8^+$ T cells.

Figure 6. Day 4 mitox-DC can prime antigen-specific $CD8^+$ T cells.
A) D4 mitox-mDC and d7 MUTZ3-mDC were loaded with $MART1_{26\text{-}35L}$ peptides and were used to prime MART1-specific CTL from HLA-A2+ donors. Shown are the percentages of MART1-tetramer positive CTL within 6 bulk cultures generated from two HLA-A2+ donors after two and three rounds of stimulation with peptide-loaded DC (of n=4 tested). **B)** $MART1_{25\text{-}36L}$ Tm-positive T cells were analyzed for IFNγ release upon target recognition. CTL bulk cultures were stimulated with JY cells pulsed with irrelevant or relevant peptide. The graph shows the percentages of IFNγ positive Tm^+ CTL that specifically recognized the relevant $MART1_{26\text{-}35L}$ epitope (experiment representative of 2).

Discussion

In this study we explored whether anthraquinone-based cytostatic drugs could be used to improve *in vitro* DC differentiation. We have shown that the cytostatic drugs mitoxantrone and doxorubicin induce cell death of DC precursors on the one hand, but differentiation of the surviving $CD34^+$ DC precursors on the other. In addition, when added at the start of differentiation in combination with DC or LC differentiation-inducing cytokines, these drugs dramatically promoted differentiation resulting in accelerated maturation of DC/LC from $CD34^+$ precursors.

In the past, systemic and local administration of carefully selected and optimally dosed cytostatic agents has been shown to enhance cellular immunity.[24] Whereas systemic administration of cyclophosphamide (CY) had severe effects like B cell depletion [25], Limpens *et al.* demonstrated that local administration of the active CY-derivative Z 7557 could prevent this B cell depletion while immunostimulatory effects were maintained.[26] In a later paper the authors showed that local administration of Z-7557 resulted in more activated DC within the regional lymph nodes.[4] Our *in vitro* data showed induction and acceleration of DC differentiation upon treatment of $CD34^+$, but not $CD14^+$, precursor cells with mitoxantrone or doxorubicin. In DC precursor cultures without added cytokines, this cell death seemed to be essential for DC differentiation induction as 16.7nM doxorubicin (IC30) was not sufficient to induce differentiation of progenitor cells, whereas 100nm (IC70) was (data not shown). Possibly, the massive cell death in the cultures provoked an endogenous danger signal (e.g. endogenous Toll-like receptor ligands[27,28]), inducing differentiation of the surviving cells. Importantly, the sensitivity to mitoxantrone-induced cytotoxicity was reduced when differentiation-inducing cytokines were present in culture (likely due to halted proliferation of $CD34^+$ progenitors) and a window was established at lower dosages of mixantrone, in which accelerated DC differentiation was achieved without excess cell death. This finding makes it possible to apply cytostatic anthraquinone-derivates (like e.g. doxorubicin and mitoxantrone, respectively) in $CD34^+$ precursor-derived DC differentiation cultures for clinical vaccination, with the express purpose to reduce the required culture time and thus increase the cost-effectiveness of this approach.

One possible cause of the observed induction and acceleration of DC differentiation could be the drug-induced expression of the ABC transporter BCRP (ABCG2), as mitoxantrone is a highly efficient BCRP substrate and we previously found that introduction of functional BCRP into MUTZ3 progenitor cells promoted accelerated LC differentiation (van de Ven et al., submitted for publication). However, no induction of BCRP expression was observed in either $CD14^+$ or $CD34^+$ MUTZ3 cells upon mitoxantrone treatment, nor could inhibition of BCRP activity with the antagonist Ko-143 prevent mitoxantrone-mediated DC differentiation of precursor cells or accelerated differentiation in the presence of cytokines (data not shown), indicating a BCRP-independent mechanism. Another possible underlying mechanism could be the induction of intracellular diacylglycerol (DAG). Bettaïeb *et al*. showed that mitoxantrone, as well as the related anthracyclin daunorubicin, induced rapid synthesis of DAG from sphingomyelin in U937 cells.[29] Previous reports had shown similar effects with doxorubicin and cisplatin.[30,31] DAG is known to activate protein kinase C (PKC) , which was previously shown to be sufficient for the induction of DC differentiation from $CD34^+$ haematopoietic progenitor cells upon their culture with the DAG-analog phorbol 12-myristate 13-acetate (PMA)[32] or with DC-inducing cytokines.[33] In this context, it is of particular interest that PKC has been implicated in the activation of the nuclear factor κB (NFκB) sub-component RelB, which in turn is linked to DC differentiation and activation.[34-36] Whether these signal transduction events are indeed induced downstream of cytostatic anthraquinone-derivatives like mitoxantrone and affect the DC differentiation-promoting effects remains to be established.

Functional capacities of *in vitro* cultured DC to be used for vaccination purposes are vitally important. The mitoxantrone-generated DC, although only differentiated for 3 days and matured with a standard maturation cocktail for twenty-four hours, were functionally equivalent to their conventionally cultured counterparts (i.e. for 7-9 days) in every way tested: they were capable of migration towards the LN-homing chemokines CCL19 and

CCL21 and capable of the induction of allogeneic T cell proliferation as well as of tumor antigen specific $CD8^+$ T cells able to secrete IFNγ upon recognition of their specific epitope. Previously we have shown that such tumor-specific $CD8^+$T cells induced by MUTZ3-DC are functional with respect to their recognition and elimination of the targeted tumor cells.[22] This method of DC differentiation could thus be a less time-consuming, more cost-effective method of generating *in vitro* cultured clinical-grade DC for tumor vaccination purposes.

Finally, our data and those of others[4,5] also suggest that local administration of cytostatic anthraquinone-derivatives like anthracyclins or anthracenediones at vaccination sites with resident DC precursors, e.g. the skin, might lead to their rapid differentiation and maturation. As such, these cytostatic drugs might act as DC potentiating vaccine adjuvants. In line with this, we found intradermal injection of mitoxantrone to lead to increased numbers of mature DC migrating from human skin explants (data not shown). In addition, the combination of anthraquinone-based chemotherapy and the administration of DC differentiation-inducing cytokines (e.g. GM-CSF) might lead to the simultaneous rapid maturation of functional DC and the release of tumor-associated antigens from dying tumor cells: a seemingly ideal scenario for in vivo tumor immunization. In line with this, Apetoh *et al*. previously showed that anthracyclins (i.e. doxorubicin) can induce immunogenic tumor cell death due to the release of the high mobility group box 1 (HMGB1) protein from dying cells, which can induce DC activation through interaction with TLR4.[28]

The optimized *in vitro* DC culture system, using low-dose mitoxantrone as a differentiation-accelerating supplement in clinical-grade media, is currently under further development for clinical purposes, as it is less time-consuming and more cost-effective than the standard culture protocols used to date for the generation of clinical-grade DC vaccines.

Acknowledgements

This work was supported by a grant from the Dutch Cancer Society (KWF) to R.J.S., G.L.S. and T.D.G. (KWF2003-2830).

Disclosures

This work was patented by DC Prime BV.

Reference List

(1) Banchereau J, Briere F, Caux C et al. Immunobiology of dendritic cells. Annu Rev Immunol. 2000;18:767-811.

(2) Gabrilovich DI, Corak J, Ciernik IF, Kavanaugh D, Carbone DP. Decreased antigen presentation by dendritic cells in patients with breast cancer. Clin Cancer Res. 1997;3:483-490.

(3) Kusmartsev S, Gabrilovich DI. Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer. Cancer Metastasis Rev. 2006;25:323-331.

(4) Limpens J, Van Meijer M, Van Santen HM et al. Alterations in dendritic cell phenotype and function associated with immunoenhancing effects of a subcutaneously administered cyclophosphamide derivative. Immunology. 1991;73:255-263.

(5) Yu B, Kusmartsev S, Cheng F et al. Effective combination of chemotherapy and dendritic cell administration for the treatment of advanced-stage experimental breast cancer. Clin Cancer Res. 2003;9:285-294.

(6) Bosch F, Ferrer A, Villamor N et al. Fludarabine, cyclophosphamide, and mitoxantrone as initial therapy of chronic lymphocytic leukemia: high response rate and disease eradication. Clin Cancer Res. 2008;14:155-161.

(7) Mike S, Harrison C, Coles B et al. Chemotherapy for hormone-refractory prostate cancer. Cochrane Database Syst Rev. 2006;CD005247.

(8) Oyan B, Koc Y, Ozdemir E et al. High dose sequential chemotherapy and autologous stem cell transplantation in patients with relapsed/refractory lymphoma. Leuk Lymphoma. 2006;47:1545-1552.

(9) Onyenadum A, Gogas H, Kosmidis P et al. Mitoxantrone plus gemcitabine in pretreated patients with metastatic breast cancer. J Chemother. 2006;18:192-198.

(10) Long HJ, III, Nelimark RA, Podratz KC et al. Phase III comparison of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) vs. doxorubicin and cisplatin (AC) in women with advanced primary or recurrent metastatic carcinoma of the uterine endometrium. Gynecol Oncol. 2006;100:501-505.

(11) Michallet AS, Coiffier B. Recent developments in the treatment of aggressive non-Hodgkin lymphoma. Blood Rev. 2008.

(12) Yardley DA, Burris HA, III, Farley CP et al. A phase II feasibility trial of dose-dense docetaxel followed by doxorubicin/cyclophosphamide as adjuvant or neoadjuvant treatment for women with node-positive or high-risk node-negative breast cancer. Clin Breast Cancer. 2008;8:242-248.

(13) Scott LJ, Figgitt DP. Mitoxantrone: a review of its use in multiple sclerosis. CNS Drugs. 2004;18:379-396.

(14) Masterson AJ, Sombroek CC, de Gruijl TD et al. MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors. Blood. 2002;100:701-703.

(15) Santegoets SJ, Masterson AJ, van der Sluis PC et al. A CD34+ human cell line model of myeloid dendritic cell differentiation: evidence for a CD14+CD11b+ Langerhans cell precursor. J Leukoc Biol. 2006;80:1337-1344.

(16) Santegoets SJ, Bontkes HJ, Stam AG et al. Inducing antitumor T cell immunity: comparative functional analysis of interstitial versus Langerhans dendritic cells in a human cell line model. J Immunol. 2008;180:4540-4549.

(17) Santegoets SJ, van den Eertwegh AJ, van de Loosdrecht AA, Scheper RJ, de Gruijl TD. Human dendritic cell line models for DC differentiation and clinical DC vaccination studies. J Leukoc Biol. 2008.

(18) van Helden SF, van Leeuwen FN, Figdor CG. Human and murine model cell lines for dendritic cell biology evaluated. Immunol Lett. 2008;117:191-197.

(19) Palucka AK, Ueno H, Fay JW, Banchereau J. Taming cancer by inducing immunity via dendritic cells. Immunol Rev. 2007;220:129-150.

(20) van Loevezijn A, Allen JD, Schinkel AH, Koomen GJ. Inhibition of BCRP-mediated drug efflux by fumitremorgin-type indolyl diketopiperazines. Bioorg Med Chem Lett. 2001;11:29-32.

(21) Bontkes HJ, de Gruijl TD, Schuurhuis GJ et al. Expansion of dendritic cell precursors from human CD34(+) progenitor cells isolated from healthy donor blood; growth factor combination determines proliferation rate and functional outcome. J Leukoc Biol. 2002;72:321-329.

(22) Santegoets SJ, Schreurs MW, Masterson AJ et al. In vitro priming of tumor-specific cytotoxic T lymphocytes using allogeneic dendritic cells derived from the human MUTZ-3 cell line. Cancer Immunol Immunother. 2006;55:1480-1490.

(23) Yssel H, De Vries JE, Koken M, Van Blitterswijk W, Spits H. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J Immunol Methods. 1984;72:219-227.

(24) Scheper RJ, Limpens J, Tan BT et al. Immunotherapeutic effects of local chemotherapy with an active metabolite of cyclophosphamide. Methods Find Exp Clin Pharmacol. 1987;9:611-615.

(25) Stockman GD, Heim LR, South MA, Trentin JJ. Differential effects of cyclophosphamide on the B and T cell compartments of adult mice. J Immunol. 1973;110:277-282.

(26) Limpens J, Garssen J, Germeraad WT, Scheper RJ. Enhancing effects of locally administered cytostatic drugs on T effector cell functions in mice. Int J Immunopharmacol. 1990;12:77-88.

(27) Messmer D, Yang H, Telusma G et al. High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization. J Immunol. 2004;173:307-313.

(28) Apetoh L, Ghiringhelli F, Tesniere A et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007;13:1050-1059.

(29) Bettaieb A, Plo I, Mansat-de Mas V et al. Daunorubicin- and mitoxantrone-triggered phosphatidylcholine hydrolysis: implication in drug-induced ceramide generation and apoptosis. Mol Pharmacol. 1999;55:118-125.

(30) Posada J, Vichi P, Tritton TR. Protein kinase C in adriamycin action and resistance in mouse sarcoma 180 cells. Cancer Res. 1989;49:6634-6639.

(31) Rubin E, Kharbanda S, Gunji H, Weichselbaum R, Kufe D. cis-Diamminedichloroplatinum(II) induces c-jun expression in human myeloid leukemia cells: potential involvement of a protein kinase C-dependent signaling pathway. Cancer Res. 1992;52:878-882.

(32) Davis TA, Saini AA, Blair PJ et al. Phorbol esters induce differentiation of human CD34+ hemopoietic progenitors to dendritic cells: evidence for protein kinase C-mediated signaling. J Immunol. 1998;160:3689-3697.

(33) St Louis DC, Woodcock JB, Franzoso G et al. Evidence for distinct intracellular signaling pathways in CD34+ progenitor to dendritic cell differentiation from a human cell line model. J Immunol. 1999;162:3237-3248.

(34) Clark GJ, Gunningham S, Troy A, Vuckovic S, Hart DN. Expression of the RelB transcription factor correlates with the activation of human dendritic cells. Immunology. 1999;98:189-196.

(35) Cejas PJ, Carlson LM, Kolonias D et al. Regulation of RelB expression during the initiation of dendritic cell differentiation. Mol Cell Biol. 2005;25:7900-7916.

(36) Ouaaz F, Arron J, Zheng Y, Choi Y, Beg AA. Dendritic cell development and survival require distinct NF-kappaB subunits. Immunity. 2002;16:257-270.