The contribution of ABC transporters to dendritic cell development and function

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Chapter 1

General introduction partly adapted from:

ABC drug transporters and immunity:

novel therapeutic targets in autoimmunity and cancer.

Submitted

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Introduction

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1. The immune system

The immune system protects against invading pathogens and altered-self and is educated to be tolerant toward self-tissue. In the immune system of higher vertebrates two components can be discriminated: the innate immunity and the adaptive immunity. The innate immunity is referred to as the non-specific, or antigen-independent, response and consists of inflammatory cells that recognize pathogenic patterns, the so-called pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) like Toll-like receptors (TLR), C-type lectins or they can sense tissue damage through NOD-like receptors.[1-5] PRRs can be found on the surface of cells belonging to the innate response like monocytes, macrophages, natural killer (NK) cells, neutrophils, granulocytes and dendritic cells (DC). Upon the encounter with a pathogen or damaged tissue, these cells are triggered and respond by uptake and killing of the pathogen or pathogen-infected cells, as well as by secretion of chemokines and cytokines in order to attract cells of the adaptive immune system.

The adaptive immune system consists of T- and B cells that will respond in an antigen-specific manner and are capable of forming an immunological memory. This memory response facilitates a rapid antigen-specific response upon re-encounter of a certain pathogen. The adaptive response is restricted to antigenic peptide signals provided by major histocompatibility (MHC) complexes.[6] There are two types of MHC complexes, i.e. the MHC class I and II molecules. MHC I molecules present peptides derived from endogenous proteins and are expressed by all nucleated cells, whereas MHC II molecules are found only on antigen presenting cells (APC) like B cells, monocytes, macrophages and DC[7] and present peptides derived from exogenous proteins. For presentation on MHC I molecules, endogenous proteins are degraded by the proteasome and are translocated into the endoplasmic reticulum (ER) through the heterodimeric transporter associated with antigen processing (TAP1/2; ABCB2/3), which belongs to the super family of ATP binding cassette (ABC) transporters.[8,9] In the ER, peptides of 8-10 amino acids are loaded onto the MHC I molecules, after which the MHC I/peptide complex is transported to the cell surface. MHC I/peptide complexes are recognized by $CD8^+$ T cells.[10] Exogenous proteins, taken up through endocytosis, are processed in the endosomal route and are loaded on MHC II molecules in so-called MHC class II compartment (MIIC).[11] MHC II/peptide complexes are subsequently transported to the cell surface, where they can be recognized by $CD4^+$ T cells. Beside the original paradigm that MHC I molecules present peptides of endogenous proteins and MHC II molecules present peptides of exogenous proteins, it is now also appreciated that professional APCs, like DC, have the capacity to present exogenous antigens on MHC I molecules. This process, which is known as cross-presentation, is believed to be important for the generation of a CD8-mediated immune response against pathogen-infected cells or tumor cells. Additionally, degraded, endogenous cytoplasmic proteins can be presented on MHC II by various means of autophagy, whereby the antigenic peptides are transported into the lysosomes which eventually fuse with the MIIC.[12,13] A transporter that has been associated with peptide transport into lysosomes is the homodimeric ABC transporter TAP-Like (TAPL/ABCB9).[14-16]

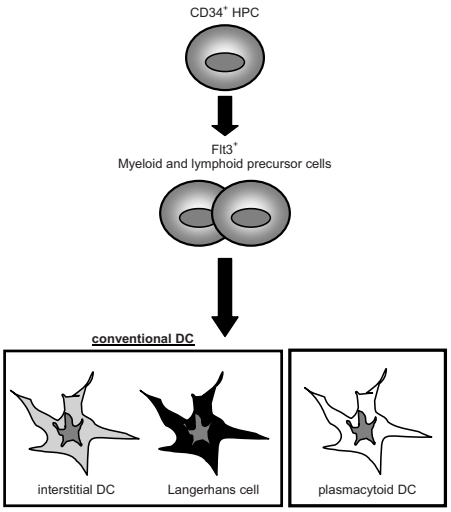

Figure 1. Differentiation of Dendritic cell subsets

Interstitial DC, Langerhans cells and plasmacytoid DC ultimately derive from a CD34+ haematopoietic progenitor cell with the capacity to differentiate into myeloid and lymphoid Flt3+ precursor cells. Both the Flt3+ myeloid and lymphoid precursors can develop into all three DC subsets, depending on environmental factors. (Adapted from Shortman and Naik, Nature.Rev.Immunology, 2007[17]).

1.1 Dendritic cells

DC are seen as the professional APC and function as bridges between the innate and the adaptive immune response.[18] DC are located at barrier sites such as peripheral tissues lining the external environment like the skin and mucosal layers and sample their surroundings for foreign antigens or danger signals. In their immature state, DC are equipped to take up and process antigens. Upon antigen encounter, DC undergo maturation which results in phenotypical and functional alterations, like a reduced capacity to take up antigens, but an increased capacity to process and present antigens. Beside that, the expression levels of chemokine receptors required for the migration to the draining lymph nodes, like CCR7 which responds to the lymph-node homing chemokines CCL-19 / MIP-3β and CCL-21 / 6Ckine [19], and integrins and adhesion molecules required for the interaction with T cells like ICAM-1 (CD54), are increased.[18,20] On the other hand, expression levels of receptors involved in recruitment of immature DC to inflamed peripheral tissues like CCR1, -5 and –6 are down-regulated.[20] With maturation, also the expression levels of MHC I and II molecules increase[7], as well as the levels of the co-stimulatory molecules CD80 (B7.1), CD86 (B7.2) and CD40, which are all required for an optimal stimulation of T cells. In addition, maturing DC gain the capacity to secrete pro-inflammatory cytokines like interleukin-12 (IL-12), which are needed for the induction of $CD8^+$ cytotoxic- (CTL) and $CD4^+$ T helper (Th) cell responses. Different effector T helper responses are required for the generation of immune responses to bacteria, viruses, parasites, fungi or tumor cells.[21] DC are involved in the skewing of effector T helper responses towards a Th1 phenotype, which give help to $CD8^+$ responses against virally infected cells and tumor cells, a Th2 phenotype, which supports antibody production by B cells and is important for controlling parasitic infections or a Th17 phenotype, which was more recently discovered and is believed to be involved in the pathogenesis of certain auto-immune diseases.[22] In addition, DC can induce the generation of T regulatory cells (Treg), through the secretion of IL-10.[23]

1.1.1 DC subsets

A variety of DC subsets has been characterized in mice.[24-29] In humans, where the *in vivo* characterization of subsets is more complicated, two myeloid DC subsets have been described in skin, i.e. interstitial/dermal DC (IDC) and epidermal Langerhans cells (LC), and two subsets have been described in blood, i.e. blood myeloid (interstitial) DC and plasmacytoid DC (pDC).[30-33] IDC and LC are also known as conventional DC. IDC, LC and pDC are ultimately derived from a $CD34^+$ haematopoietic progenitor cell (HPC). Initially it was believed that this $CD34^+$ HPC gave rise to a myeloid precursor, the predecessor of IDC and LC, and a lymphoid precursor, being the predecessor of pDC. Recently, Shortman and Naik postulated that conventional DC as well as pDC can be generated from either a myeloid or a lymphoid FMS-related tyrosine kinase 3 (Flt3)-positive precursor [Figure 1].[34,35] The majority of the DC will be derived from the myeloid precursor, but depending on the circumstances or the availability of the precursors, all subsets can also be generated from the lymphoid precursor.[17,36]

LC, which were first observed and described 140 years ago by Paul Langerhans, reside in epithelia and in skin epidermis [Figure 2]. Under steady state conditions, LC are *in* vivo derived from precursors present in the epidermis.[37] *In vitro*, LC can be generated from $CD34^+$ blood or bone-marrow precursors using the cytokines GM-CSF, TNFα and TGFβ.[38-40] Also *in vivo* TGFβ is crucial for the generation of LC, as LC are absent in mice lacking this cytokine or lacking the TGFβ receptor.[41] Under inflammatory conditions *in vivo*, generation of LC is believed to occur through the influx of $CD14^+$ inflammatory monocytes from blood.[42] IDC can be found in the sub-epithelia and in the dermis of the skin [Figure 2]. IDC can be cultured *in vitro* from $CD34^+$ blood or bone marrow precursor cells by culturing the cells with GM-CSF, TNFα and IL-4 or they can be generated from $CD14^+$ monocytes with GM-CSF in combination with IL4 [43], IL-15 [44-46], TSLP [47] or IFN-α/β [48,49], giving rise to IDC with distinct phenotypes and functions. PDC are mostly present in blood and in lymphoid organs. They have the capacity to rapidly secrete vast amounts of type I interferons when they encounter viruses.[50] In human, pDC can be generated *in vitro* from $CD34^+$ haematopoietic progenitors by culturing with Flt3-L [51] and thrombopoietin.[52] However, most extensive characterization of pDC has been done in mice.[53]

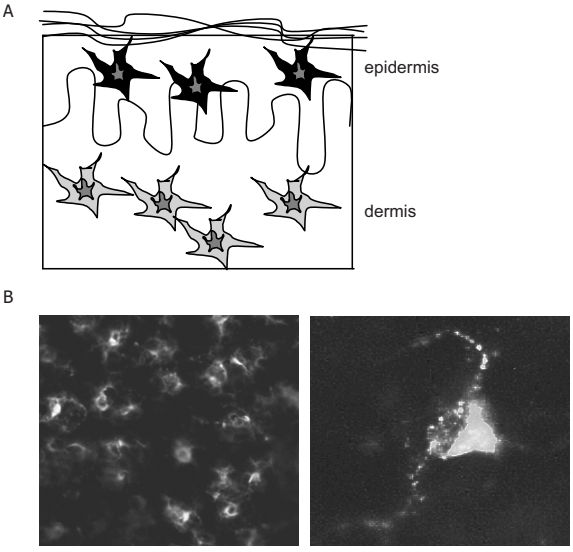

Figure 2. Skin DC

A) schematic representation of human skin with LC (black) localized in the epidermal skin layer and dermal/interstitial DC (grey) localized in the dermal skin layer. **B)** CD1a staining of epidermal skin sheets, showing the typical network of LC (left) and an enlargement of a Langerin+ cell present within the epidermis, clearly showing the protruding dendrites (right).

1.1.2 MUTZ3 as an in vitro model to culture human LC and IDC

The emergence of *in vitro* culture systems for human LC and IDC, as described above from blood- or bone marrow derived precursor cells, but also the development and characterization of several human DC cell lines [54,55], has made it easier to investigate human DC development and functioning. One such DC cell line that was introduced by our group as a model for DC development, is the acute myeloid leukemia-derived cell line MUTZ3.[56] MUTZ3 consists of three subsets i.e. a $CD34^+$ proliferating subset, a $CD34^-CD14^-$ 'intermediate' subset and a $CD14^+$ differentiating subset.[57] This cell line can be cultured into Langerhans-like cells (MUTZ3-LC) by culturing the progenitor cells in the presence of GM-CSF, TNFα and TGFβ or interstitial DC (MUTZ3-IDC) by culturing with GM-CSF, TNFα and IL-4. MUTZ3-LC and –IDC can be further matured into functional mature DC by adding maturation-inducing cytokines like TNFα, IL-1β, IL-6 and prostaglandin E_2 (PGE_2) [Figure 3A]. The MUTZ3-LC express LC-specific molecules like the C-type lectin Langerin, have high expression levels of the DC-marker CD1a and contain LC-specific Birbeck granules.[58] MUTZ3-IDC express the typical IDC C-type lectin DC-SIGN and have lower expression levels of CD1a compared to the MUTZ3-LC [Figure 3A and B]. MUTZ3-LC and –IDC have been extensively studied in our lab, and were found to be functional with respect to T cell activation, cytokine production, migration and the capacity to prime antigen-specific T cells [59] and in many ways resemble their *in vivo* skin counterparts based on gene-expression profiles and functionality.[60,61]

1.1.3 Immunoediting and immunotherapy

If both the innate and adaptive immune responses would function flawlessly, immune cells would recognize cancerous cells as 'altered self' and would eradicate them. Unfortunately, tumor cells have developed numerous mechanisms in order to fool the immune system and escape immune surveillance.[62] The effect that the immune system has on growing tumors and vice versa has been termed immunoediting.[63] Three stages (also referred to as the three "E's") were postulated: elimination, equilibrium and escape. In this model, the immune system surveys the body and is capable of eradicating tumor cells (elimination). However, surveillance by the immune system also leads to the generation/selection of less immunogenic tumors. Tumor cells that mutate under pressure of the immune system may survive the elimination phase and enter the equilibrium phase were they will eventually develop into tumors that can exist in the presence of an intact immune response.[64] Tumors that have escaped immune surveillance often secrete immune inhibitory mediators like vascular endothelial growth factor (VEGF), TGFβ, IL-6 and IL-10, resulting in a suppressive environment.[65-67] In such an environment, DC differentiation and/or activation is hampered, subsequently leading to the induction of T cell anergy or tolerance against tumor-associated antigens (TAA).[68] Indeed in most cancer patients, DC were found to be inefficiently activated at the site of the tumor.[69,70] Currently, when surgery, radiotherapy and chemotherapy fail, mostly clinical trial-based, anti-tumor treatment regimens focus on immunotherapy, whereby the aim is to (re-)activate the immune system. This can either be done in a passive way (passive immunotherapy), by introducing effector components like specific antibodies or antigen-specific T cells [71-73], which will result in a fast anti-tumor response without inducing a memory response or in an active way (active immunotherapy), where the aim is to induce a memory response against the tumor cells e.g. by vaccination with autologous or allogeneic tumor cells, TAA-presenting DC or fusions of tumor cells and DC.[74-76] As orchestrators of the adaptive immune response, DC are believed to be ideal tools for immunotherapy.[77] Today's challenge is to find the most optimal therapy, which is likely to involve a combination of chemo- or radiotherapy with immunotherapy focusing on generating effector T cell responses, combined with strategies to brake tolerance or block immunosuppressive signals like Treg depletion or the infusion of anti-CTLA4 antibodies to promote T cell proliferation by removing inhibitory check-points.[78-82]

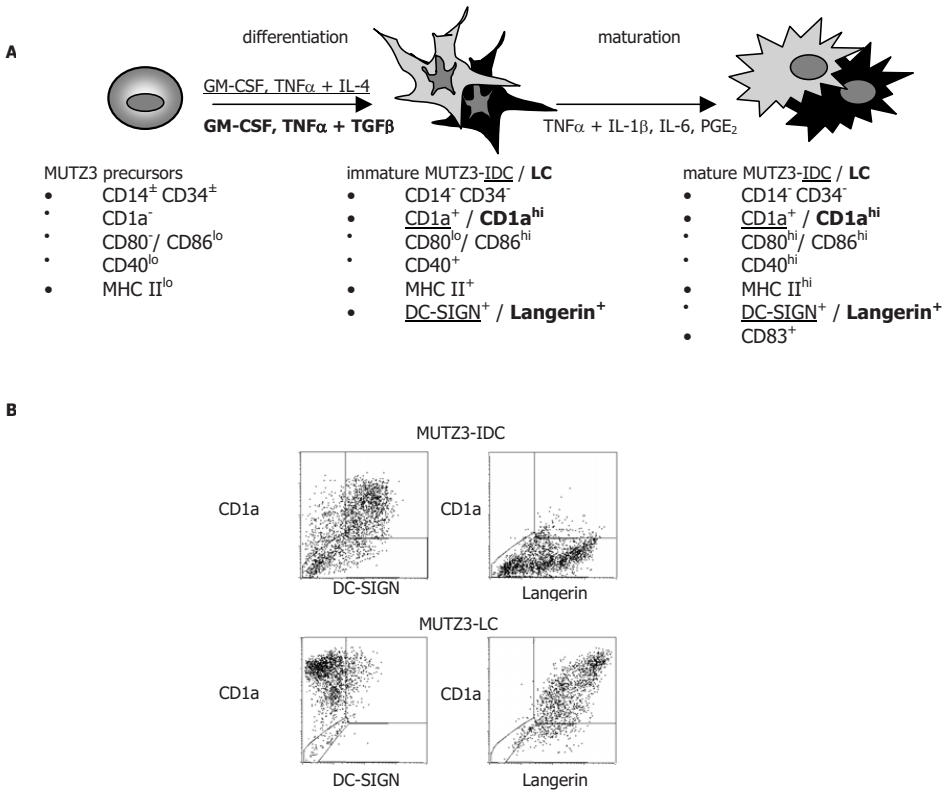

Figure 3. MUTZ3-IDC and –LC differentiation from MUTZ3 precursors.

A) MUTZ3-IDC (grey) and –LC (black) differentiation and maturation from MUTZ3 precursor cells with typical surface marker expression profiles (typical IDC: underlined, typical LC; bold, common expression on both IDC and LC; normal). **B)** Flowcytometric dot plots showing typical MUTZ3-IDC (top) and –LC (bottom) phenotype for the markers CD1a, DC-SIGN and Langerin.

1.2 Multidrug resistance

1.2.1 Cytotoxic drugs

Next to surgery and radiotherapy, cancer patients are often treated with chemotherapeutic agents either as a single drug or in a combination therapy of more than one toxic drug.[83,84] These treatments are very aggressive and along with destruction of the tumor cells, also result in destruction of healthy tissue and in suppression of the immune response by depletion of immune cells. However, there seems to be a window where the treatment with cytotoxic drugs can lead to enhancement of DC activation and positive effects on anti-tumor immunity. In the late 80s/early 90s Scheper and Limpens published several papers showing that whereas systemic administration of the drug cyclophosphamide induced severe immunosuppressive side-effects like B cell depletion, local administration of the active derivative of this drug (Z 7557) prevented this B cell depletion and resulted in highly activated DC within the regional lymph nodes.[85-87] Also studies on combination therapies of DC vaccination with

local low-doses of chemotherapy were found to enhance the anti-tumor immune response.[88] These observations justify further research for the use of combined DC/drug treatments, where the cytotoxic drugs are explored as immune-stimulating adjuvants.

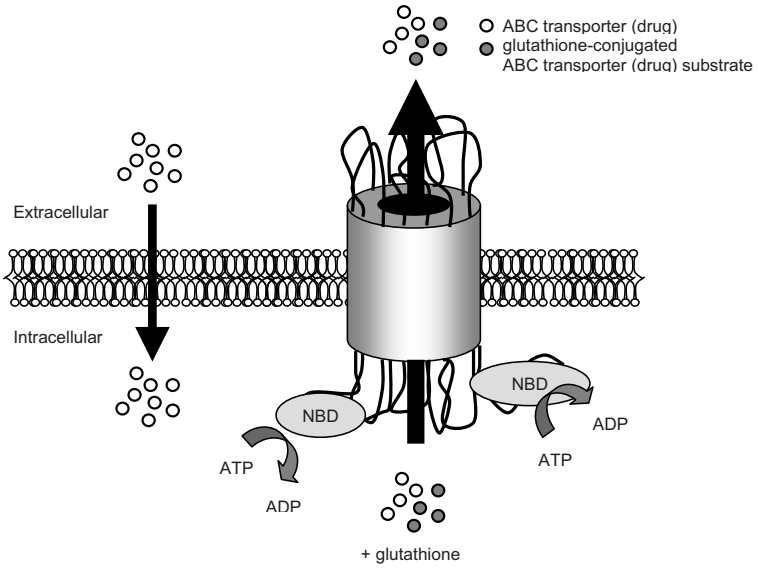

Figure 4. Energy dependent substrate transport by ABC transporters.

The ABC trans-membrane transporters require energy that becomes available from the hydrolysis of ATP into ADP in order to transport their (drug) substrates across membranes. Most often this transport is from the intracellular environment to the extracellular environment, but it can also be from the cytosol into organelles -when the ABC transporter is present on intracellular membrane structures like the ER (ABCB2/3)[9] or lysosomes (ABCB9),[14-16] or from organelles into the cytosol in case of ABCB10, which is located on mitochondria.[89] Some transporters require co-transport of glutathione. Figure adapted from Jansen et al.[90] ATP/ADP: adenosine tri/di phosphate, NBD: nucleotide binding domain.

Another complicating phenomenon resulting from chemotherapy treatment, is the induction of so-called clinical multidrug resistance (MDR). MDR is characterized as the resistance to multiple functionally and structurally, unrelated anti-cancer agents. It manifests itself by a loss in therapy efficacy upon repetitive drug exposure and by ineffectiveness of consecutive treatment with unrelated drugs.[91] It was even found that some tumors already have intrinsic resistance against cytotoxic drugs at the start of the treatment. There can be several causes of MDR: 1) defects in drug uptake, 2) enhanced drug detoxification, 3) increased drug target levels, 4) defects in apoptotic pathways and 5) the presence of active drug-efflux transporters.

1.2.2 ABC transporters

Resistance to anti-cancer drugs is a common problem faced in the treatment of cancer. Over the last decades research has shown that some ATP-Binding Cassette (ABC) transporters play an important role in MDR.[92-94] ABC-transporters represent a large family of around 50 trans-membrane proteins, which actively transport compounds across membranes, including the transport of peptides as mentioned above for TAP and TAPL [Figure 4]. The total ABC-transporter family is divided in 7 sub-families named ABCA to ABCG (according to the

official HUGO nomenclature) and many transporters exhibit wide substrate specificity by which one transporter can provoke resistance to multiple types of anticancer drugs.[95] Several transporters in the ABCB, -C and -G families have been linked to resistance against anti-cancer agents: P-glycoprotein (P-gp; MDR1; ABCB1), the MDR/TAP member ABCB5, Multidrug Resistance Protein 1 to 8 (MRP1-8; ABCC1-5, ABCC10-11) and the Breast Cancer Resistance Protein (BCRP; ABCG2).[92,96-98] Based on vesicle studies characterizing ABC transporter (drug) substrates and their expression and localization in tissues with barrier functions, these transporters are thought to play critical roles in protection against endogenous and exogenous toxic compounds. Several diseases have been linked to defective MDR transporters, and this could point to relevant physiological functions of these transporters to maintain homeostasis.[92] In recent years, expression and contributory roles of ABC transporters in the development and functioning of various immune cells have been described [Figure 5].

P-glycoprotein (ABCB1) and ABCB5

The prototype MDR transporter, P-glycoprotein (P-gp; MDR1; ABCB1) was discovered in the 1970s by Juliano *et al.*[99] and its physiological functions and role in clinical MDR have been broadly studied. The MDR1 gene on chromosome 7q21 encodes a 170kD protein and is a family member of the TAP transporter (ABCB2/3). P-gp contains two membrane spanning domains (MSDs) and two nucleotide binding domains (NBDs) required for ATP hydrolysis [Figure 6].[100,101] P-gp is located on apical membranes, mainly in organs with barrier functions such as the blood brain barrier [102,103], the kidneys [104], liver [105], intestine [106] and placenta.[107] Basal P-gp expression has been reported in several cancers, with highest expression in colon, renal, adrenal, mammary and pancreatic tumors.[108] Although this expression may contribute to primary resistance to cytotoxic agents, P-gp is often induced in tumors upon chemotherapy treatment. High P-gp expression has been correlated with poor treatment outcome in adult patients with acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL), whereas in pediatric patients P-gp expression had no prognostic impact.[109]

With a role for P-gp in drug resistance, multiple studies were initiated to characterize or design P-gp inhibitors.[108,110] Clinical trials using first generation P-gp antagonists, like verapamil [111], tamoxifen [112] and cyclosporine A [113,114] proved ineffective due to low plasma concentrations unable to inhibit P-gp *in vivo*, unexpected drug interactions, or severe clinical toxicity. In addition, the concentrations needed to inhibit P-gp activity in tumor cells *in vivo* blocked the function of P-gp in the kidneys and the liver, resulting in severe toxicities. Also studies with second generation antagonists (e.g. PSC-833 [115], S9788 [116] and VX-710 [117]) and third-generation antagonist like Tariquidar [108] revealed toxic side effects due to a reduction in the systemic clearance of the anti-cancer drugs.[108,118-120] Another inhibitor that is currently being tested in phase II and III trials is the plant alkyloid CBT-1, which can inhibit both P-gp and MRP1.[121] Reports of phase I trials with CBT-1 combined with doxorubicin or paclitaxel revealed no alterations in drug pharmacokinetics and some tumor regression was observed.[122,123] The P-gp family member ABCB5 was more recently discovered to be responsible for a drug-resistant phenotype in melanoma cells. ABCB5, which physiological function is to maintain membrane hyperpolarization, was reported by Frank et al. to be a molecular marker and a putative therapeutic target for melanoma cancer stem cells.[98]

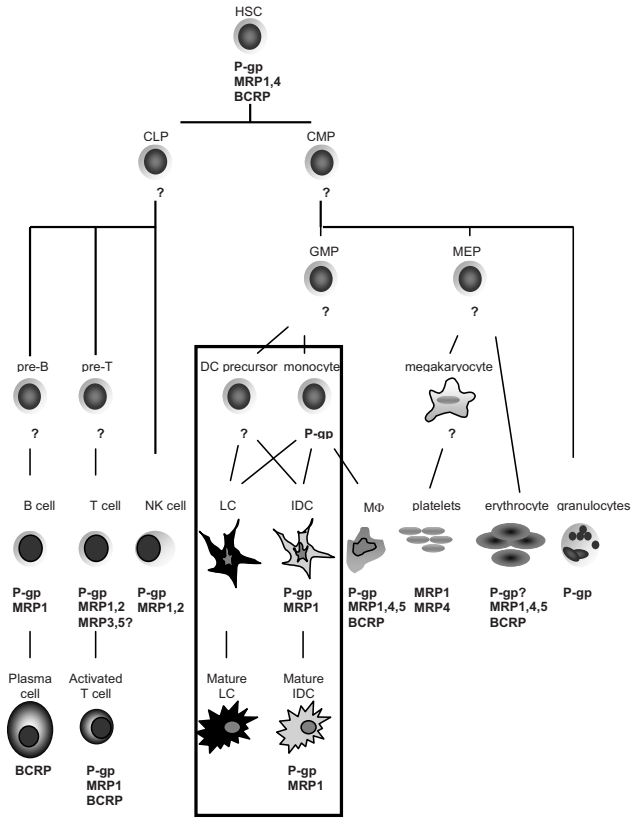

Figure 5. Expression of MDR-related ABC transporters on haematopoeietic cells.

A schematic representation of haematopoietic lineages, with for each cell type indicated which ABC transporter was found to be expressed either at mRNA level or protein level. Expression of ABC transporters on LC/IDC and their precursors, as depicted by the black box, is further investigated in this thesis. HPS: haematopoietic progenitor cell, CLP: common lymphoid precursor, CMP: common myeloid precursor, GMP: granulocyte/macrocyte progenitor, MEP: megakeryocyte/erythroid progenitor, NK: natural killer cell, DC: dendritic cell, MΦ: macrophage (In part adapted from Kock et al.[124]).

Multidrug Resistance Proteins

Thirteen ABCC family members have been characterized thus far, of which MRP1-8 are recognized for their role in MDR.[92,125] Regarding the structure of ABCC-family proteins two classes can be defined [Figure 6]: proteins with 2 membrane spanning domains (MRP4, 5 and 8), which confer resistance to nucleoside-based compounds, and proteins comprised of 3 membrane spanning domains (MRP1, 2, 3, 6 and 7), which mainly confer resistance to some natural agents. MRPs are widely expressed in the human body and can provoke resistance to a variety of established anti-cancer agents and other compounds.[126,127] High expression can be found in tissues that are frequently exposed to toxic compounds and have protective or barrier functions such as liver, kidney, blood-brain barrier and intestine.[128] Currently, several MRP antagonists have been identified, but so far only one phase I clinical trial was reported, testing the non-steroid anti-inflammatory drug (NSAID) sulindac as potential MRP1 antagonist, which was originally used to treat auto-immune diseases like rheumatoid arthritis.[129-132] In this non-randomized trial, escalating doses of sulindac were combined with the cytostatic drug epirubicin in patients with cancers of various origin. Sulindac had no effect on epiribicin pharmacokinetics and did not induce toxicity at a dose level of 600 mg. Of 15 patients with an evaluable tumor, two patients displayed a partial response and a prolonged stable disease was observed in four patients, but due to the small patient group, clinical outcome could not be related to MRP1 expression or MRP1 inhibition by sulindac.[133] It is of interest to note that beyond sulindac, several other NSAIDs turned out to possess MRP1 antagonistic activity.[134]

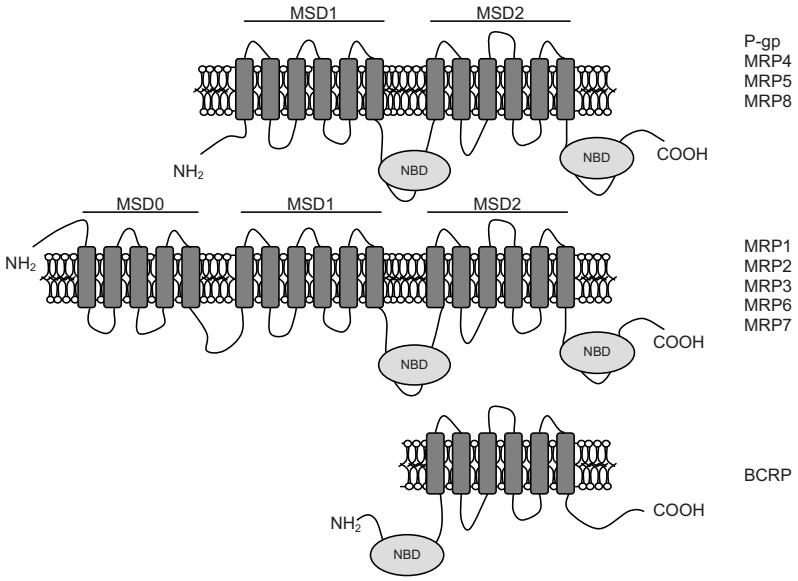

Figure 6. ABC transporter topology.

Typical topology of the ABC transmembrane proteins. MSD: membrane spanning domain. NBD: nucleotide binding domain.

Breast Cancer Resistance Protein

The Breast Cancer Resistance Protein (BCRP; ABCG2) was originally identified in the MCF7/AdrVp cell line, which displayed resistance to the anti-cancer drugs mitoxantrone, doxorubicin and daunorubicin, but did not express P-gp or MRP1.[135] BCRP is a so-called half-transporter with only one MSD and one NBD [figure 6] and functions as a homodimer[136] or possibly in multimeric forms.[137] BCRP expression has been described in various cancers [138] and drug-resistant cell lines.[139-142] Beside its role in MDR, it is anticipated that BCRP's physiological function is to protect cells from toxic stress, given its expression on numerous tissues like mammary glands, placenta, the blood brain barrier, testis and intestine.[143,144] Similar to P-gp (Abcb1 a/b) and Mrp1 knockout mice, Bcrp knockout mice displayed no clear phenotype when unchallenged. The animals did however develop severe phototoxicity upon administration of a chlorophyl-rich diet due to accumulation of the chlorophyll breakdown product protoporphyrin IX.[145] Krishnamurthy et al. observed that BCRP protects cells from hypoxic conditions via interactions with heme molecules.[146] BCRP expression is high in the so-called side-population of stem cells derived from various tissues.[147,148] The characterization of this side-population is based on the extrusion of Hoechst 33342 dye by BCRP [149,150] and BCRP is believed to protect these cells against various toxins. Several BCRP antagonists have been developed over the past decade (e.g. Fumetrimorgin-C, KO-143, GF120918), but thus far none of them have been thoroughly evaluated in clinical trials.

1.2.3 Physiological ABC transporter substrates related to the immune response

ABC transporters can play critical roles in the innate and adaptive immune response, as several small pro-inflammatory molecules have been described to be substrates for ABC transporters. Groups of such molecules are eicosanoid lipid mediators like prostaglandins and leukotrienes [151] and sphingolipids [152] [Figure 7] but also steroids or tripeptides like glutatione (GSH). The cysteinyl leukotriene C_4 (LTC_4), which is derived from arachidonic acid, exerts a pro-inflammatory effect on inflammatory cells [153] i.e. by facilitating the migration of immune cells to lymph nodes.[154,155] LTC_4 is the glutathione-conjugated form of LTA_4 and can be transported by MRP1 [156], MRP2 [157], MRP3 [158], MRP4 [159], MRP6 [160], MRP7 [161] and MRP8.[162] MRP1 has also been implicated in the transport of LTD_4 and LTE_4, which are extracellularly metabolized forms of LTC_4 [156] and MRP4 was shown to transport LTB_4 [163], which is the hydrolyzed form of the precursor LTA_4 [Figure 7]. The other group of eicosanoids, the prostaglandins are *de novo* synthesized upon stress, cytokine- or growth factor-mediated stimulation or other stimuli.[164] Different ABC transporters can transport prostaglandins: MRP4 can export prostaglandin E_1 (PGE_1), PGE_2 and $PGF_{2\alpha}$ [165] while MRP2 can export prostaglandin A_1 (PGA_1).[166] The tripeptide GSH (L-y-glutamyl-L-cysteinyl-glycine) is utilized for various cellular detoxification pathways and several ABC transporters transport GSH or GSH S-conjugates or require co-transport of GSH to transport specific drug substrates like alkylating agents.[167] Other key inflammatory components transported by MDR transporters are platelet activating factor (PAF), which is secreted by P-gp [168,169], the cyclic nucleotides cGMP and cAMP, transported by MRP4, MRP5 [170,171] and MRP8 [172], sphingosine 1-phosphate (S1P) transported by P-gp [173] or MRP1 [174] [Figure 7] and steroids, which are potential substrates for P-gp, MRPs and BCRP.[92,175-178]

A controversial issue remains whether MDR transporters are involved in the secretion of (pro)-inflammatory cytokines and chemokines with relatively low molecular mass (5-17kD). Potential protein substrates for MDR transporters could be secretory proteins that are not secreted through the classical exocytic pathway and lack a hydrophobic leader peptide. These proteins, also referred to as leaderless proteins, are secreted through a distinct secretory pathway in which ABC family member A plays a role, rather than ABCB/C/G (MDR-

related) family members.[179,180] During the last decade several studies have postulated that MDR proteins like P-gp could function as efflux transporters of cytokines such as TNFα, interleukins (IL-2, IL-4, IL-12)[181-183] and IFNγ [184] and that P-gp inhibition could reduce the secretion of several cytokines.[185] However, results from these studies are contradicted by *in-vivo* models of Abcb1 a/b knockout mice, which revealed no differences in cytokine profiles compared to their wild type counterparts.[186] These conflicting data and the currently known physical limitations of ABC transporters like TAP to transport only small (oligo) peptides of up to 40-mer residues [187,188], do not seem compatible with a direct role in the transport of cytokines with molecular masses exceeding 17kD. It may however be possible that by extruding some relevant physiological substrates, alterations in cytokine secretion may result as secondary effects.[189]

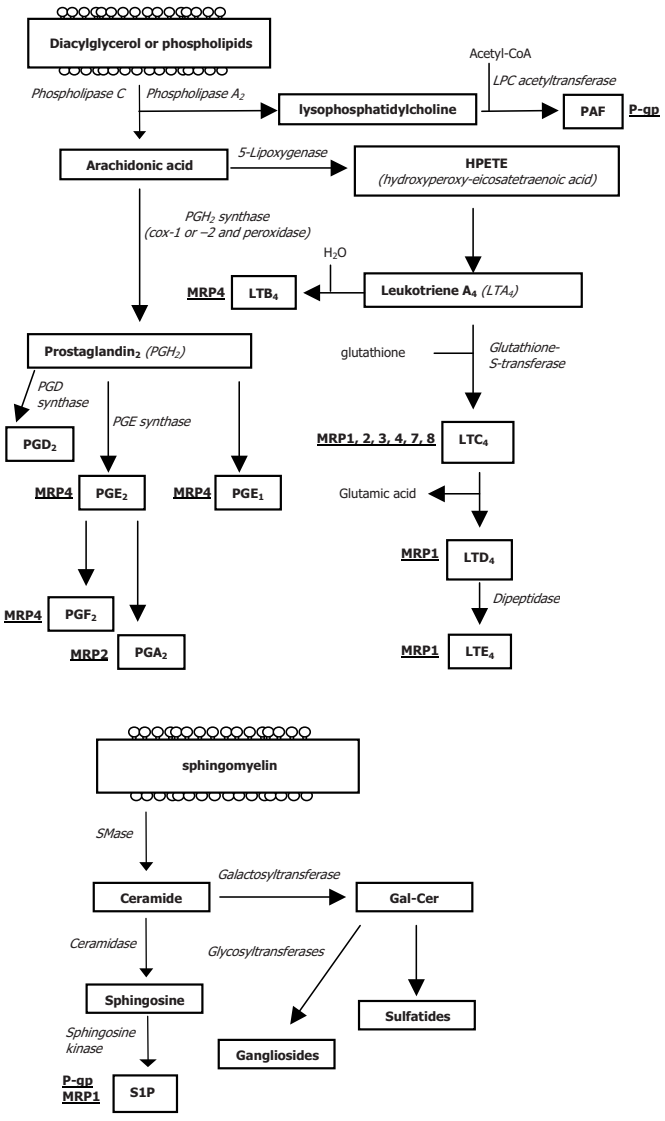

Figure 7. Synthesis of lipid mediators of inflammatory responses and ABC transporters by which they may be pumped.

Plasma membrane lipids are converted into a variety of lipid mediators involved in many immunological processes like prostaglandins, leukotrienes and sphingosines. Many of these lipid mediators have been described to be substrates for MDR-related ABC transporters, as indicated in the scheme. PAF: platelet activating factor, $LTB_4/C_4/D_4/E_4$: leukotriene $B_4/C_4/D_4/E_4$, $PGA_2/D_2/E_1/E_2/F_2$: prostaglandin $A_2/D_2/E_1/E_2/F_2$, GalCer: galactocyl-ceramide, S1P: sphingosine-1-phosphate, SMase: sphingomyelinase.

1.2.4 ABC transporters and DC

Both mouse and human DC were described to express P-gp [190,191] and Randolph *et al.* reported that DC require P-gp activity for their migration to lymphatic vessels, as P-gp neutralizing antibodies and the P-gp antagonist Verapamil reduced migration of DC and retained the DC within the epidermis.[190] It remains unclear which physiologic P-gp substrate(s) actually drive DC migration. Possible candidates are the P-gp substrates PAF [169] and S1P. The latter has been studied for its role in T lymphocyte migration.[192] Conceptually, a model for T cell homing towards the LN might involve S1P transport by P-gp and LTC_4 transport by MRP1. As MRP1 was found to be expressed by DC and to be involved in DC migration through the transport of LTC_4 [156,193], a similar model could apply for LN homing by DC. A direct contribution of MRP1 in DC migration was shown in *Mrp1* knockout mice, which required the exogenous addition of the MRP1 substrate LTC_4 or its derivative LTD_4 to restore DC migration. In light of these observations and with ABC transporters transporting pro-inflammatory substrates, we felt there was a clear rationale to explore the expression patterns and relative importance of ABC transporters in the development and the functioning of the sentinels of the immune system, the DC.

1.3 Scope of this thesis

The main aims of the project leading to this thesis were to

i) Clarify the role(s) of ABC transporters in DC physiology and function.
ii) Delineate effects of cytotoxic drugs on DC and immune functions.
iii) Provide clues for augmenting DC functions in immuno-suppressed cancer patients.

For these studies we made use of several relevant DC models, i.e. human monocyte-derived DC (MoDC), the AML-derived cell line MUTZ3, human $CD34^+$ blood precursor cells, human skin explants and DC isolated thereof, and murine bone marrow-derived DC (BMDC).

In **Chapter 2** we studied the role of the two best-characterized ABC transporters, P-gp (ABCB1) and MRP1 (ABCC1) in DC differentiation. In our study MoDC, MUTZ3-DC and -LC were differentiated in the presence of P-gp and MRP1 antagonists and effects on DC phenotype and functions were analyzed.

Chapter 3 describes the effects of introduction of BCRP (ABCG2) in MUTZ3 precursor cells on DC differentiation. In addition, human skin sections, isolated skin DC and blood DC precursors and DC derived thereof were stained for BCRP to study the expression levels and patterns of this transporter.

In **Chapter 4** we examined the DC-differentiating effects of the cytotoxic drugs mitoxantrone and doxorubicin, as typical drug substrates of ABC transporters. We describe a fast protocol for the generation of functional DC/LC using the cytotoxic drug mitoxantrone. The use of low-dose cytostatic agents as immuno-adjuvant or in combined chemo-immunotherapies is discussed.

As opposed to the short-term immunostimulating effects of cytotoxic drugs discussed in Chapter 4, **Chapter 5** illustrates the putative negative side effects of long-term use of these drugs on the capacity of precursor cells to differentiate into DC.

In **Chapter 6** the expression of the ABC transporter MRP4 (ABCC4) on human skin DC and its necessity for human skin DC migration is described and discussed. These observations made in human *in vitro* and *ex vivo* models were further evaluated in a murine *in vivo* setting as described in **Chapter 7**.

Chapter 8 shows the use of the adenoviral vector Ad5/3 as a novel tool to selectively target mature human skin and lymph node DC '*in vivo'*, e.g. applied in immunotherapeutic strategies aiming to introduce or knock down ABC transporters in order to improve/reduce DC functionality.

In **Chapter 9** the data described in this thesis are summarized, the relevance of ABC transporters for DC and the immune system and the consequences for the treatment of cancer are discussed and future perspectives are given.

Chapter 10 contains the English and Dutch summaries. The author's CV, publication list and 'Dankwoord / Acknowledgements' can be found in **Chapter 11**.

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