

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.I.]. 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



Chapter 3

**The Breast Cancer Resistance Protein (BCRP; ABCG2) promotes
Langerhans cell differentiation from CD34⁺ progenitor cells**

To be submitted

Rieneke van de Ven
Jelle J. Lindenberg
Anneke W. Reurs
Hester van Crujisen
Rik J. Scheper
George L. Scheffer
Tanja D. de Gruijl

Chapter 3

Abstract

Epidermal Langerhans cells (LC) and dermal interstitial dendritic cells (IDC) were found to express the ATP-Binding Cassette (ABC) transporter Breast Cancer Resistance Protein (BCRP; ABCG2). Also, low BCRP expression was present on CD34⁺ blood DC precursors and expression was increased upon their differentiation to LC. The CD34⁺ AML-derived DC cell line MUTZ3 was used to study the effect of BCRP on LC/IDC development. MUTZ3 cells can be cultured into LC or IDC, depending on the cytokine cocktail used. Introduction of functional BCRP in MUTZ3 progenitor cells via retroviral transduction, resulted in accelerated LC differentiation kinetics with elevated Langerin levels and gave comparable subcellular BCRP localization as detected in skin LC or IDC. Moreover, in wtMUTZ3 cultures IDC differentiation resulted in typical CD1a⁺DC-SIGN⁺ cells, whereas in MUTZ3-BCRP IDC cultures the majority of cells remained negative for the IDC-specific C-type lectin DC-SIGN, but rather displayed typical LC characteristics like enhanced Langerin expression and high expression levels of CD1a. BCRP-induced acceleration of, and skewing towards, LC-like differentiation coincided with early RelB expression, was independent of Notch signaling, and depended on endogenous TGF β production. Together these data support a role for BCRP in rapid and preferential LC differentiation from CD34⁺ myeloid DC progenitors.

Introduction

Antigen presentation by professional antigen presenting cells (APC) is a crucial step in the initiation of an effective immune response. The most potent APC are dendritic cells (DC) which, when fully matured, can efficiently activate antigen-specific T cells in the lymph nodes.¹ In order to make optimal use of DC as a therapeutic tool, it is important to identify proteins that can enhance their potency. We are studying whether members of the ATP-binding cassette (ABC) transporter family, which are known to play a role in chemotherapy-induced multidrug resistance (MDR), like P-glycoprotein (P-gp:ABCB1), the Multidrug resistance proteins (MRP1-5: ABCC1-5) and the breast cancer resistance protein (BCRP; ABCG2), affect DC functions and may be exploited to improve DC-based immunotherapy. Previous reports by us and others showed expression of the ABC transporters P-gp, MRP1 and MRP4 on DC²⁻⁴, revealed their roles in DC migration⁴⁻⁶, and highlighted MRP1 activity as important for optimal DC differentiation.⁷

This report focuses on BCRP in DC. BCRP was first characterized by Doyle et al. in 1998 in the multidrug resistant breast cancer cell line MCF7/AdrVp⁸, which displayed ATP-dependant resistance to the anti-cancer agents mitoxantrone, doxorubicin and daunorubicin, but did not express P-gp or MRP1. Beside being over-expressed in several cancers and drug-selected cancer cell lines, BCRP has been described to be abundantly expressed on a sub-population of haematopoietic and non-haematopoietic stem cells^{9,10}, and was shown to be responsible for the extrusion of Hoechst dye, characterizing the so-called side-population. However, BCRP does not seem to have a crucial role in haematopoiesis as Bcrp1 knock-out mice had no severely altered phenotype and displayed normal haematological parameters.^{9,11} Accordingly, there were no effects on haematopoiesis in wt mice when treated with Bcrp1 inhibitors.¹²

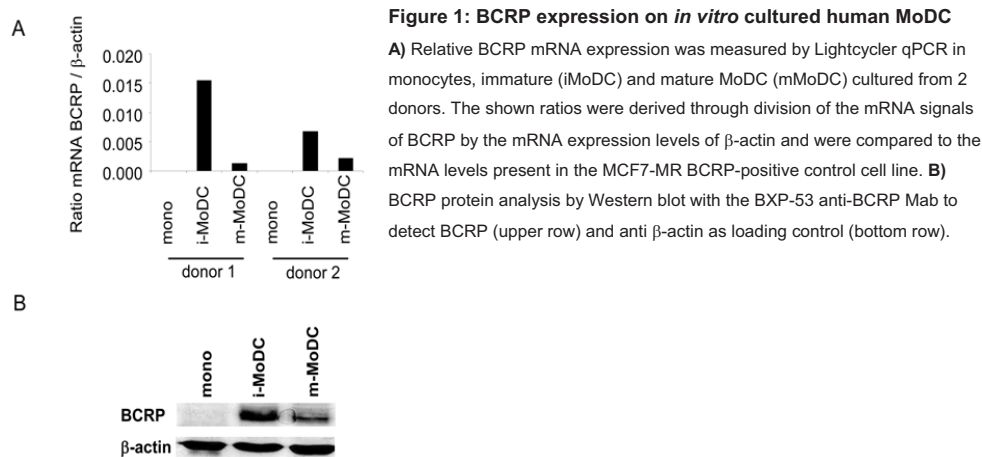
BCRP expression can be regulated by various environmental and physiological factors, consistent with a role in cellular defense mechanisms. Transcription-regulatory regions have been described within the BCRP promoter region that respond to a.o. hypoxia inducible factor (HIF)-1 α ¹³, activating protein-1 (AP-1)¹⁴, estrogen¹⁵, the nuclear hormone receptor peroxisome proliferator-activated receptor- γ (PPAR γ)¹⁶, and Notch signaling.¹⁷

To study DC development and functioning, we made use of the human acute myeloid leukemia (AML)-derived DC cell line MUTZ3¹⁸ from which both interstitial DC (IDC) and Langerhans cells (LC) can be generated in

BCRP promotes LC differentiation from CD34⁺ progenitor cells

the presence of IL-4 and TGF β , respectively. The progenitor population consists of a heterogeneous population of CD34⁺, CD34⁻ CD14⁻ (double negative) and CD14⁺ cells.¹⁹ LC, but not IDC, generated from MUTZ3 were shown to express LC-specific Langerin and Birbeck granules.²⁰ Indeed, extensive phenotypic and functional comparative studies showed MUTZ3-LC and -IDC to be equivalent to their *in vivo* counterparts and support the use of MUTZ3 as a valid DC differentiation model.²¹

In this study we analyzed BCRP expression on human skin-derived IDC/LC and *in vitro* generated IDC/LC and assessed whether the presence of this transporter influenced DC development and functionality. The data presented here provide evidence for a contributory role of BCRP to LC development.



Results

BCRP expression on human skin DC and monocyte-derived DC

Monocyte-derived DC (MoDC) were used as an *in vitro* model for interstitial DC (IDC) and were analyzed for the expression of BCRP by Lightcycler RT-PCR [Figure 1A] and western blot analysis [Figure 1B]. BCRP mRNA and protein expression were detected in immature (iMoDC) and mature MoDC (mMoDC), but were undetectable in monocytes. Both mRNA and protein expression were found to be highest in immature DC.

To study whether BCRP activity was required for IDC differentiation, as was previously found for MRP1⁷, MoDC were cultured in the absence or presence of the BCRP antagonist Ko-143 (200nM). Blocking BCRP activity during MoDC differentiation and/or maturation had no effect on DC morphology, phenotype, or T cell stimulatory ability in an allogeneic mixed leukocyte reaction (MLR) (data not shown).

BCRP expression on human skin DC and CD34⁺ blood precursors

To study BCRP expression on fresh, *ex vivo* DC, human skin biopsies were analyzed. In the epidermis, BCRP expressing cells with a clear DC morphology were detected in 3/5 donors [Figure 2A], while in 2/5 donors detection of BCRP positive LC was complicated by strong positive staining of surrounding epithelial cells. BCRP expressing cells were also present in the dermis of all donors tested [Figure 2A]. To confirm that the BCRP-positive cells were indeed DC, CD1a⁺ cells were isolated from epidermis (LC) and dermis (IDC) by CD1a-guided magnetic bead sorting. Isolated skin LC expressed high levels of CD1a and Langerin, whereas isolated skin IDC

Chapter 3

expressed lower levels of CD1a and were negative for Langerin [Figure 2B]. In the LC, BCRP staining was most prominent within the cytoplasm of the cells, whereas in IDC a nuclear staining pattern was observed [Figure 2B]. Next, medium alone or medium with the BCRP antagonist Ko-143 (200nM) was i.d. injected into human skin to study a role for BCRP in skin DC migration. No significant differences were found in the number of migrated cells (migrated cells per biopsy: 2291 ± 487 vs 2516 ± 863 for medium and medium plus Ko-143, respectively), suggesting that BCRP, in contrast to P-gp⁵, MRP1⁶, and MRP4⁴, does not play a role in human DC migration. As no BCRP protein expression was detected in blood monocytes [Figure 1B], CD34⁺ precursor cells were isolated from human blood and were *in vitro* expanded with stem cell factor (SCF) and Flt3-ligand in order to obtain sufficient cells.²² Weak BCRP expression could be detected on cytospin preparations of expanded CD34⁺ cells [Figure 2C]. Interestingly, BCRP expression was increased early during LC differentiation as shown in figure 2C on cells 72 hours after the initiation of LC differentiation with GM-CSF, TGFβ and TNFα.

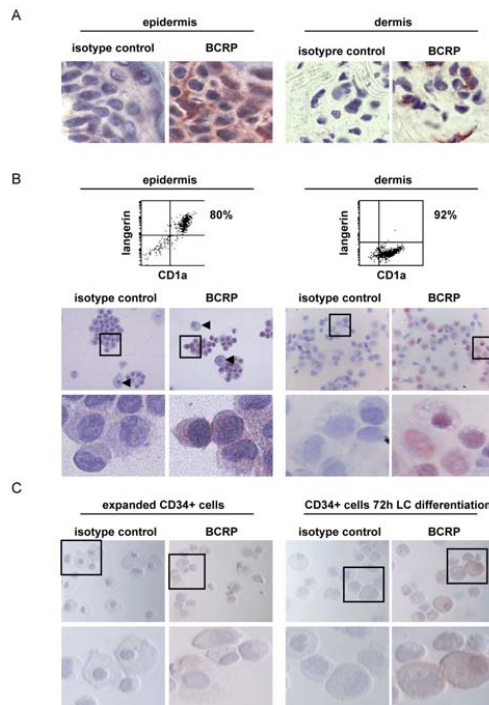


Figure 2: BCRP expression on human skin DC and *in vitro* expanded CD34⁺ blood precursors and early CD34⁺ blood-LC

A) Immunohistochemistry on human skin sections; shown are the epidermis (left) and dermis (right) (magnification 400x). Skin was stained with isotype control IgG1 or BXP-21 to detect BCRP protein expression. **B)** CD1a⁺ epidermal LC (left) and dermal IDC (right) were isolated from human epidermal and dermal sheets by CD1a-guided MACS. Cells were tested for purity by flow cytometry, using anti CD1a- and Langerin antibodies. Cytospin preparations were stained for BCRP protein using the rat Mab BXP-53 or isotype control. The arrowheads identify remaining keratinocytes in the epidermal suspensions. Top row (magnification 200x), bottom row enlargement of cells. **C)** Cytospin preparations of *in vitro* expanded CD34⁺ blood precursor cells (left) and CD34⁺ blood LC after seventy-two hours of differentiation (right) were stained with isotype control and BXP-53 Mabs. Top row overview (magnification 200x), bottom row detail (additional 3x magnification).

BCRP promotes LC differentiation from CD34⁺ progenitor cells

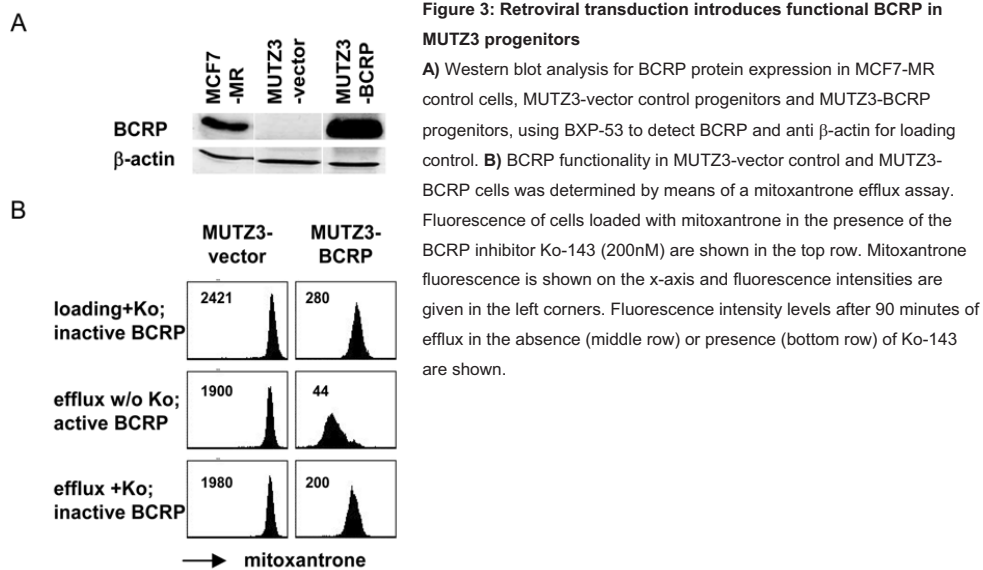


Figure 3: Retroviral transduction introduces functional BCRP in MUTZ3 progenitors

A) Western blot analysis for BCRP protein expression in MCF7-MR control cells, MUTZ3-vector control progenitors and MUTZ3-BCRP progenitors, using BXP-53 to detect BCRP and anti β -actin for loading control. **B)** BCRP functionality in MUTZ3-vector control and MUTZ3-BCRP cells was determined by means of a mitoxantrone efflux assay. Fluorescence of cells loaded with mitoxantrone in the presence of the BCRP inhibitor Ko-143 (200nM) are shown in the top row. Mitoxantrone fluorescence is shown on the x-axis and fluorescence intensities are given in the left corners. Fluorescence intensity levels after 90 minutes of efflux in the absence (middle row) or presence (bottom row) of Ko-143 are shown.

Introduction of BCRP in MUTZ3 progenitors promotes LC differentiation

Since human skin LC and IDC, as well as early LC cultures from CD34⁺ blood precursor cells showed expression of BCRP, we decided to analyze a possible role for BCRP in differentiation of these DC subsets. As it is very laborious to obtain sufficient amounts of CD34⁺ DC precursors from blood, we took advantage of the CD34⁺ MUTZ3 cell line¹⁸, which we showed to be a relevant model to study both LC and IDC development.¹⁹⁻²¹ Hence BCRP expression was analyzed on MUTZ3 progenitors and on *in vitro* cultured MUTZ3-derived LC and IDC. BCRP proved undetectable in these MUTZ3 cultures: only a very weak BCRP protein signal was detected in iLC by Western blot and mRNA levels were much lower than found for MoDC (data not shown). We next investigated whether the introduction of functional BCRP in the CD34⁺ MUTZ3 progenitor cells would influence LC/IDC development. BCRP-expressing MUTZ3 progenitor cells were therefore generated via retroviral transduction. The BCRP protein levels in the generated MUTZ3-vector control and MUTZ3-BCRP progenitor cells are shown in figure 3A. Specific and functional expression of BCRP in MUTZ3-BCRP cells was confirmed by the demonstration of a BCRP-dependent mitoxantrone efflux [Figure 3B]. Accordingly, toxicity assays verified that the MUTZ3-BCRP cells were better protected against mitoxantrone-induced cell death compared to MUTZ3 or MUTZ3-vector control cells (data not shown).

A contribution of BCRP to LC differentiation was observed when performing a differentiation kinetics experiment with the MUTZ3-vector control and MUTZ3-BCRP cell lines: higher rates of CD1a⁺Langerin⁺ MUTZ3-BCRP-derived LC were found in the cultures on days 4 and 7 as compared to MUTZ3-vector control conditions, while on day 10 LC contents were comparable between both cultures [Figure 4A and 4B]. Also, significantly more MUTZ3-BCRP-derived LC expressed the co-stimulatory molecules CD80 [Figure 4B] and CD86 (not shown) at earlier time points, consistent with enhanced differentiation.

Of note, at all time points Langerin expression levels were higher on the MUTZ3-BCRP LC [Figure 4A and 4C], but only significantly so on day 4 [Figure 4C]. These data demonstrate that introduction of BCRP expression at the CD34⁺ progenitor state accelerates LC differentiation. No differences in the induction of T cell proliferation were found when fully mature MUTZ3-vector control and -BCRP LC were analyzed in an allogeneic

Chapter 3

MLR (data not shown). In accordance with this, marker expression analysis after maturation revealed no differences between the MUTZ3-vector control and -BCRP cultures, suggesting that BCRP expression in precursor cells only provides an advantage in early stages of LC differentiation.

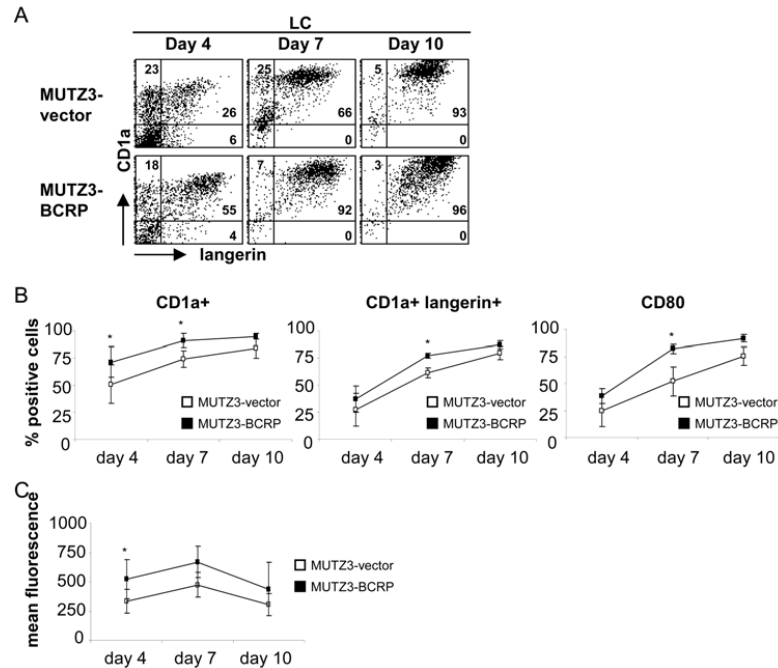


Figure 4: BCRP expression in MUTZ3 progenitors accelerates LC differentiation

A) LC differentiation kinetics for MUTZ3-vector control (upper row) and MUTZ3-BCRP cells (bottom row). Flowcytometric phenotypic analyses were performed on days 4, 7 and 10 of differentiation culture. Shown are CD1a and Langerin expression. Numbers listed in the plots represent the percentages of positive cells. **B)** Kinetics of LC differentiation: graphs show the average percentage of CD1a (left), Langerin (middle) and CD80 (right) positive cells from 4 experiments (* $p < 0.05$). **C)** Average mean fluorescence intensity levels for Langerin on CD1a⁺Langerin⁺ cells ($n = 4$) on days 4, 7 and 10 of culture (* $p < 0.05$).

Presence of BCRP blocks IDC development and promotes LC differentiation; accelerated LC-like differentiation correlates with early RelB expression

Kinetics experiments were also performed for MUTZ3-derived IDC cultures. Despite the presence of exogenous IL-4 and the absence of exogenous TGF β in these cultures, the MUTZ3-BCRP failed to adopt a typical DC-SIGN⁺ IDC phenotype. Instead, IDC cultures contained a significantly larger CD1a^{hi} DC-SIGN⁻ population at day 3 and 7 compared to vector control cells [Figure 5A and B ($n=3$; * $p < 0.05$)]. In addition, compared to MUTZ3-vector control IDC, unexpected expression of Langerin was observed [Figure 5C] and mean fluorescence intensity levels for CD1a and Langerin were significantly higher on MUTZ3-BCRP IDC [Figure 5D] ($p = 0.03$), implying that the presence of BCRP in CD34⁺ precursor cells interferes with IDC differentiation in favor of more LC-like development.

BCRP promotes LC differentiation from CD34+ progenitor cells

To confirm accelerated LC-like development at the signal transduction level, expression of RelB was examined. The transcription factor RelB is a sub-component of the nuclear factor κ B (NF κ B) and has been described to be essential for DC/LC development and to correlate with DC activation.²³⁻²⁵ MUTZ3-vector control and MUTZ3-BCRP progenitor cells, as well as IDC/LC derived thereof, were analyzed for the expression of RelB by Western blot. RelB was not expressed by the progenitor cells (data not shown) but day 3 MUTZ3-BCRP IDC/LC generated in the presence of either exogenous IL-4 or TGF β expressed more RelB than MUTZ3-vector IDC/LC (shown for the IL-4-containing IDC cultures in Figure 5E), indicative of a more differentiated/activated phenotype.

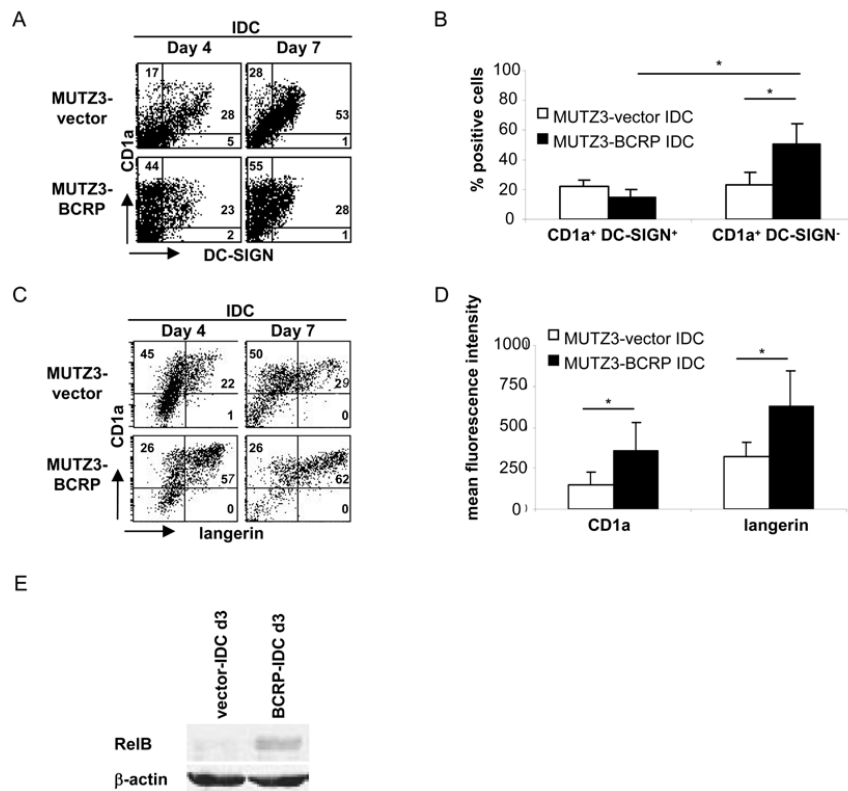


Figure 5: BCRP expression at the progenitor stage favors LC-like differentiation and accelerated differentiation correlates with early RelB expression

A) IDC-differentiation kinetics of MUTZ3-vector control (upper row) and MUTZ3-BCRP cells (bottom row). Phenotypic analyses were performed on days 4 and 7. Shown are CD1a and DC-SIGN expression. Numbers in the plots represent the percentages positive cells. **B)** Mean percentages (n=3) of CD1a⁺ DC-SIGN⁺ cells and CD1a⁺ DC-SIGN⁻ cells in day 3 MUTZ3-vector control IDC (white bars) or MUTZ3-BCRP IDC (black bars) (* p < 0.05). **C)** Plots show the percentages CD1a⁺ and Langerin⁺ cells on day 4 and 7 of IDC cultures. **D)** Average mean fluorescence intensity values (n=4) for CD1a and Langerin on day 3 in MUTZ3-vector control IDC (white bars) or MUTZ3-BCRP IDC (black bars) (* p < 0.05). **E)** Western blot analysis of protein expression of the NF κ B subunit RelB (upper row) in day 3 MUTZ3-vector control vs. MUTZ3-BCRP IDC. Relative loading was verified by β -actin staining (bottom row).

Chapter 3

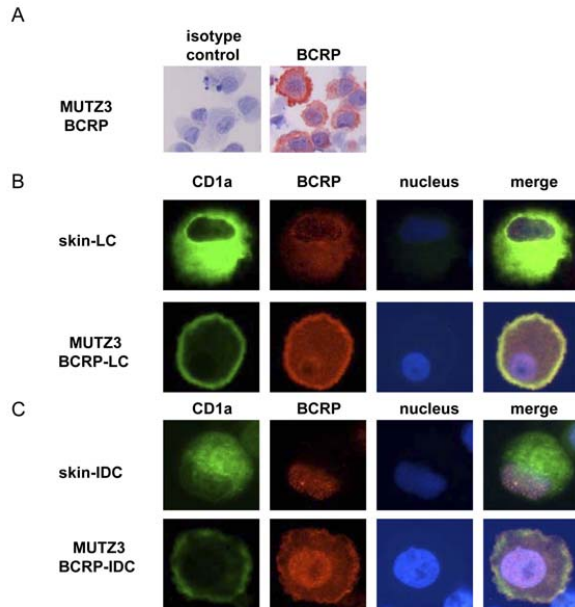


Figure 6: Comparative BCRP subcellular localization analysis between skin LC/IDC and MUTZ3-BCRP LC/IDC

A) BCRP expression and isotype control in MUTZ3-BCRP progenitor cells. B and C) Immunocytochemical analysis of CD1a (green) and BCRP (red) expression and nuclear staining (DAPI: blue) on cytospin preparations of human skin LC (B; top row) or IDC (C; top row), day 3 MUTZ3-BCRP LC (B; bottom row) or MUTZ3-BCRP IDC (C; bottom row) Merged images of CD1a, BCRP and nuclear staining are shown on the right (B and C) (original magnification 400x).

BCRP localization in isolated skin LC/IDC versus MUTZ3-BCRP LC/IDC

Isolation of CD1a⁺ skin LC and IDC, revealed differential subcellular BCRP localization within the two DC subsets (i.e. cytoplasmic localization in skin LC and nuclear localization in skin IDC; see Figure 2B). To check whether the introduction of BCRP in the MUTZ3 progenitor led to a similar distribution of BCRP in LC and IDC, day 3 differentiated MUTZ3-BCRP LC and IDC were analyzed. Whereas BCRP was localized throughout the cytoplasm and nucleus of MUTZ3-BCRP progenitor cells [Figure 6A], expression patterns in differentiated MUTZ3-BCRP IDC and LC were distinctly different and in part reflected the expression patterns observed in their primary skin-derived counterparts (emigrated from dermal and epidermal layers, respectively Figure 6B). Enforced BCRP expression in the LC and IDC cultures resulted in a clear membranous localization (as compared to a more cytoplasmic localization in the primary LC), but most strikingly, a similar and characteristic nuclear localization was apparent in the dermally derived IDC and the MUTZ3-BCRP IDC. Co-localization of BCRP (in red) and CD1a (in green) in both skin-derived and MUTZ3-BCRP LC, as well as in MUTZ3-BCRP IDC, which adopt LC-like characteristics, is highly suggestive of a functional correlate between (membranous) BCRP and CD1a co-localization, and LC differentiation [Figure 6B].

Accelerated LC differentiation is independent of HIF-1 α , PPAR γ and Notch but depends on endogenously derived TGF β

We next set out to determine what environmental conditions and associated regulatory processes might induce BCRP-enforced LC differentiation. Hence we analyzed the contribution of various regulatory mechanisms previously described to be involved in BCRP- or LC differentiation regulation.^{13,16,26-28} As the BCRP promoter contains a hypoxia responsive element to which HIF-1 α can bind, wtMUTZ3 progenitor cells were cultured under hypoxic (1% O₂) or normoxic conditions (21% O₂) for one passage and were subsequently differentiated into LC under hypoxia or normoxia. No accelerated or enhanced LC differentiation was observed under hypoxic

BCRP promotes LC differentiation from CD34+ progenitor cells

conditions compared to normoxic conditions as demonstrated by the average percentage of CD1a⁺ Langerin⁺ cells at day 3 [Figure 7A]. A second regulatory mechanism that had previously been reported to induce BCRP expression and affect DC differentiation from monocytes was the ligation of the nuclear hormone receptor PPAR γ .¹⁶ Treatment of MUTZ3 progenitor cells with the PPAR γ ligand rosiglitazone (RSG) did not induce BCRP expression (data not shown) and had no effect on MUTZ3-LC differentiation [Figure 7A].

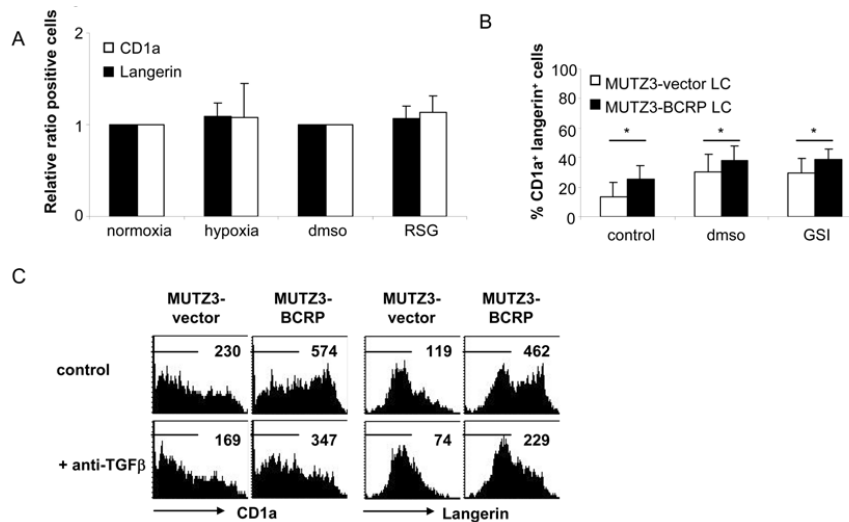


Figure 7: Regulation of accelerated and skewed LC differentiation: CD1a^{hi} and Langerin expression on MUTZ3-BCRP IDC depends on endogenous TGF β

(A) Hypoxic culture conditions (1% O₂) (n = 3), or ligation of the PPAR γ receptor with 2.5 μ M RSG (n = 3) had no effect on conventional MUTZ3-LC differentiation. Shown are the relative ratios for CD1a and Langerin expression against the normoxia (21% O₂) control for hypoxia or the vehicle (dms) control for RSG (B) Accelerated LC differentiation in MUTZ3-BCRP cells is independent of Notch signaling as inhibition of Notch signaling with 30 μ M GSI did not block accelerated differentiation (n = 3) (* p < 0.05). Shown are the percentages of CD1a⁺ Langerin⁺ cells on day 3 MUTZ3-BCRP LC cultures. (C) CD1a and Langerin fluorescence levels were determined in day 3 MUTZ3-vector control IDC or MUTZ3-BCRP IDC, cultured in the absence (top row) or presence (bottom row) of 5 μ g/ml TGF β -neutralizing antibodies. Results shown are from one experiment of three. The mean fluorescence intensities (MFI) are listed. The position of the isotype control is indicated in the histogram plots.

Two mechanisms involved in LC differentiation were subsequently analyzed, i.e. Notch receptor ligation and endogenous TGF β .^{26,28} To determine whether BCRP-induced accelerated LC differentiation in the MUTZ3 model depended on Notch signaling, a γ secretase inhibitor (GSI) was added during LC cultures from MUTZ3-vector control and -BCRP cells. At day 4 marker expression was analyzed. Inhibition of Notch signaling did not affect LC differentiation, as the percentage of CD1a⁺ Langerin⁺ cells remained the same. There was a slight, DMSO-dependent increase in the percentage of CD1a⁺ Langerin⁺ cells [Figure 7B], but the difference between the MUTZ3-vector control LC and MUTZ3-BCRP LC remained significant.

CD1a expression and LC development were previously reported to depend on endogenous TGF- β .²⁸ Since IDC cultured from MUTZ3-BCRP cells failed to adopt a typical DC-SIGN⁺ phenotype, but rather developed into LC-like cells with high CD1a and upregulated Langerin expression levels, we hypothesized that this could be due to increased endogenous TGF- β levels. To test this, the emergence of the CD1a^{hi}Langerin⁺ population in MUTZ3-

Chapter 3

BCRP IDC cultures, observed in the absence of exogenously added TGF β [Figure 5C], was analyzed in the absence or presence of TGF- β neutralizing antibodies. Neutralization of endogenous TGF- β indeed reduced CD1a as well as Langerin expression levels [Figure 7C], demonstrating its importance in BCRP-related induction of LC development.

Discussion

The ABC transporter BCRP was detected by immuno-staining on human skin LC and IDC *in situ*, on *in vitro* expanded CD34⁺ blood precursors and LC differentiated thereof and on *in vitro* cultured MoDC. Due to variable staining of epithelial cells, consistent with a previous report on epidermal BCRP expression,^{29,30} BCRP-expressing LC were not discernable *in situ* in all donors (i.e. in 3/5 only). However, BCRP expression was definitively confirmed on isolated CD1a⁺ LC and IDC in 3/3 donors. In rat epithelia, BCRP expression was recently reported on MHC II positive cells, identified as small, slow-cycling LC.³¹

In order to study whether BCRP expression played a role in human LC or IDC development, functional BCRP was introduced in the MUTZ3 cell line, which can be cultured into IDC or LC, the latter with typical Langerin, CD1a^{hi} and Birbeck granule expression.^{19,20} Introduction of functional BCRP in CD34⁺ MUTZ3 precursor cells accelerated LC differentiation and enhanced expression of the LC-typifying C-type Lectin Langerin. This accelerated induction of differentiation was accompanied by early expression of RelB, a NF- κ B subunit, previously described to be of particular importance for DC and LC development, activation and functionality.^{23,25,32} It is evident that expression of BCRP somehow predisposes myeloid precursors to differentiate into LC rather than IDC, as, regardless of the presence of adequate amounts of IL-4 to induce IDC differentiation and halt LC development, CD1a⁺ cells developing in the IDC cultures never adopted a typical DC-SIGN⁺ IDC phenotype, but rather acquired abnormally high CD1a and Langerin expression levels. Similarly, in two experiments where only GM-CSF and TNF α were added to MUTZ3-vector or MUTZ3-BCRP cultures, the percentages of CD1a⁺Langerin⁺ cells were 3- and 5-fold higher and mean fluorescence intensities of Langerin were 2-fold higher in MUTZ3-BCRP vs MUTZ3-vector control cultures on day 4 (data not shown). Overall, these data clearly support a role for BCRP in skewing the differentiation of CD34⁺ DC precursors away from the IDC lineage and towards the LC lineage.

Prevailing hypoxic conditions in the epidermis²⁷ and the fact that BCRP expression can be induced under hypoxia³³, prompted us to test whether hypoxic conditions could be a physiological trigger by which induced BCRP expression on LC precursors might lead to accelerated LC differentiation. Only in 1/5 experiments accelerated differentiation under hypoxic culture conditions of wtMUTZ3 precursors was observed correlating with an increase in BCRP expression (data not shown). Another BCRP regulatory mechanism is through ligation of the nuclear hormone receptor PPAR γ .¹⁶ Unlike in DC differentiation from monocytes where PPAR γ ligation with RSG was shown to increase BCRP expression and reduce CD1a expression on MoDC cultures¹⁶, MUTZ3-LC differentiation from MUTZ3 progenitors pre-treated with RSG or differentiated in the presence of RSG, was not hampered and did not induce BCRP expression. Possibly PPAR γ ligation has different effects on CD34⁺ (MUTZ3) vs CD14⁺ (monocyte) progenitors. It should also be kept in mind that earlier studies have been published showing a suppressive effect of PPAR γ ligation on DC development, DC functions and immunity³⁴⁻³⁷; it seems unlikely that all these defects are caused by induction of BCRP expression. Finally, since LC differentiation has been linked to Notch receptor ligation²⁶, a GSI was added during MUTZ3-BCRP LC cultures to analyze whether the observed accelerated differentiation depended on Notch signaling. The BCRP-induced accelerated differentiation appeared to be Notch-independent.

BCRP promotes LC differentiation from CD34⁺ progenitor cells

Endogenous TGF β is known to be required for CD1a expression and *in vitro* LC development from CD34⁺ precursors.²⁸ As the addition of exogenous TGF β is required for the differentiation of precursor cells into LC²⁸, the contribution of possibly enhanced levels of endogenously produced TGF β could not be analyzed in LC cultures by means of a neutralizing antibody. However, we did find the observed high percentages of CD1a^{hi} and Langerin LC-like cells in MUTZ3-BCRP IDC cultures to be dependent on endogenous TGF β as neutralizing TGF β antibodies reduced CD1a and Langerin expression to near-control levels. In concordance with the data described by Caux et al., we also observed a slight reduction in CD1a expression in the MUTZ3-vector control IDC upon neutralization of TGF β . Based on studies that showed ABC transporter substrates to be of low molecular weight (MW<1 kDa)^{38,39}, it is unlikely that BCRP itself excretes TGF β .⁴⁰ However, it could be that BCRP transports a substrate that induces TGF β production and/or secretion or facilitates the splicing and subsequent activation of TGF β . Thus, the presence of BCRP on the membrane of CD34⁺ precursors might indirectly increase endogenous TGF β production. We were unable to detect secreted TGF β in the supernatants of MUTZ3-vector control and – BCRP cultures compared to culture medium (data not shown). One explanation for this might be that TGF β is not secreted, but rather remains bound to the outer plasma membrane of the cells, and can stimulate differentiation via cell/cell contact, or that small amounts of secreted TGF β are rapidly bound to its receptor in an autocrine or paracrine fashion and depleted from the medium.

The localization of BCRP within the cytoplasm or on the plasma membrane of skin LC, as opposed to the nuclear localization in skin IDC [Figure 2B and 6], is certainly consistent with a possible contribution of BCRP to LC differentiation through the (vesicular) transport of substrates to the extra-cellular environment. The abundant presence of BCRP on or near the plasma membrane might facilitate secretion of an, as yet unknown, substrate responsible for the observed accelerated LC differentiation kinetics and concomitant high CD1a and Langerin expression on the cell membrane. The observed co-localization of BCRP and CD1a in LC, but not in IDC, is particularly thought-provoking in this regard.

In conclusion, we have demonstrated that skin-resident LC and IDC, as well as *in vitro* expanded CD34⁺ blood precursors and LC cultured thereof and *in vitro* cultured MoDC express the ABC transporter BCRP. Introduction of the transporter in CD34⁺ MUTZ3 progenitors affected differentiation by accelerating LC differentiation and interfering with IDC differentiation through the promotion of LC development. This acquired predisposition of CD34⁺ precursor cells for LC differentiation was shown to be independent of Notch signaling, but dependent on endogenous TGF β activity. From a therapeutic perspective, one could envision the use of BCRP induction on LC precursors to enhance and accelerate LC differentiation as a tool to optimize immunization through the skin. Since epidermal LC express BCRP and are known to be (at least in part) replenished from skin-resident cells⁴¹ and in light of the presence of a BCRP⁺CD34⁺ side population in the epidermis^{29-31,42}, this would seem feasible. In this regard, further research is warranted to confirm a role for BCRP in epidermal LC development, to investigate the underlying molecular mechanisms, and to identify specific BCRP substrates that might be involved.

Material and Methods

Chemicals:

Unless stated otherwise, all chemicals and drugs were obtained from Sigma Chemical Co. (St. Louis, MO) except for Ko-143 which was kindly provided by Dr. JD. Allen and has been described before.⁴³

Tumor cell lines:

Chapter 3

The breast carcinoma cell line MCF7 and its BCRP over-expressing subline MCF7/MR⁴⁴ were used as control cell lines where indicated.

Retroviral constructs:

The retroviruses LZRS-IRES-GFP (vector) and LZRS-BCRP-IRES-GFP (BCRP) were kind gifts of Prof. P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). Retroviruses were propagated in Phoenix A cells by plasmid DNA transfection using Lipofectamine 2000 (Invitrogen) in serum free medium for 3 hours. Medium was refreshed after 24 hours and viral supernatants were harvested 48 hours post transfection and were either directly used for transduction or stored at -80°C in 1ml aliquots.

MUTZ3 and dendritic cell cultures:

The AML-derived MUTZ3 cell line was cultured as described before.¹⁹ In short, MUTZ3 progenitors were cultured in MEM- α (Minimum essential medium, Lonza, Belgium) containing 20% fetal calf serum (FCS), 100 IU/ml sodium-penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 50 μ M β -mercaptoethanol (2ME) and 10% 5637 (renal cell carcinoma) conditioned medium (MUTZ3 routine medium) in 12-well plates (Costar) at a concentration of 0.2 million cells/ml and were passaged twice weekly. Langerhans cells (MUTZ3-LC) were cultured from MUTZ-3 progenitors with 10ng/ml TGF- β 1 (Biovision, Mountain View, CA), 100ng/ml rhGM-CSF (Sagramostim, Berlex), and 120 IU/ml TNF α (Strathmann Biotec) for 10 days to obtain immature MUTZ3-LC as described previously.⁷ Interstitial DC (MUTZ3-IDC) were cultured from MUTZ3 progenitors with 20ng/ml IL-4, 100ng/ml rhGM-CSF and 120 IU/ml TNF α for 6 days as described.¹⁹ Interstitial DC (IDC) were also generated *in vitro* from human peripheral blood monocytes from healthy donors (MoDC) by adding 10ng/ml IL-4 and 100ng/ml rhGM-CSF for 6 days as described before.³ Where indicated, MoDC cultures received the BCRP antagonist Ko-143 (200nM) on day 0 and day 3. Where applicable, immature MoDC, MUTZ3-IDC and MUTZ3-LC were matured at day 6 (MoDC / MUTZ3-IDC) or day 10 (MUTZ3-LC) by adding a maturation cocktail containing 2400 IU/ml TNF α , 100ng/ml IL-6 (Strathmann Biotec), 25ng/ml IL-1 β (Strathmann Biotec) and 1 μ g/ml prostaglandin E2 (PGE₂) (Sigma Aldrich) for 2 days. For the hypoxia cultures, MUTZ3 progenitors were cultured at 1% O₂ for one passage (3-4 days) before starting a MUTZ3-LC culture under hypoxia. Phenotypic analyses were done at days 3, 6 and 10. For PPAR γ activation, MUTZ3 progenitor cells were cultured for 1-2 passages in the presence of 2.5 μ M rosiglitazone maleate (RSG; Alexis Biochemicals, San Diego, CA) [¹⁶] and were differentiated into MUTZ3-LC in the absence or presence of RSG.

CD34⁺ haematopoietic progenitor cells

CD34⁺ haematopoietic progenitor cells were isolated from blood of healthy donors and cultured for 2-5 weeks with 25ng/ml *flms*-like tyrosine kinase-3 ligand (Flt3-L) and 10ng/ml stem cell factor (SCF) to expand their numbers while maintaining their ability to differentiate to DC, as described previously.²² Expanded CD34⁺ progenitor cells were analyzed for BCRP expression by immunocytochemistry (see below). To study BCRP expression during differentiation, expanded CD34⁺ progenitors were cultured for 72 hours in the presence of 100ng/ml rhGM-CSF, 10ng/ml TGF β and 120IU/ml TNF α . After 72 hours, phenotypic analysis was performed by flow cytometry and cytospin preparations were made for immunocytochemistry.

MUTZ3 retroviral transduction

For the generation of MUTZ3 cells expressing BCRP, MUTZ3 progenitor cells were transduced with the LZRS-IRES-BCRP-GFP retrovirus. LZRS-IRES-GFP was used as a control. MUTZ3 retroviral transduction was performed as described previously.⁴ In short, 0.5 million MUTZ3 progenitor cells were transferred to retronectin-coated plates (40 μ g/ml retronectin, Takara, Japan) in 1ml viral supernatant. Plates were centrifuged for 90 minutes at 2000rpm at 25°C. Cells were re-transduced with 1ml fresh viral supernatant after 24 hours. After 3 days, cells were analyzed for GFP expression and the GFP⁺ cells were sorted using a FACS STAR flowcytometer (Becton and Dickinson, San Jose, CA). The retrovirus specifically infected and transduced the CD34⁺ proliferative progenitor population of the MUTZ3 cell line. Sorted GFP⁺ cells transduced with LZRS-IRES-GFP were designated "MUTZ3-vector control". LZRS-BCRP-IRES-GFP transduced cells were further selected for the cells with high BCRP expression by adding 0.25 μ g/ml puromycin, an antibiotic recently described to be a substrate for BCRP.⁴⁵ These cell were designated "MUTZ3-BCRP".

BCRP promotes LC differentiation from CD34+ progenitor cells

Phenotypic characterization of MUTZ3-vector (i.e. control cells) and –BCRP DC/LC was performed after 3 or 4, 7 and 10 days (LC) of differentiation. Where indicated, 30 μ M γ 40-secretase inhibitor II (GSI; Calbiochem, Darmstadt Germany) or the vehicle control DMSO were added during MUTZ3-vector and MUTZ3-BCRP LC differentiation in order to inhibit Notch-receptor signalling. Neutralizing TGF β antibodies (clone 1D11, R&D systems) were added to MUTZ3-vector and –BCRP IDC cultures at day 0 in a concentration of 5 μ g/ml (diluted in PBS) to study the contribution of endogenous TGF β .

Flow cytometric immunophenotypical analyses

IDC/LC were immunophenotyped using FITC- and/or PE-conjugated Mabs directed 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). MUTZ3-vector and –BCRP cells were phenotyped using APC-conjugated CD1a (BD Biosciences, San Jose, CA) and the above mentioned PE-conjugated antibodies. In short, 2.5 to 5 \cdot 10⁴ cells were washed in PBS supplemented with 0.1% BSA and 0.02% NaN₃ and incubated with specific or corresponding isotype-matched control Mabs for 30 minutes at 4°C. Cells were washed and analyzed with a FACS-Calibur flow cytometer (Becton and Dickinson, San Jose, CA) equipped with CellQuest analysis software and the results were expressed as mean or median fluorescence intensities, the percentage of positive cells or the relative ratios of positive cells compared to the control cultures.

Lightcycler PCR:

RNA was isolated using RNA-Bee (Bio-connect, Huissen, The Netherlands), following the manufacturer's instructions. The RNA concentrations were analyzed on a Nanodrop spectrophotometer. cDNA was synthesized from 2 μ g RNA using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen). cDNA samples were diluted 1:15 and 5 μ l cDNA was used in a Lightcycler PCR reaction using Lightcycler FastStart DNA Master^{plus} HybProbe (Roche, Mannheim, Germany) using fluorescent-labeled Taqman probes for BCRP and β -actin. (BCRP Forward 5'→3' primer: AGATGGGTTTCCAAGCGTTCAT. BCRP Reverse 5'→3' primer: CCAGTCCCAGTACGACTGTGACA. BCRP Probe: TGCTGGTAATCCCCAGGCCTCTATAGC) (β -actin forward 5'→3' primer: TCACCCACACTGTGCCCATCTACGA; β -actin reverse 5'→3' primer: CAGCGGAACCGCTCATTGCCAATGG; β -actin probe: ATGCCCTCCCCATGCCATCCTGCGT). The relative ratios given are the concentration of BCRP mRNA divided by the concentration of β -actin mRNA for the given samples, based on a titration curve of BCRP mRNA levels of the BCRP-positive cell line MCF7-MR.

Western blotting

Cell pellets were lysed in ice-cold lysis buffer (1 mM EDTA; 1% NP-40; 1 mM PMSF), kept on ice for an hour, sonicated and stored at -20°C until further use. Protein concentrations were determined with a Bio-Rad protein assay (Bio-Rad, Richmond, CA). Proteins were fractionated on an 8% polyacrylamide gel and subsequently transferred to nitrocellulose filter by electroblotting. For protein detection, filters were blocked (PBS containing 1% BSA, 1% milk powder and 0.05% tween-20) overnight and were subsequently incubated in blocking buffer for two hours at room temperature with the BXP-53 (0.5 μ g/ml) rat Mab to detect BCRP¹¹, RelB rabbit polyclonal IgG (1 μ g/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) to detect RelB and MAB1501R mouse Mab (0.3 μ g/ml) (Chemicon International, Temecula, CA) to detect β -actin. Immuno-reactivity was visualized with rabbit-anti-rat immunoglobulin conjugated to HRP (Dako A/S, Denmark) for BXP53, rabbit-anti-mouse immunoglobulin conjugated to HRP (Dako A/S, Denmark) for β -actin and swine-anti-rabbit conjugated to HRP (Dako A/S, Denmark) to detect RelB (all 1:1000), followed by color development with 0.5g l⁻¹ 3,3'-diamino-benzidine tetrahydrochloride, 0.15 g l⁻¹ chloronaphthol and 0.02% H₂O₂ in PBS or with enhanced chemiluminescence (ECL, Roche Diagnostics, Almere, The Netherlands).

Efflux assay

2 \cdot 10⁵ cells were washed and taken up in culture medium supplemented with FCS, L-glutamine, penicillin and streptomycin. Cells were loaded with mitoxantrone (20 μ M) with or without Ko-143 (200 nM) for 30 minutes at 37°C. Cells were washed twice in ice-cold PBS, taken up in complete culture medium and allowed to efflux for 90 minutes at 37°C either in the absence or the presence of Ko-143. Finally, cells were washed twice in ice-cold PBS, taken up in 300 μ l of ice-cold PBS and kept on ice until fluorescence was analyzed on a FACS-Calibur flowcytometer. Mitoxantrone was measured in the FL-4 channel with a 633 nm

Chapter 3

laser and a 660 nm bandpass filter. BCRP activity ratios were obtained by dividing the median drug fluorescence in the presence of inhibitor by the median drug fluorescence in the absence of inhibitor, after subtraction of the median fluorescence of unloaded control cells as described previously.⁴⁶

Immunocytochemistry

Human skin tissue was obtained from patients undergoing corrective breast or abdominal plastic surgery at the VU University medical center (Amsterdam, The Netherlands), the Tergooi hospital (Hilversum, The Netherlands) or the Slotervaart hospital (Amsterdam, The Netherlands), after informed consent and following hospital guidelines. Sections were made from frozen skin biopsies using a cryostat such that both epidermal and dermal layer were present in the slides. Cytospin preparations were made of cell suspensions by centrifugation for 4 minutes at 650rpm. Skin sections and cytospins were air-dried overnight and fixed in 100% acetone for 10 minutes. For BCRP detection, samples were incubated with the anti-BCRP mouse mab BXP-21 (5 µg/ml)⁴⁷ or the rat mab BXP-53 (5 µg/ml)¹¹ at room temperature for 90 minutes. Antibody binding was detected with biotinylated rabbit anti-mouse F(ab')₂ fragments (1:300, Dako A/S, Denmark) or rabbit anti-rat F(ab')₂ fragments (1:100, Dako A/S, Denmark) and streptavidin conjugated to HRP (1:500, Dako A/S, Denmark). Bound peroxidase was developed with 0.02% (w/v) 3-amino-9-ethylcarbazole (AEC) and 0.02% (v/v) H₂O₂ in 0.1M sodium acetate pH5.0. Slides were counterstained with haematoxylin and mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany). For negative controls, slides were stained with isotype-matched mouse or rat IgG antibodies. Fluorescent double-stainings of cytospins were performed as previously described⁷, using the BXP-53 antibody to detect BCRP.

Skin explant DC and isolation of CD1a⁺ skin LC and IDC

Skin explant cultures and skin DC migration assays, with subsequent analysis, were performed as described previously.⁴⁸ For BCRP blocking during migration, 200nM Ko-143 was injected intradermally (i.d), after which 6mm punch biopsies were taken and DC were allowed to migrate from the biopsies for 2 days in culture medium in 48-well plates before harvesting, quantification with flow-count fluorospheres (Beckman Coulter, Fullerton, CA). For cell isolation, full thickness skin biopsies (2-3mm), consisting of dermis and epidermis, were collected from healthy donor skin with a dermatome (Aesculap, Tuttlingen, Germany). Biopsies were incubated in 50ng/ml dispase II (Roche, Penzberg, Germany) for 20 minutes at 37°C after which the epidermal and dermal layers were separated with tweezers. To isolate epidermal DC (LC), epidermal sheets were incubated in 0.05% trypsin at 37°C for 15 min and resuspended in IMDM + 10% FCS. Single cell suspensions were made by filtering twice through a 100µm sterile filter. Dermal IDC were isolated by incubating dermal skin layers in collagenase (200mg collagenase diluted in 26.6ml PBS and 6.6ml dispase II) for 90 minutes at 37°C. Single cell suspensions were made by filtering twice through 100µm sterile filters. Viable cells were counted by trypan blue exclusion and CD1a⁺ cells were purified by magnetic bead sorting (MACS) using Myltenyi anti-CD1a-magnetic beads (Myltenyi Biotec, Bergisch Gladbach, Germany). After MACS, the populations were checked for purity, based on CD1a expression in conjunction with Langerin. Cytospins were made of the CD1a⁺ LC and IDC and were stained for BCRP with BXP-53 as described above.

Statistical analysis

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

Acknowledgements

The authors would like to thank R. Oerlemans (VUmc, The Netherlands) for his help with the Lightcycler qPCR and Dr. H. Bontkes for the expanded CD34⁺ blood precursors. 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) and partly supported by the Netherlands Organization for Scientific Research (NWO VIDI grant 917-56-32 to T.D.G).

Disclosures

The authors have no financial conflict of interest.

BCRP promotes LC differentiation from CD34+ progenitor cells

Reference List

- (1) Schuurhuis DH, Fu N, Ossendorp F, Melief CJ. Ins and outs of dendritic cells. *Int Arch Allergy Immunol.* 2006;140:53-72.
- (2) Laupeze B, Amiot L, Bertho N et al. Differential expression of the efflux pumps P-glycoprotein and multidrug resistance-associated protein in human monocyte-derived dendritic cells. *Hum Immunol.* 2001;62:1073-1080.
- (3) Schroeijers AB, Reurs AW, Scheffer GL et al. Up-regulation of drug resistance-related vaults during dendritic cell development. *J Immunol.* 2002;168:1572-1578.
- (4) van de Ven R, Scheffer GL, Reurs AW et al. A role for multidrug resistance protein 4 (MRP4; ABCC4) in human dendritic cell migration. *Blood.* 2008.
- (5) Randolph GJ, Beaulieu S, Pope M et al. A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. *Proc Natl Acad Sci U S A.* 1998;95:6924-6929.
- (6) Robbiani DF, Finch RA, Jager D et al. The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes. *Cell.* 2000;103:757-768.
- (7) van de Ven R, de Jong MC, Reurs AW et al. Dendritic cells require multidrug resistance protein 1 (ABCC1) transporter activity for differentiation. *J Immunol.* 2006;176:5191-5198.
- (8) Doyle LA, Yang W, Abruzzo LV et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A.* 1998;95:15665-15670.
- (9) Zhou S, Schuetz JD, Bunting KD et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med.* 2001;7:1028-1034.
- (10) Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood.* 2002;99:507-512.
- (11) Jonker JW, Buitelaar M, Wagenaar E et al. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci U S A.* 2002;99:15649-15654.
- (12) Allen JD, Schinkel AH. Multidrug resistance and pharmacological protection mediated by the breast cancer resistance protein (BCRP/ABCG2). *Mol Cancer Ther.* 2002;1:427-434.
- (13) Krishnamurthy P, Schuetz JD. The ABC transporter Abcg2/Bcrp: role in hypoxia mediated survival. *Biomaterials.* 2005;18:349-358.
- (14) Bailey-Dell KJ, Hassel B, Doyle LA, Ross DD. Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene. *Biochim Biophys Acta.* 2001;1520:234-241.
- (15) Ee PL, Kamalakaran S, Tonetti D et al. Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene. *Cancer Res.* 2004;64:1247-1251.
- (16) Szatmari I, Vamosi G, Brazda P et al. Peroxisome proliferator-activated receptor gamma-regulated ABCG2 expression confers cytoprotection to human dendritic cells. *J Biol Chem.* 2006;281:23812-23823.
- (17) Bhattacharya S, Das A, Mallya K, Ahmad I. Maintenance of retinal stem cells by Abcg2 is regulated by notch signaling. *J Cell Sci.* 2007;120:2652-2662.
- (18) Hu ZB, Ma W, Zaborski M et al. Establishment and characterization of two novel cytokine-responsive acute myeloid and monocytic leukemia cell lines, MUTZ-2 and MUTZ-3. *Leukemia.* 1996;10:1025-1040.
- (19) 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.
- (20) 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.
- (21) 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.
- (22) 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.
- (23) Ouaz F, Arron J, Zheng Y, Choi Y, Beg AA. Dendritic cell development and survival require distinct NF-kappaB subunits. *Immunity.* 2002;16:257-270.
- (24) 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.

Chapter 3

- (25) 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.
- (26) Hoshino N, Katayama N, Shibasaki T et al. A novel role for Notch ligand Delta-1 as a regulator of human Langerhans cell development from blood monocytes. *J Leukoc Biol*. 2005;78:921-929.
- (27) Evans SM, Schrlau AE, Chalian AA, Zhang P, Koch CJ. Oxygen levels in normal and previously irradiated human skin as assessed by EF5 binding. *J Invest Dermatol*. 2006;126:2596-2606.
- (28) Caux C, Massacrier C, Dubois B et al. Respective involvement of TGF-beta and IL-4 in the development of Langerhans cells and non-Langerhans dendritic cells from CD34+ progenitors. *J Leukoc Biol*. 1999;66:781-791.
- (29) Triel C, Vestergaard ME, Bolund L, Jensen TG, Jensen UB. Side population cells in human and mouse epidermis lack stem cell characteristics. *Exp Cell Res*. 2004;295:79-90.
- (30) de Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells*. 2005;23:63-73.
- (31) Chen W, Hara K, Tian Q, Zhao K, Yoshitomi T. Existence of small slow-cycling Langerhans cells in the limbal basal epithelium that express ABCG2. *Exp Eye Res*. 2007;84:626-634.
- (32) Platzter B, Jorgl A, Taschner S, Hocher B, Strobl H. RelB regulates human dendritic cell subset development by promoting monocyte intermediates. *Blood*. 2004;104:3655-3663.
- (33) Krishnamurthy P, Ross DD, Nakanishi T et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. *J Biol Chem*. 2004;279:24218-24225.
- (34) Gosset P, Charbonnier AS, Delerive P et al. Peroxisome proliferator-activated receptor gamma activators affect the maturation of human monocyte-derived dendritic cells. *Eur J Immunol*. 2001;31:2857-2865.
- (35) Nencioni A, Grunebach F, Zobywalski A et al. Dendritic cell immunogenicity is regulated by peroxisome proliferator-activated receptor gamma. *J Immunol*. 2002;169:1228-1235.
- (36) Angeli V, Hammad H, Staels B et al. Peroxisome proliferator-activated receptor gamma inhibits the migration of dendritic cells: consequences for the immune response. *J Immunol*. 2003;170:5295-5301.
- (37) Appel S, Mirakaj V, Bringmann A et al. PPAR-gamma agonists inhibit toll-like receptor-mediated activation of dendritic cells via the MAP kinase and NF-kappaB pathways. *Blood*. 2005;106:3888-3894.
- (38) Momburg F, Roelse J, Hammerling GJ, Neeffes JJ. Peptide size selection by the major histocompatibility complex-encoded peptide transporter. *J Exp Med*. 1994;179:1613-1623.
- (39) Robey RW, Polgar O, Deeken J, To KW, Bates SE. ABCG2: determining its relevance in clinical drug resistance. *Cancer Metastasis Rev*. 2007;26:39-57.
- (40) Miyazono K, Hellman U, Wernstedt C, Heldin CH. Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. *J Biol Chem*. 1988;263:6407-6415.
- (41) Merad M, Manz MG, Karsunky H et al. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol*. 2002;3:1135-1141.
- (42) Yano S, Ito Y, Fujimoto M et al. Characterization and localization of side population cells in mouse skin. *Stem Cells*. 2005;23:834-841.
- (43) 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.
- (44) Taylor CW, Dalton WS, Parrish PR et al. Different mechanisms of decreased drug accumulation in doxorubicin and mitoxantrone resistant variants of the MCF7 human breast cancer cell line. *Br J Cancer*. 1991;63:923-929.
- (45) Takenaka K, Morgan JA, Scheffer GL et al. Substrate overlap between Mrp4 and Abcg2/Bcrp affects purine analogue drug cytotoxicity and tissue distribution. *Cancer Res*. 2007;67:6965-6972.
- (46) van der Pol MA, Pater JM, Feller N et al. Functional characterization of minimal residual disease for P-glycoprotein and multidrug resistance protein activity in acute myeloid leukemia. *Leukemia*. 2001;15:1554-1563.
- (47) Maliepaard M, Scheffer GL, Faneyte IF et al. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res*. 2001;61:3458-3464.
- (48) de Gruijl TD, Luykx-de Bakker SA, Tillman BW et al. Prolonged maturation and enhanced transduction of dendritic cells migrated from human skin explants after in situ delivery of CD40-targeted adenoviral vectors. *J Immunol*. 2002;169:5322-5331.