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van de Ven, R.

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Chapter 7

Unimpaired immune functions in the absence of Mrp4 (Abcc4)

Submitted

Rieneke van de Ven

Jan de Groot

Anneke W. Reurs

Pepijn G.J.T.B. Wijnands

Koen van de Wetering

John D. Schuetz

Tanja D. de Gruijl

Rik J. Scheper

George L. Scheffer

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Abstract

Dendritic cell (DC) migration to draining lymph nodes is important for the initiation of an effective immune response. Recently we reported that the human ATP-binding cassette (ABC) transporter multidrug resistance protein 4 (MRP4; ABCC4) is required for the migration of human DC. Since the ABC transporter MRP1 (ABCC1) was previously shown to play a role in both human and mouse DC migration, we here studied whether Mrp4 is similarly required for DC migration in mice and whether the absence of Mrp4 interferes with the generation of an immune response. Immunological responses were compared in wild-type FVB (FVBwt), Mrp4 knockout (KO) or Mrp4/5 double knockout (dKO) mice. Skin, a preferred immunization site, was analyzed for DC markers, as well as for Mrp1 and Mrp4 expression. Whereas Mrp1 was abundantly present within FVBwt skin, only few Mrp4 expressing cells were detected. In addition, no Mrp4 protein expression was detected on in vitro cultured FVBwt bone-marrow derived DC (BM-DC). DC migration from murine ear skin was unaltered between FVBwt and MRP4/5 dKO animals. The absence of Mrp4 also had no effect on immune responses upon allergen sensitization, immunization or oral tolerance induction. We thus conclude that in contrast to its human counterpart, murine Mrp4 is not involved in DC migration, nor indeed, in the generation of an effective immune response. These data reveal disparities in the physiological role of ABC transporters between species, which may derive from differences in substrate specificity.

Introduction

Mice lacking the ATP-binding cassette (ABC) transporter Multidrug resistance protein 4 (Mrp4; Abcc4) have been generated for *in vivo* drug sensitivity experiments¹⁻³ and to unravel physiological substrates for this transporter.^{4,5} Although human MRP4 has been reported to transport prostaglandins E₁ and E₂ (PGE₁ and PGE₂)⁶, which are known immune modulators⁷⁻⁹, no reports have been published on the effect of MRP4 deficiency on the immune response *in vivo*. Recently we showed that human skin dendritic cells (DC) express MRP4 and that interference with MRP4 expression or activity hampered their migratory capacity.¹⁰ The MRP4 substrate guiding human DC migration remains to be identified, as relevant concentrations of the known substrates PGE₂, cGMP¹¹, leukotriene B₄ (LTB₄) or the LTC₄-derivative LTD₄¹², could not restore migration of *in vitro* cultured Langerhans cells (LC) with RNAi-mediated reduced expression of MRP4.

DC are the most potent antigen presenting cells (APC) and are crucial for the initiation of an effective immune response via activation of antigen specific T cells.¹³ Both the ABC transporters P-glycoprotein (P-gp; Abcb1) and the Multidrug resistance protein 1 (Mrp1; Abcc1) were previously reported to play a role in the migration of murine DC towards the lymph node-homing chemokines CCL19 and CCL21.^{14,15} The mechanism whereby P-gp influences DC migration is still poorly understood. MRP1 transports the inflammatory mediator LTC₄, which is known to regulate inflammatory responses and DC migration.¹⁵⁻¹⁷ Robbiani et al. showed that DC migration towards CCL19 was hampered in DC obtained from Mrp1 knock-out mice and could be restored by the exogenous addition of LTC₄ or its derivative LTD₄.¹⁵ They also showed that inhibition of MRP1 with the antagonist MK-571 in human monocyte-derived DC or human skin explants reduced DC migration.¹⁵ We recently found that interference with MRP4 expression in human skin DC induced even stronger inhibition of DC migration than interference with MRP1 expression.¹⁰ Hence we decided to study the consequences of Mrp4 deficiency for the *in vivo* immune response in Mrp4 knockout (Mrp4 KO) mice. Our hypothesis was that Mrp4 deficiency in mice would result in reduced DC migration and consequently impaired immune responsiveness.

It is known that MRP4 and MRP5 (ABCC5) are very homologous and share several substrates.^{18,19} Although we never observed MRP5 expression on human skin DC (unpublished data), we did observe an

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increase in MRP5 mRNA expression in MUTZ3-shMRP4 cells.¹⁰ To exclude compensatory mechanisms, we tested the immune response of Mrp4 KO mice, as well as Mrp4/Mrp5 double knock-out (Mrp4/5 dKO) mice. Several immunological parameters were analyzed, assessing the *in vivo* and *in vitro* functionality of DC, T cells and B cells. Contrary to our expectations, no indication was found that the lack of Mrp4 expression in mice resulted in altered DC migration or altered immunological responses. This study, combined with our previous report on the necessity of MRP4 for human DC migration¹⁰, thus emphasizes the differences between species (i.e. human and mice), with respect to the importance of ABC transporter proteins for the functioning of DC.

Materials and Methods

Mice

FVB Mrp4/Mrp5 double knockout (Mrp4/5 dKO) were generated in the Netherlands cancer institute (NKI) and FVB Mrp4 single knockout (Mrp4 KO) mice were generated in the lab of Dr. J. Schuetz. Animals were kept under conventional conditions at the animal facility at the department of pathology, VU University medical center, Amsterdam. All animal experiments were carried out after consent and approval of the ethical board of the Animal Experimental Commission from the VU University.

Bone marrow-derived DC cultures

Bone marrow-derived DC (BM-DC) were generated as described previously.²⁰ In short, bone marrow was flushed with RPMI medium using a 19 gauge needle from femur and tibia of FVBwt and Mrp4 KO mice. Single cell suspensions were made by resuspension of the bone marrow in the medium and filtration over a 100µm pore-sized sterile cell strainer. Mononuclear cells were separated from the bone marrow cells by density-gradient centrifugation over Lympholyte-M (Cedarlane Laboratories, Hornby, Canada) for 20 minutes at 1500g. 2×10^6 mononuclear cells were seeded in 5ml of RPMI supplemented with 10% FCS, 100 IU/ml sodium-penicillin, 100µg/ml streptomycin, 2 mM L-glutamine in 100x20mm petri dishes, with the addition of 20ng/ml recombinant mouse GM-CSF (rmGM-CSF, Peprotech, Huissen, The Netherlands). Medium and rmGM-CSF were refreshed at days 5 and 8. At day 10 cells were matured by adding 1mg/ml lipopolysaccharide (LPS; Sigma, St. Louis, USA) for 24 hours.

Flow cytometry

Phenotypic characterization of (BM)DC, blood and spleen samples was performed by flow cytometry, using PE- and FITC-labeled anti-mouse monoclonal antibodies against CD3 (1:100), CD8 (1:100), CD11c (1:100), CD86 (1:100) (BD Biosciences Europe, Erembodegem, Belgium), CD80(1:100) (Southern Biotechnology Associates Inc., Birmingham, USA), F4/80 (1:100) (Invitrogen, San Diego, USA) and MHC II (1:1500) (eBiosciences, San Diego, USA), unconjugated CD19 (1:100) (BD Biosciences Europe, Erembodegem, Belgium) with donkey-anti-rat IgG-PE (1:200) (Jackson Immunoresearch, Suffolk, UK) as secondary antibody.

Immunocytochemistry

Cytospin preparations from BM-DC or murine skin sections were analyzed for ABC transporter expression. In short, samples were incubated overnight at 4°C with 5 µg/ml rat monoclonal antibodies (Mabs); MRP1 to detect Mrp1 (Abcc1); M₄-10 to detect Mrp4 (Abcc4) and M₅-10 to detect Mrp5 (Abcc5).^{1,21,22} The M₅-10 Mab was generated in our lab against a fusion protein of the bacterial maltose binding protein and the N-terminal region (amino acid 1-38) of mouse Mrp5 (unpublished data). A rat Mab against mouse MHC II (clone M5/114.15.2, eBiosciences, San Diego, USA) was taken along as a positive control to stain DC. Antibody binding was detected with biotinylated 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). As negative control, isotype-matched rat IgG antibody were used.

Split ear migration

Ears were obtained from 3 sacrificed FVBwt and Mrp4/5 dKO mice after the *in vivo* experiments (3 experiments with 3 mice were performed). Dorsal and ventral ear-halves were separated using tweezers and a scalpel and after carefully scraping the cartilage from the dorsal half, each half was left floating on 1ml IMDM supplemented with 10% FCS, 100 IU/ml sodium-penicillin,

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100µg/ml streptomycin, 2 mM L-glutamine in a 24-well culture plate. To the medium of either the dorsal or the ventral ear-half, 20ng/ml rmGM-CSF was added. Cells were allowed to migrate from the ears for 48 hours after which migrated cells from 3 ear-halves were pooled (medium or rmGM-CSF separately), quantified by trypan blue exclusion and by means of flow-count fluorospheres (Beckman Coulter, Fullerton, CA) and were phenotyped by flow cytometry. As the addition of rmGM-CSF to the medium did not significantly alter the amount of migrated DC, the medium and rmGM-CSF values were taken together in the overall analysis.

Polyclonal cell proliferation

Single cell suspensions were generated from mouse spleens by applying the tissue to 100µm pore-sized sterile cell strainers (Beckton-Dickinson, Franklin Lakes, NJ, USA). 1.0×10^5 splenocytes were stimulated with 1, 10 and 100ng/ml PMA (Sigma, St.Louis, USA), 0.1, 1 and 10µg/ml PHA (Murex, Paris, France) or 0.1, 1 and 10µM LPS for 24 or 48 hours, after which 2.5µCi/ml [³H]-thymidine (6.7Ci/mmol, MP Biomedicals, Irvine, CA) was added per well for 16 hours. Plates were harvested onto glass fiber filtermats (Packard Instruments, Groningen, The Netherlands) using a Skatron cell harvester (Skatron Instruments, Norway). [³H]-thymidine incorporation was counted and quantified using a Topcount NXT Microbetacounter (Packard, Meriden, CT). Responses are shown as mean counts per minute (cpm) from triplicate wells averaged over 3 mice per group.

Oxazolone sensitization

All handlings were performed under anesthetics with isoflurane. For these experiments twelve FVBwt and twelve FVB Mrp4/5 dKO mice were used. On day -1 blood was drawn for FACS analysis and ear thickness was measured. On day 0 the abdomen was shaved and 6 FVBwt and 6 FVB Mrp4/5 dKO mice were sensitized by epi-cutaneous (e.c) application of 20µl 5% oxazolone (OXA; Sigma, St. Louis, USA) in acetone / olive oil (8:1) at 0h and 8h. Six control animals received 20µl vehicle solution. After 5 days, all mice were challenged by e.c. application of 10µl 5% OXA in EtOH on the left ear. The right ear received vehicle. Swelling of the ear was measured 5, 24, 48 and 72 hours after the challenge. Mice were sacrificed and spleen and bone marrow were removed for further analysis.

Ovalbumin immunization

Six FVBwt and six FVB Mrp4/5 dKO mice received a subcutaneous (s.c.) injection of 50µg ovalbumin (OVA; Sigma, St. Louis, USA) in dimethyldioctadecyl ammonium bromide (DDA; 30nM in 50µl) (Tramedico, Weesp, The Netherlands). All handlings were performed under anesthesia with isoflurane. Control animals (6 per group) received s.c. vehicle injection. On day 7, ear thickness was measured and all animals were challenged by intra cutaneous (i.c.) injection of 10µg OVA in 30µl PBS in the left ear. The right ear received vehicle. Ear swelling was measured at 5-72 hours after challenge. After 72 hours, blood was drawn from the mice to screen for OVA-specific antibodies in the serum and animals were sacrificed.

Oral tolerance induction

Six FVBwt and six FVB Mrp4/5 dKO mice received intra gastric OVA at day 1, 3 and 8 (10mg OVA in 200µl PBS each time). Control mice were fed only PBS. On day 13, all mice were s.c. immunized with 50µg OVA in DDA (30nM). After 7 days (day 20), ear thickness was measured before challenging the mice with an i.c. injection of 10µg OVA in 30µl PBS in the left ear, followed by measurements of ear swelling after 5-72 hours. The right ear received vehicle as a control.

Antibody isotype shift

Sera from control and OVA immunized FVBwt and FVB Mrp4/5 dKO mice were tested for OVA-specific antibodies by enzyme-linked immunosorbent assay (ELISA). Flat bottom 96-well microtiter plates were coated overnight with 1µg/well OVA. Each mouse serum was diluted 1:50, 1:200 and 1:800 in PBS and applied to the wells in triplicate. An in-house made mouse-anti OVA antiserum was taken along as positive control. Anti-OVA antibodies in the serum were detected with goat-anti-mouse immunoglobulin (1:1000) against IgG (total), IgG1, IgG2a, IgG2b, IgG3, IgM, IgA or IgE (Sigma, St.Louis, USA), followed by HRP-conjugated rabbit-anti-goat immunoglobulins (1:1000) (DAKO, Glostrup, Denmark). Between successive steps, plates were washed 3x with PBS containing 0.02% Tween-20 (Fagron, Uitgeest, The Netherlands). Bound antibody was detected by a color reaction using 10 µg/ml TMB (3,3',5,5'-tetramethylbenzidine) with 1:1000 3% H₂O₂. The color reaction was stopped by adding 50µl of 2.5M H₂SO₄. The absorbance was determined at an optical density (OD) of 450nm.

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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 $p < 0.05$.

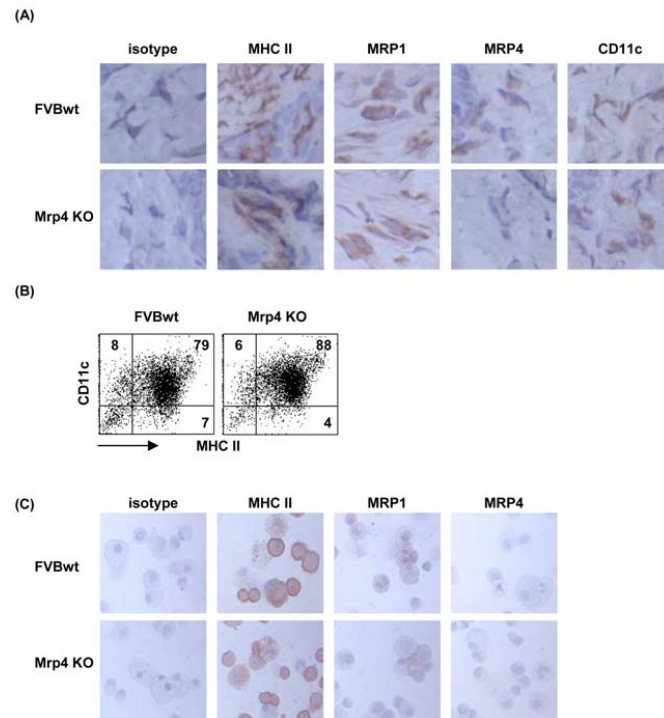


Figure 1: Immunohistochemical analysis of murine skin and BM-DC for ABC transporters.

A) Immunohistochemical analysis of skin sections from FVBwt and Mrp4 KO mice stained for the markers MHC II, CD11c, MRP1 and Mrp4 (magnification 200x). **B)** Flow cytometric analysis showing CD11c and MHC II expression levels on BM-DC cultured from FVBwt or Mrp4 KO cells. Percentages positive cells are indicated in the corresponding quadrants. **C)** Immunohistochemical analysis for MHC II, MRP1 and Mrp4 expression on BM-DC from FVBwt or Mrp4 KO mice.

Results

Mrp4 and Mrp1 expression on murine skin- and BM-DC

As we have reported, human epidermal skin DC, and at lower levels dermal DC, express MRP4.¹⁰ When immunohistochemical analysis was performed on mouse skin, comparable amounts of cells with DC morphology stained with antibodies directed against the DC/macrophage-marker MHC II, the DC-marker CD11c and the ABC transporter Mrp1 [Figure 1A]. Mrp4 expression was detected at lower levels and in fewer cells compared to MHC II and Mrp1 in dermal cells of FVBwt mice [Figure 1A]. In skin from Mrp4 KO mice, MHC-II, CD11c and Mrp1 were expressed to comparable levels as in the FVBwt skin, suggesting normal DC differentiation *in vivo*, in the absence of Mrp4 protein. Apparently, the absence of Mrp4 also had no effect on *in vitro* bone-marrow DC (BM-DC) differentiation. In figure 1B CD11c and MHC II expression is depicted on *in vitro* generated wt and Mrp4 ko BM-DC (representative of 4 experiments). Figure 1C shows immunocytochemical stainings of BM-DC cytospin preparations for MHCII, Mrp1 and Mrp4. No Mrp4 (or Mrp5, data not shown) protein expression was detected in

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the FVBwt BM-DC. As expected, weak but distinct Mrp1 expression was observed on *in vitro* cultured FVBwt BM-DC. Mrp1 protein was present in similar levels in BM-DC from Mrp4 KO mice as from wt mice. Given the lack of Mrp4 expression on wt BM-DC further experiments on a potential role of Mrp4 in DC functioning focused on skin DC.

Unaltered skin DC migration, blood cell subset distribution and polyclonal cell proliferation in the absence of Mrp4

To examine whether the absence of Mrp4 influenced murine skin DC migration, DC migration from split-ear skin was assessed. Dorsal and ventral ear-halves were split and left floating on medium with or without the addition of recombinant murine GM-CSF (rmGM-CSF) for 48 hours (3 animals per group). The amount of CD11c⁺ MHC II⁺ CD86⁺ migrated cells was quantified by flow cytometry. Due to the low numbers of migrated cells, migrated cells from 3 mice per group were pooled. Figure 2A shows the total amount of MHC II⁺ CD86⁺ migrated cells for the 3 experiments. All CD86⁺ cells expressed the DC marker CD11c (data not shown). Despite considerable inter-experimental differences in the numbers of migrated skin-DC DC from Mrp4/5 dKO mice migrated clearly as good as the wtDC. Nor was there any alteration in the percentages of CD11c⁺ CD86⁺ MHC II⁺ cells within the migrated populations [Figure 2B]. These results indicate that murine skin-DC do not require Mrp4 (or -5) for migration.

The fact that Mrp4, unlike in man, did not appear to be important for murine DC migration, did not rule out an alternative role for this transporter in the generation of an effective immune response in mice. We therefore decided to further analyze immune parameters and *in vivo* immune responses in these animals. In order to avoid any possible compensation by Mrp5 of Mrp4 functions *in vivo*, Mrp4/5 dKO mice were used for most of these experiments. First, immune cell subsets were determined in the blood [Figure 2C] and in the spleen (data not shown) of FVBwt and Mrp4/5 dKO mice to see whether putative effects on the immune response could have their origin in altered cell-to-cell ratios. Blood from 6 FVBwt and 6 Mrp4/5 dKO animals was analyzed for CD3 (total T cell pool), CD8 (cytotoxic T cell subset and NK cells), CD19 (B cells) and F4.80 (macrophages and DC). No differences in the percentages of cells was observed for the analyzed lineages and subsets [Figure 2C]. Also, when studying the same lineages and subsets of cells within single cell suspensions from spleen, no differences were found between FVBwt and Mrp4/5 dKO mice (data not shown).

Putative differences on *in vivo* immune responses could also have their origin in altered cell proliferation. Hence we studied polyclonal cell proliferation of isolated splenocytes in reaction to PMA, PHA or LPS stimulation. Figure 2D shows the average polyclonal cell proliferation (plus standard deviation) upon 100 ng/ml PMA stimulation of FVBwt, Mrp4/5 dKO and Mrp4 KO splenocytes (n=6). Also, no differences in polyclonal cell proliferation was observed upon stimulation with PHA or LPS (data not shown).

***In vivo* immune responses**

FVB wild-type (FVBwt) and Mrp4/5 dKO mice were compared for the adaptive immune responsiveness after skin sensitization, subcutaneous immunization or oral tolerance induction.

First, the allergen oxazolone (OXA) was used in a delayed type hypersensitivity (DTH) assay, to study T cell immune reactions to allergens. OXA was applied on the flank of the animals on days 0 and 1. On day 5, animals were challenged by applying OXA on the ear, and the response was measured by ear-thickness measurements 5, 24, 48 and 72 hours after the challenge. The graph in figure 3A displays the average increase in ear thickness (plus standard deviation) after OXA application at the 5, 24 and 48 hour time points (6 animals per group). Any responses to the vehicle control (i.e. ethanol) were subtracted from the OXA response. No significant differences in response to OXA were observed between the FVBwt and Mrp4/5 dKO mice at any of the time points, suggesting a normal immune response and memory T cell induction to allergens entering through the skin. Essentially identical results were found when another allergen, di-nitrochlorobenzene (DNCB), was used (data not

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shown). Next we analyzed how the animals responded in an immunization setting. In this experiment, the mice received subcutaneous (s.c.) immunization with ovalbumine (OVA) at day 0, and were challenged 7 days later. Ear thickness was measured 5, 24 and 48 hours after the challenge [Figure 3B]. Control mice only received the challenge and no immunization, and any responses in these control animals were subtracted from the OVA-immunized responses. As with the skin sensitization experiment, no significant differences were observed between wt and dKO animals in response to OVA immunization, indicating that the generation of a memory response to foreign antigens was not hampered due to the absence of Mrp4 (and –5).

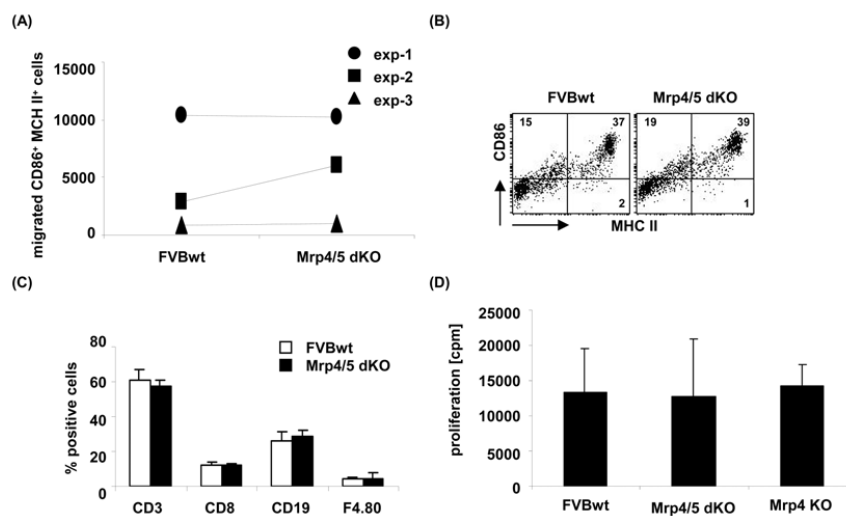


Figure 2: Mrp4 deficiency does not impair skin DC migration, immune cell subset distribution or polyclonal cell proliferation.

A) Mouse ears were split and DC were allowed to migrate from both ear-halves towards medium alone, or medium supplemented with rGM-CSF. Absence of Mrp4/5 did not influence DC migration (shown are data from 3 separate experiments, pooling the ears of 3 mice per experiment). **B)** Phenotypic analysis of migrated ear-skin DC for the DC markers CD86 and MHC II. Percentages of positive cells are listed in the quadrants. **C)** Whole blood was analyzed by flow cytometry for the percentages of T cells (CD3 and CD8), B cells (CD19) and antigen presenting cells (macrophages/DC;F4/80) (n = 6 animals per group). **D)** Splenocytes from FVBwt, Mrp4/5 dKO and Mrp4 KO mice were tested for polyclonal cell proliferation in response to PMA (100ng/ml) stimulation (3 mice per group).

It has been reported that in mice Mrp4 is present within the gastrointestinal (GI)-tract.²³ In order to see whether the absence of Mrp4 (and –5) in the GI-tract would influence the uptake and processing of orally-delivered antigens, tolerance induction towards an orally administered antigen was analyzed. Control animals did not receive intra-gastric OVA, but did receive OVA immunization and the OVA challenge (immunization group in Figure 3C). Figure 3C shows that oral tolerance was induced to similar levels despite the absence of Mrp4 and –5, implying normal uptake and processing of oral antigens in the GI-tract in Mrp4/5 dKO mice. As expected, small scale tolerance induction experiments with Mrp4 KO mice gave similar results (data not shown).

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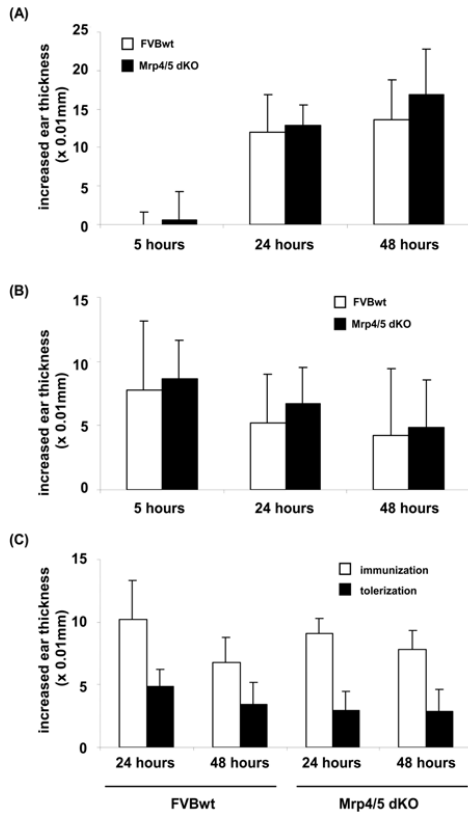


Figure 3. Unimpaired immune response in mice lacking Mrp4 and Mrp5 expression.

A) Mice were sensitized on the flank with oxazolone at days 0 and 1 and were challenged on the ear at day 5. Shown is the increase in ear thickness after 5, 24 and 48 hours. Control ears received ethanol instead of oxazolone and control values were subtracted from the OXA-induced values (n = 6 mice). **B)** Animals were immunized s.c. with PBS (control) or ovalbumine (OVA) at day 0 and were challenged with OVA 7 days later. Similar ear swelling was observed in FVBwt and Mrp4/5 dKO mice (5h, 24h and 48h time points). PBS control values were subtracted from the OVA-induced values (n = 6 mice). **C)** Mice received intra-gastric PBS (immunization: white bars) or OVA (tolerization: black bars) 3 times and were immunized with OVA 5 days after the last feeding. Seven days post immunization animals were challenged on the ears with OVA to study oral tolerance induction and ear thickness was measured (24 and 48 hour time points are shown) (n = 6 mice per group).

Humoral response profile after OVA immunization in FVBwt and Mrp4/5 dKO mice

With memory T cell responses being unaltered, we finally analyzed whether there might be differences at the Th1/Th2 and humoral level between FVBwt and MRP4/5 dKO mice by looking at the production of Th1/Th2-associated antibodies. Serum was collected from mice (5 animals per group) after PBS (control) or OVA immunization and was analyzed for the presence of anti-OVA Th1 and Th2-induced immunoglobulins. The presence of the Th1-associated isotypes IgG2a and IgG3 (both IFN γ induced) within the serum are shown in figure 4A and Th2-associated isotypes IgG1 (IL-4 induced) and IgG2b (TGF β induced) are shown in figure 4B. No differences could be detected in the humoral immune response between FVBwt and Mrp4/5 dKO mice and no clear skewing towards either a Th1 or Th2 response upon OVA immunization was observed.

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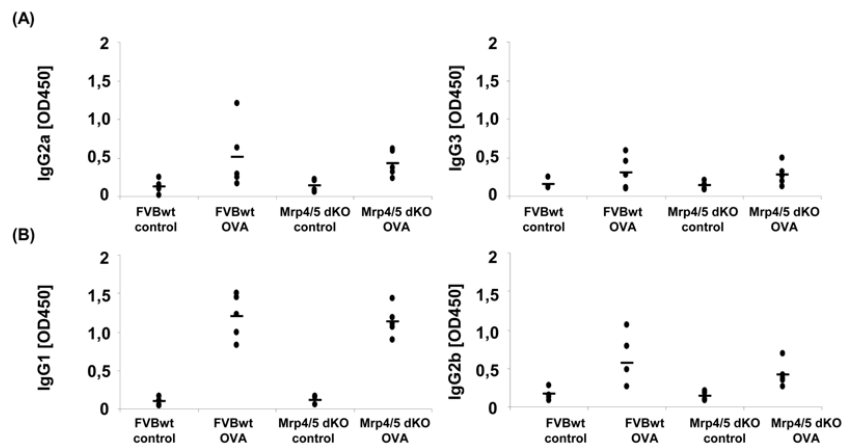


Figure 4. Similar humoral response to OVA in FVBwt and Mrp4/5 dKO mice.

Serum was collected from mice immunized with PBS (control) or OVA (5 mice per group) and was analyzed for the presence of the Th1 cytokine-induced IgG2a and IgG3 isotypes (A) and the Th2 cytokine-induced IgG1 and IgG2b isotypes (B). No differences were observed in the levels of Th2 or Th1 cytokine-induced antibody isotypes between FVBwt and Mrp4/5 dKO mice.

Discussion

In this manuscript we demonstrate that mice do not require Mrp4 for a proper functional immune response. Earlier, we found human skin DC to express MRP4, and interference with MRP4 activity or expression in human skin or *in vitro* cultured Langerhans cells reduced the migratory capacity of DC.¹⁰ If Mrp4 would have similar importance in mice, Mrp4 KO mice would represent an attractive model to further study the importance of MRP4 in DC migration and in the *in vivo* generation of an effective immune response. Hence FVBwt, Mrp4/5 dKO and Mrp4 KO mice were analyzed in this study for their responses to model allergens and antigens under different experimental settings. Although expression of Mrp4 protein could be detected on a small percentage of cells in FVBwt skin samples, there seemed to be no functional role for this transporter in murine DC functioning. The presence of CD11c and MHCII expressing cells in mouse skin suggested normal DC differentiation. Indeed normal *in vitro* BM-DC differentiation was observed using bone-marrow cells from Mrp4/5 dKO mice. The results also indicated that skin-DC migration was unimpaired in Mrp4/5 dKO animals, as comparable migration was observed between wtDC and Mrp4/5 dKO DC in a split-ear migration assay.

Before studying whether Mrp4 might be relevant for the induction of an *in vivo* immune response, we first analyzed whether Mrp4/5 dKO mice displayed altered cellular subset distributions in the blood and the spleen and studied whether the absence of Mrp4 influenced normal polyclonal cell proliferation. Neither in the blood, nor in the spleen was there a different distribution in percentages of T-, B- or antigen presenting cells (DC and macrophages). Also, normal polyclonal cell proliferation was observed when stimulating Mrp4/5 dKO splenocyte populations with PMA, PHA or LPS. Furthermore, no differences between FVBwt or knockout animals were found in response to i) skin sensitization with the allergens oxazolone and DNCB, ii) s.c. immunization with ovalbumine or iii) oral tolerance induction with ovalbumine. In addition, no effects on the humoral response could be observed, with similar levels of specifically Th1 and Th2-induced immunoglobulins in OVA-immunized FVBwt and Mrp4/5dKO animals.

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Based on the data described in this manuscript, we conclude that MRP4 has dissimilar functions in man and mice. This discrepancy between these two species is in analogy with studies on the importance of the major vault protein (MVP/LRP) in human DC *in vitro* and the generation of an immune response in mice *in vivo*.^{24,25} In our study, murine BM-DC appeared negative for Mrp4 but did express Mrp1. Also in murine skin, Mrp1 expressing cells were much more abundant than Mrp4 expressing cells suggesting that in mice Mrp1, rather than Mrp4, is more important for DC migration. In contrast, MRP4 expression in human skin DC was more pronounced than MRP1 expression and DC-targeting of an adenovirus encoding a shRNA construct against MRP4 resulted in a significant reduction in human skin DC migration, whereas targeting an adenovirus encoding a shRNA against MRP1 only moderately reduced DC migration.¹⁰ In mice, Robbiani et al. showed complete restoration of Mrp1 KO BM-DC migration upon addition of the Mrp1 substrate LTC₄.¹⁵ However, for human DC this has not been fully elucidated. Although human DC were found to migrate less towards the chemokine CCL19 after exposure to the MRP-family inhibitor MK-571¹⁵, no data are available showing that the addition of LTC₄ or its derivative LTD₄, could restore this migration. In our study on MRP4 in human LC with RNAi-reduced MRP4 expression, LTC₄ and LTD₄ could not restore migration towards CCL19 or CCL21.¹⁰ These LC did express MRP1, suggesting that LTC₄ secretion through MRP1 is not sufficient for human DC migration. Altogether, these data thus suggest that the presence of abundant Mrp1 on murine DC is sufficient for optimal DC migration and that these cells do not require the additional contribution of Mrp4, whereas in humans MRP4 seems to play a more dominant role.

It is unknown whether the human MRP4 and the murine Mrp4 have similar substrate specificities. Recent papers showed that there can be marked differences in substrate profiles between murine and human ABC transporters.^{26,27} Such differences could perhaps explain the relative differences in importance of human or murine MRP4 and MRP1 analogues in DC migration.

In conclusion, the lack of Mrp4 (and -5) did not affect the generation of an *in vivo* immune response in mice, arguing against an important role for Mrp4 in murine DC migration, or murine immunity in general. It also points out the substantial differences in this respect between human and murine DC. These results stress the importance of human models, such as relevant DC cell lines²⁸⁻³¹ and human skin explant and skin equivalent models, to study human DC physiology in a close-to-*in vivo* setting, as mice do not always constitute a suitable *in vivo* model.

Acknowledgements

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Reference List

- (1) Leggas M, Adachi M, Scheffer GL et al. Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. *Mol Cell Biol.* 2004;24:7612-7621.
- (2) Imaoka T, Kusuhaara H, Adachi M et al. Functional involvement of multidrug resistance-associated protein 4 (MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir. *Mol Pharmacol.* 2007;71:619-627.
- (3) Kruh GD, Belinsky MG, Gallo JM, Lee K. Physiological and pharmacological functions of Mrp2, Mrp3 and Mrp4 as determined from recent studies on gene-disrupted mice. *Cancer Metastasis Rev.* 2007;26:5-14.
- (4) Zamek-Gliszczyński MJ, Nezasa K, Tian X et al. Evaluation of the role of multidrug resistance-associated protein (Mrp) 3 and Mrp4 in hepatic basolateral excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in Abcc3^{-/-} and Abcc4^{-/-} mice. *J Pharmacol Exp Ther.* 2006;319:1485-1491.

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- (5) Hasegawa M, Kusuhara H, Adachi M et al. Multidrug resistance-associated protein 4 is involved in the urinary excretion of hydrochlorothiazide and furosemide. *J Am Soc Nephrol.* 2007;18:37-45.
- (6) Reid G, Wielinga P, Zelcer N et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci U S A.* 2003;100:9244-9249.
- (7) Luft T, Jefford M, Luetjens P et al. Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. *Blood.* 2002;100:1362-1372.
- (8) Legler DF, Krause P, Scandella E, Singer E, Groettrup M. Prostaglandin E2 is generally required for human dendritic cell migration and exerts its effect via EP2 and EP4 receptors. *J Immunol.* 2006;176:966-973.
- (9) Scandella E, Men Y, Legler DF et al. CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. *Blood.* 2004;103:1595-1601.
- (10) 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;112:2353-2359.
- (11) Giordano D, Magaletti DM, Clark EA. Nitric oxide and cGMP protein kinase (cGK) regulate dendritic-cell migration toward the lymph-node-directing chemokine CCL19. *Blood.* 2006;107:1537-1545.
- (12) Rius M, Hummel-Eisenbeiss J, Keppler D. ATP-dependent transport of leukotrienes B4 and C4 by the multidrug resistance protein ABCC4 (MRP4). *J Pharmacol Exp Ther.* 2008;324:86-94.
- (13) Schuurhuis DH, Fu N, Ossendorp F, Melief CJ. Ins and outs of dendritic cells. *Int Arch Allergy Immunol.* 2006;140:53-72.
- (14) 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.
- (15) 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.
- (16) Leier I, Jedlitschky G, Buchholz U et al. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J Biol Chem.* 1994;269:27807-27810.
- (17) Goulet JL, Snouwaert JN, Latour AM, Coffman TM, Koller BH. Altered inflammatory responses in leukotriene-deficient mice. *Proc Natl Acad Sci U S A.* 1994;91:12852-12856.
- (18) Reid G, Wielinga P, Zelcer N et al. Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. *Mol Pharmacol.* 2003;63:1094-1103.
- (19) Borst P, de Wolf C, van de WK. Multidrug resistance-associated proteins 3, 4, and 5. *Pflugers Arch.* 2007;453:661-673.
- (20) Lutz MB, Kukulski N, Ogilvie AL et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods.* 1999;223:77-92.
- (21) Flens MJ, Izquierdo MA, Scheffer GL et al. Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res.* 1994;54:4557-4563.
- (22) Scheffer GL, Kool M, Heijn M et al. Specific detection of multidrug resistance proteins MRP1, MRP2, MRP3, MRP5, and MDR3 P-glycoprotein with a panel of monoclonal antibodies. *Cancer Res.* 2000;60:5269-5277.
- (23) Belinsky MG, Guo P, Lee K et al. Multidrug resistance protein 4 protects bone marrow, thymus, spleen, and intestine from nucleotide analogue-induced damage. *Cancer Res.* 2007;67:262-268.
- (24) Mossink MH, de Groot J, van Zon A et al. Unimpaired dendritic cell functions in MVP/LRP knockout mice. *Immunology.* 2003;110:58-65.

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- (25) 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.
- (26) de Wolf CJ, Yamaguchi H, van der Heijden I et al. cGMP transport by vesicles from human and mouse erythrocytes. *FEBS J.* 2007;274:439-450.
- (27) Zimmermann C, van de WK, van de SE et al. Species-dependent transport and modulation properties of human and mouse multidrug resistance protein 2 (MRP2/Mrp2, ABCC2/Abcc2). *Drug Metab Dispos.* 2008;36:631-640.
- (28) 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.
- (29) Santegoets SJ, Schreurs MW, Masterson AJ et al. In vitro priming of tumor-specific cytotoxic T lymphocytes using allogeneic dendritic cells derived from the human MUTZ-3 cell line. *Cancer Immunol Immunother.* 2006;55:1480-1490.
- (30) 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.
- (31) van Helden SF, van Leeuwen FN, Figdor CG. Human and murine model cell lines for dendritic cell biology evaluated. *Immunol Lett.* 2008;117:191-197.