The contribution of ABC transporters to dendritic cell development and function

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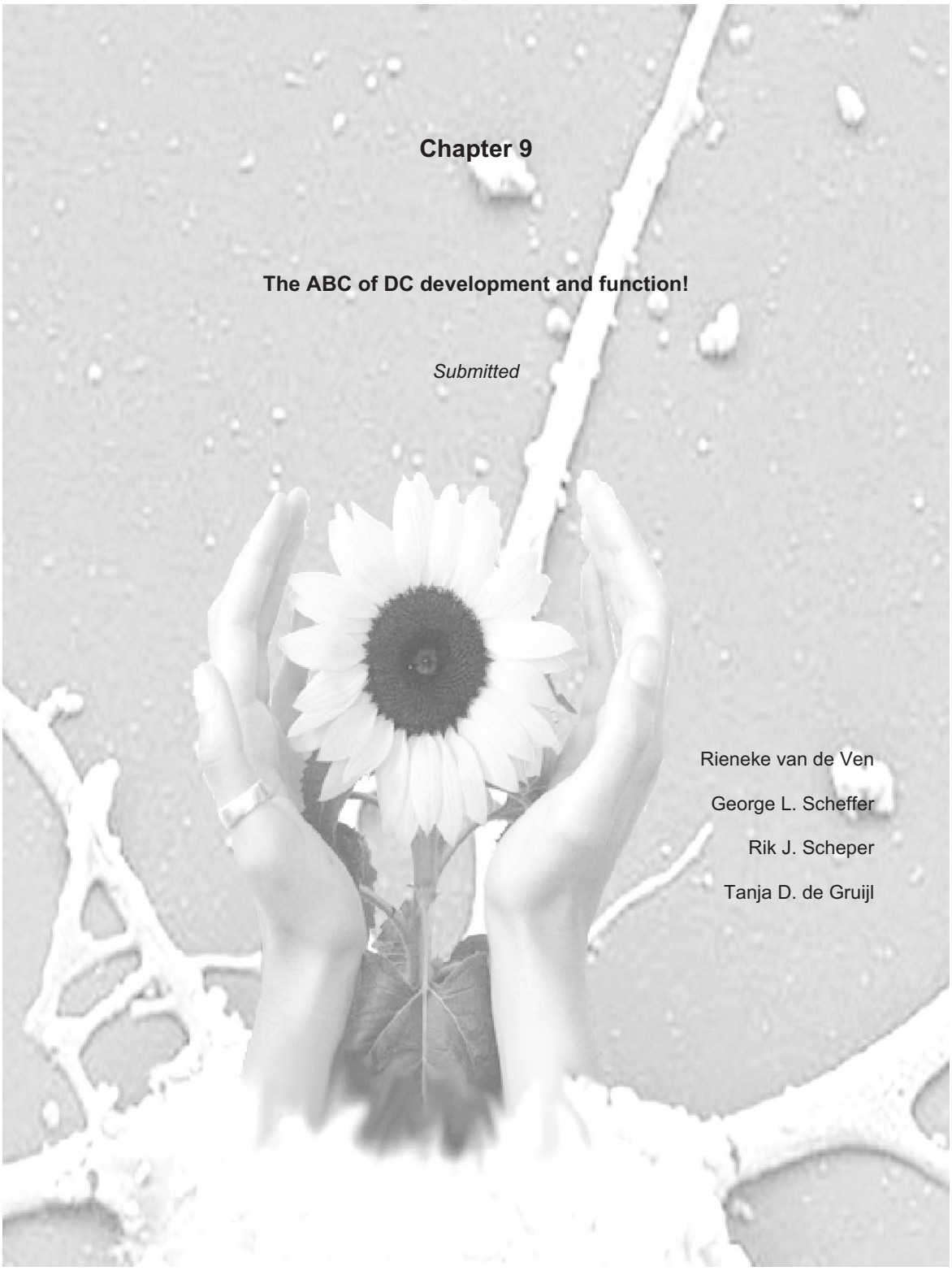

Chapter 9

The ABC of DC development and function!

Submitted

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Abstract

Dendritic cells (DC) are professional antigen presenting cells (APC) that bridge the innate and adaptive immune system. Characterization of factors that optimize their efficiency may increase the efficacy of DC-based immunotherapeutic strategies. Here we give an overview of the contribution of ABC transporters to DC development and function and discuss how these pumps can mediate specific functions in DC.

Efficient treatment of tumors with chemotherapeutic agents is frequently hampered by acquired multidrug resistance (MDR), which can be caused by the presence of ATP-binding cassette (ABC) transporters.[1,2] These transmembrane proteins are able to transport many different drugs and toxic compounds, thereby reducing the toxic load in tumor cells. In theory, inhibition of ABC transporter activity should result in increased efficacy of drug treatment. However, various clinical trials exploiting ABC transporter antagonists in combination with cytotoxic drugs gave disappointing results, often due to severe toxicity.[3] The reason for this turned out to be that ABC transporters are not only expressed by tumor cells, but in addition have important functions on barrier organs and tissues like the kidneys, the liver and the blood brain barrier.[4-6] In recent years several reports were published on the expression and functions of ABC transporters in immune cells.[7-14] Beside suppressive pressure by the tumor environment and toxic effects of cytotoxic drugs on immune cells, the inhibition of ABC transporters in conjunction with drug application could thus further dampen the effectiveness of the immune system.

Cancer immunotherapy, which aims to (re)-activate the patient's immune system against tumors, may present an attractive adjuvant therapy option.[15] DC are ideal tools for immunotherapeutic strategies due to their intrinsic capacity to efficiently present antigens to T cells and thereby induce both $CD8^+$ cytotoxic T (CTL) cells as well as $CD4^+$ T helper (Th) cells and promote cytotoxic anti-tumor responses and anti-tumor memory responses.[16,17] On the other hand, DC are also involved in the maintenance of tolerance to self antigens[18,19] and can induce tolerance or T cell anergy to tumor antigens when insufficiently activated due to environmental suppression.[20] To optimally use DC to improve the immune response in immunocompromised cancer patients, a better understanding of their development and function is warranted. Here, we discuss the contribution of ABC transporters to DC development and function and the consequences and possibilities for future immunotherapeutic strategies.

ABC transporter expression on DC

Expression of ABC transporter family members on DC was first reported by Randolph *et al.*, who described P-glycoprotein (P-gp; ABCB1) and multidrug resistance protein 1 (MRP1; ABCC1) expression on DC.[13,14] Those data prompted us to study expression patterns of ABC transporters on different subsets of human DC. Table I presents an overview of the expression of P-gp, MRP1, -3 and –4 (ABCC1,3,4) and breast cancer resistance protein (BCRP; ABCG2) on isolated skin interstitial DC (IDC) and Langerhans Cells (LC), immature and mature monocyte-derived (interstitial) DC (MoDC) and immature and mature IDC and LC derived from the acute myeloid leukemia cell line MUTZ3. MUTZ-3 represents a well characterized cell line model for human DC and LC differentiation, reflecting all the physiologically relevant cytokine-induced maturation states and showing a high phenotypic and functional homology to primary DC and LC counterparts.[29-31] As an internal control, all DC types expressed high mRNA levels of one of the molecules forming the transporter associated with antigen processing (TAP1; ABCB2).[32] In contrast to the data reported by Randolph et al., no P-gp protein or mRNA expression was detected on any of the DC populations tested. All DC types expressed high mRNA and protein levels of MRP1. It must be noted however that MRP1 protein expression was hardly detected *in situ* on human skin IDC and LC (data not shown), suggesting that perhaps the high expression on isolated cells was partly caused by the isolation procedure. Expression of MRP4 and BCRP was confirmed at the protein level in skin IDC and LC and confirmed

in situ in skin biopsies (van de Ven et al., submitted for publication).[24] Beside expression of the ABC transporters shown in Table I, array analysis also revealed mRNA expression of MRP5 (ABCC5) and MRP7 (ABCC10) in immature and mature MoDC and in immature MUTZ3-IDC and LC (our own unpublished observations, data not shown). No MRP7 mRNA was present in the isolated human skin IDC/LC, and MRP5 mRNA expression was not analyzed. MRP5 and MRP7 protein expression were not further studied. A profile of ABC transporter expression on DC and their precursor cells, based on these expression analyses and additional analysis of $CD14^+$ and $CD34^+$ precursor cells, is presented in Figure 1.

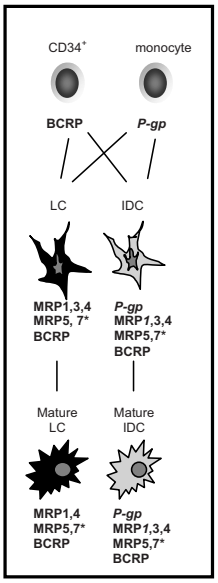

Figure 1. ABC transporter expression on DC.
P-gp expression on monocytes and P-gp and MRP1 expression on immature and mature IDC had already been reported in literature (*in italics).*[13,14,28,33] Our own studies have now expanded knowledge of the ABC transporter repertoire expressed on DC (see Table I). $CD34^+$ blood DC precursors were found to express BCRP (van de Ven et al., submitted for publication). * MRP5 and MRP7 mRNA expression unpublished data van de Ven et al.

Table I. Protein and mRNA expression on skin-isolated and *in vitro* generated DC[1]

	P-gp[2]		MRP1		MRP3		MRP4		BCRP	
	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA
skin IDC	-	-	80% + 20% -	+	nd	+	10% + 75%-/+ 15% -	+	80% + 20% -	nd
skin LC	-	-	90%++ 10% -	++	nd	++	90%+++ 10%+	++	90% + 10% -	nd
immature MoDC	-	-/+	100% +	+	10% + 90% -	++	-	-	60% + 40% -	++
mature MoDC	-	-/+	100%++	++	10% + 60%-/+ 30% -	+++	-	-	30% + 20%-/+ 50% -	+
immature MUTZ3-IDC	-	-	90% + 10%-	+	-	-	10%+ 80%-/+ 10% -	++	-	-
mature MUTZ3-IDC	-	-	75%++ 15% + 10% -	+	-	-	60%++ 25% + 15% -	++	-	-
immature MUTZ3-LC	-	-	90% ++ 5% + 5% -	++	5% -/+ 95% -	+	80%++ 15% + 5% -	++	-	-
mature MUTZ3-LC	-	-	90%++ 5% + 5% -	++	-	-	60%++ 30% + 10% -	++	-	-

[1]Shown are the levels of protein expression as determined by immunocytochemistry for P-gp, MRP1, MRP3, MRP4 and BCRP. The indicated percentages refer to the average percentage of cells having that staining intensity within cytospin preparations of the respective DC type. For skin Interstitial DC (IDC) and Langerhans Cells (LC), only the expression on CD1a+ isolated skin IDC/LC is shown. mRNA levels were determined by micro-array analysis. Isolated skin IDC/LC and immature MUTZ3-DC/LC were directly compared on Affymetrix Human Genome U133A arrays as described.[21] Immature and mature Monocyte-derived DC (MoDC), immature and mature MUTZ3-DC and -LC were compared by means of a low-density micro-array as described.[22-24] (nd = not determined. Antibodies used for immunocytochemistry: C219 for P-gp, MRPr1 for MRP1[25], M_3II-9 for MRP3 [4], M_4I-10 for MRP4 [26] and BXP-53 for BCRP.[27] (– : no expression, -/+: weak expression, +: clear expression, ++ strong expression, +++: very strong expression).

[2] P-gp was previously reported to be expressed on human MoDC.[13,28]

Roles for ABC transporters in DC physiology

The presence of ABC transporters on DC suggests that these pumps contribute to either the development or to functional characteristics of DC. As mentioned, the first link between ABC transporter expression and DC function was reported by Randolph *et al.* who observed a reduced migration of human skin LC from the epidermis when P-gp activity was blocked.[13] Although never fully elucidated, possible P-gp substrates responsible for this migration could include platelet activating factor (PAF) or sphingosine-1-phosphate (S1P).[7,34] The same group later reported

MRP1 expression on both mouse and human DC, with considerably higher expression levels in mice, and showed that DC migration was hampered in mice lacking Mrp1 expression.[14] Hampered migration of Mrp1 deficient murine bone marrow-derived DC (BM-DC) was restored by exogenous addition of the MRP1/Mrp1 substrate leukotriene C_4 (LTC_4) or its derivative LTD_4. In human DC, MRP1 was also reported to contribute to migration in response to CCL19 and -21, but we found a relatively stronger involvement of MRP4.[24] Human skin DC express high protein levels of MRP4 (Table I and van de Ven et al.[24]). Intradermal injection of the MRP4 antagonist sildenafil revealed a possible contribution to human skin DC migration, as a drastic reduction in the number of migrated skin DC was observed. This was confirmed by knock-down of MRP4 expression by means of RNA interference (RNAi) in MUTZ3-LC (by retroviral transduction) or in human skin DC *in situ* (by targeted adenoviral transduction), which significantly decreased the capacity of DC to migrate.[24] In contrast, DC-targeted adenoviral delivery of MRP1 short-hairpin RNA (shRNA) only resulted in a moderate, non-significant reduction of human skin DC migration. These data demonstrate the dominance of MRP4 over MRP1 in facilitating human DC migration. Since the MK-571 antagonist used by Robbiani *et al.* can also inhibit MRP4 activity at the concentrations used in their study, inhibitory effects they observed may at least in part have been caused by MRP4 inhibition. Studies conducted in Mrp4 knockout mice revealed that in mice Mrp4 is not required for DC migration, thus clearly demonstrating inter-species differences in the functional significance of ABC transporters (van de Ven et al., submitted). Although the known MRP4 substrates PGE_2, cGMP, LTB_4 and the LTC_4-derivative LTD_4, have all been implicated in DC migration, the actual MRP4 substrate responsible for human DC migration remains to be identified, as addition of none of these substrates could restore the migratory capacity of LC generated from MUTZ3-shMRP4 cells.[24]

Reports on the role of P-gp and MRP1 in mature DC migration[14] did not rule out additional roles of these transporters at other developmental stages of DC. In our hands, inhibition of P-gp activity with PSC-833 did not result in altered DC differentiation, in contrast to the study reported by Pendse et al.[35] We showed that addition of the antagonists probenecid or MK-571, which both can block MRP1 activity, during DC differentiation from either monocytes or $CD34^+$ MUTZ3 precursor cells, resulted in hampered IDC and LC differentiation.[36] Laupèze et al. had shown that addition of a low-affinity MRP1 antagonist significantly reduced expression levels of CD40 on MoDC.[33] The underdeveloped state of the DC due to the inhibition of MRP1 with potent antagonists was apparent based on the morphology of the cells, i.e. small and round, rather than large and 'dendritic', as well as on their phenotype with a lack of typical DC markers like CD1a, CD1c, or Langerin (for LC), and low levels of the co-stimulatory markers CD40, CD54, CD86 and HLA-DR.[36] These observations were recently confirmed by Skazik *et al* .[37] In line with this underdeveloped phenotype, cells cultured in the presence of MK-571 were also hampered in their capacity to induce T cell proliferation and to secrete the pro-inflammatory cytokine IL-12. MRP1 and the DC marker CD1a were found to co-localize within DC, and by means of MRP1 vesicle studies, the glycosphingolipid CD1a-ligands sulfatide and GM1 (ganglioside M1) were identified as MRP1 substrates (Figure 2). However, sulfatide was unable to restore DC differentiation upon exogenous addition. As human MoDC do not express MRP4 (Table I), and introduction of a shRNA against MRP4 in MUTZ3 progenitor cells did not affect MUTZ3-IDC or –LC differentiation [24], the effects observed with MK-571 on DC differentiation could be definitively attributed to MRP1.

Expression of BCRP had already been described on haematopoietic stem cells, where it is responsible for the generation of the side-population phenotype, and is believed to be important for the protection of these cells against various toxic compounds.[38-43] BCRP was found to be strongly expressed by human skin IDC and LC, as well as IDC generated from human monocytes (Table 1). Inhibition of BCRP activity during MoDC differentiation did not affect DC development, nor did intradermal injection of the BCRP antagonist Ko-143 into human skin hamper DC migration (van de Ven et al., submitted). Whereas $CD14^+$ monocytes did not express BCRP mRNA or protein, protein expression was observed on *in vitro* expanded human $CD34^+$ blood precursors.

BCRP protein expression was found to increase during $CD34^+$-LC differentiation. Introduction of functional BCRP in $CD34^+$ MUTZ3 precursor cells led to accelerated LC differentiation kinetics and skewed differentiation in the presence of IDC-inducing cytokines towards a more LC-like phenotype. This occurred even in the presence of IL-4, which normally prohibits LC differentiation and effectively drives DC development to the IDC subset. This accelerated and enforced LC differentiation was shown to coincide with early expression of the transcription factor Rel-b and to be dependent on endogenous transforming growth factor-β (TGF-β) levels, as addition of TGF-β neutralizing antibodies in cultures without exogenously added TGFβ reduced $CD1a^{hi}$ and Langerin expression (van de Ven et al., submitted).

Although MRP3 mRNA and protein expression was detected in human DC, a functional role for MRP3 in DC remains to be explored, as no MRP3-specific antagonists or fluorescent substrates are currently available that would allow functional characterization.

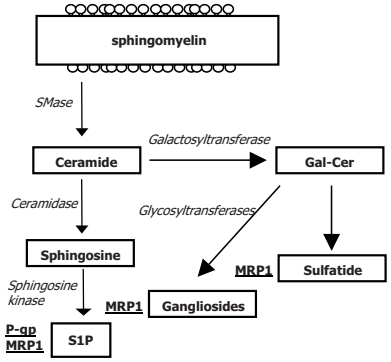

Figure 2. MRP1 transports glycosphingolipids
Using MRP1-carrying vesicles, the glycosphingolipids sulfatide and GM1 were identified as substrates of MRP1.[36] This adds two more small molecules derived from the sphingolipid synthesis pathways to the ever growing list of ABC transporter substrates.[34,44]

Implications for tumor immunotherapy

In the treatment of cancer, chemotherapy is the third most frequently used option after surgery and radiotherapy. It is recognized that chemotherapy regimens are often hampered by the development of chemoresistance during treatment.[5,45] Over the past decades, the discovery of the involvement of ABC transporters in the resistant phenotype initiated extensive research for potent ABC transporter antagonists in order to overcome drug resistance.[46,47] However, at that time little was known about the putative physiological functions of these transporters. Presently, several of these transporters have been linked to physiologically important processes, e.g. detoxification processes in the kidneys and the liver, integrity of the blood-brain-barrier and placenta in the protection against endogenous (and exogenous) toxic compounds[4-6], and the secretion of substrates needed for effector functions of immune cells.[9-12,34,48-50] It has therefore become clear that clinicians should observe caution when inhibiting the activity of these transporters, as this could evoke unwanted side effects. Given the poor clinical benefit and severe toxicity of systemic application of ABC transporter antagonists in combination with systemic chemotherapy, novel strategies should aim for more targeted or localized application of MDR antagonists. Selective modulation of MDR transporters could be achieved with targeted delivery-systems containing antagonists or shRNAs.

With respect to the immune system and cancer, it has been established that treatment with chemotherapeutic agents dramatically hampers the immune response, as many immune effector cells are collaterally targeted by this treatment. Patients receiving chemotherapy are therefore susceptible to viral and bacterial infections. Inhibition of MDR-related ABC transporters on top of chemotherapy treatment to bypass MDR

could further compound this immune-suppression and increase the risk of infection. It is also appreciated that the immune system is important for anti-tumor effects. In Figure 3 an overview is given of the known involvement of ABC transporters in each of the steps that lead to an effective anti-tumor immune response. In the adaptive response, functional APC (and DC in particular) are needed to take up tumor cell fragments and present derived tumor associated antigens (TAA) to T cells. In order for APC precursors to fully differentiate into DC they require functional MRP1[36,51] and perhaps P-gp.[35] Increased expression of BCRP at the precursor state might further accelerate the differentiation process (van de Ven et al., submitted). Upon uptake of TAA by the DC, these need to be processed and presented in the context of MHC-I and –II molecules. These processes require transport of peptides over (endoplasmic reticulum or lysosomal) membranes, in which the ABC transporters TAP1/2 (ABCB2/3) and TAP-like (TAPL; ABCB9) are known to play vital roles.[32,52-57] Subsequently, mature DC need to migrate to the draining lymph nodes in order to present the TAA-derived epitopes and activate T cells. For this migration the presence of P-gp[13], MRP1[14] and MRP4[24] is critical. For the homing of T-cells inside the lymph nodes, both MRP1 and P-gp are equally required.[34] Activated T cells subsequently leave the lymph nodes, in order to home to the site of the tumor where they utilize P-gp and MRP1 activity to secrete cytotoxic compounds from granules and, possibly, effector cytokines.[50,58,59] Within the innate immune system, NK cells are known to recognize MHC class I down regulation on tumor cells,[60] which is a trigger for these cells to eradicate the tumor cell. For this, NK cells need functional cytotoxic granules for which P-gp activity may serve as a modulator.[61-63] Inhibition of ABC transporters, together with suppressive cytokines produced by tumor cells, could potentially completely block the development of DC and stop the presentation of TAA to T cells and in addition can hamper NK cell and T cell effector functions. On the other hand, knowledge on how to potentiate ABC transporters in their functions might enhance the efficacy of the anti-tumor response.

Cumulative data underline critical functional roles for MDR-related ABC transporters in DC (see Figure 3). This knowledge should be considered and exploited for the development of new strategies for the treatment of cancer. When increased expression of a transporter leads to enhanced development and function of DC and consequently the improvement of an immune response, this could be an interesting tool for combined chemo-immunotherapies in cancer patients. Indeed, we have observed accelerated differentiation of IDC/LC from $CD34^+$ precursors upon short-term exposure to cytostatic drugs (Van de Ven *et al*, submitted). We were however, unable to pinpoint this effect to the involvement of a particular ABC transporter. Alternative ways in which to accomplish upregulated ABC transporter expression might entail the application of DC precursor- or DC-targeted gene therapy with adenoviral vectors.[64-66] Based on our own recent findings, introduction of BCRP in $CD34^+$ DC precursor cells would accelerate LC development (van de Ven et al. submitted), while increased MRP1 expression could facilitate both DC differentiation[36] and migration[14] and enhancement of MRP4 expression on DC might also increase their capacity to migrate to lymph nodes.[24] The DC will thus gain in functionality and at the same time will be more resistant to toxic side effects of chemotherapy. In light of this observation, the potential of combined immuno-and chemotherapy strategies to enhance anti-tumor responses deserve further exploration.

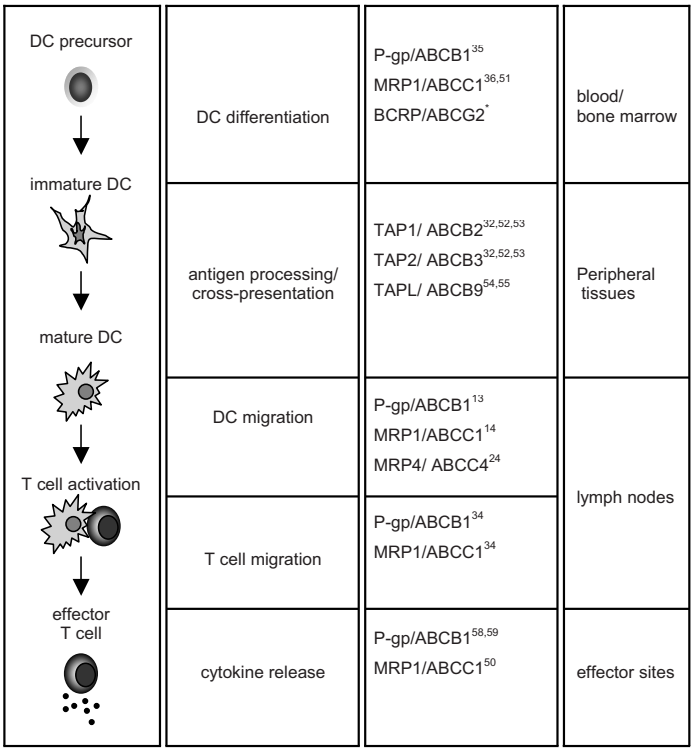

Figure 3. ABC transporters involved in the initiation of an effective immune response.
Schematic presentation of the development of an immune response and the various roles ABC transporter family members have been reported to play therein. DC; dendritic cell, TAP; transporter associated with antigen processing, TAPL; TAP-like. * van de Ven et al., submitted for publication.

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