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Dendritic Cells Require Multidrug Resistance Protein 1 (ABCC1) Transporter Activity for Differentiation


Dendritic cells (DC) express the ATP-binding cassette (ABC) transporters P-glycoprotein (ABC1) and multidrug resistance protein 1 (MRP1; ABCC1). Functionally, both these transporters have been described to be required for efficient DC and T cell migration. In this study, we report that MRP1 activity is also crucial for differentiation of DC. Inhibition of MRP1, but not P-glycoprotein, transporter activity with specific antagonists during in vitro DC differentiation interfered with early DC development. Impaired interstitial and Langerhans DC differentiation was characterized by 1) morphological changes, reflected by dropped side scatter levels in flow cytometric analysis and 2) phenotypic changes illustrated by maintained expression of the monocytic marker CD14, lower expression levels of CD40, CD86, HLA-DR, and a significant decrease in the amount of cells expressing CD1a, CD1c, and Langerin. Defective DC differentiation also resulted in their reduced ability to stimulate allogeneic T cells. We identified the endogenous CD1 ligands sulfatide and monosialoganglioside GM1 as MRP1 substrates, but exogenous addition of these substrates could not restore the defects caused by blocking MRP1 activity during DC differentiation. Although leukotriene C4 was reported to restore migration of murine Mrp1-deficient DC, the effects of MRP1 inhibition on DC differentiation appeared to be independent of the leukotriene pathway. Though MRP1 transporter activity is important for DC differentiation, the relevant MRP1 substrate, which is required for DC differentiation, remains to be identified. Altogether, MRP1 seems to fulfill an important physiological role in DC development and DC functions.


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4 Abbreviations used in this paper: DC, dendritic cell; ABC, ATP-binding cassette; MDR, multidrug resistance; P-gp, P-glycoprotein; MRP1, multidrug resistance protein 1; LT(C4), leukotriene C4; MCM, monocye-conditioned medium; LC, Langerhans-like DC; EZG, [3H]estradiol-17β-glucuronide; GSL, glycosphingolipid; LTD4, leukotriene D4; 5-LO, 5-lipoxygenase; iDC, immature DC; mDC, mature DC; SSC, side scatter.
DC, namely during DC differentiation. Immunochemical analysis showed distinct expression of MRP1 on both immature DC and mature DC (mDC). Our results show that pharmacological interference with MRP1 transporter activity during differentiation from precursor cell to iDC leads to defective DC differentiation. Further analysis excluded the newly identified MRP1 substrates sulfatide and monosialoganglioside (GM1) and the well-known MRP1 substrate LTC₄ from being the MRP1 substrates required for DC differentiation. Thus, unlike in DC migration, effects caused by inhibition of MRP1 transporter activity during differentiation seem independent of the leukotriene pathway.

Materials and Methods

Chemicals

All chemicals and drugs were obtained from Sigma-Aldrich except for doxorubicin which was purchased from Farmitalia Carlo Erba, MK571 (L-660,711) which was purchased from Alexis, and PSC833 which was obtained from Novartis.

Tumor cell lines

For controls in immunocytochemical staining, Western blot, and drug efflux experiments, the following couples of drug-sensitive parent and MDR tumor cell lines were used: for P-gp, the non-small cell lung carcinoma cell line SW-1573 and its P-gp-overexpressing subline SW-1573/2R160 (16) for MRP1, the small cell lung carcinoma cell line GLC4 and its MRP1-overexpressing subline GLC4/ADR (17) or the MRP1 overexpressing subline SW-1573/2R120 (18).

Interstitial DC cultures

Monocyte-derived DC (interstitial DC) were cultured from human PBMC of healthy donors as described before (19, 20). In brief, monocytes were obtained by plastic adherence for 1 h at 37°C and were cultured in IMDM containing 10% FCS (HyClone), 100 IU/ml sodium penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 100 U/ml rIL-4 (CLB), and 100 ng/ml recombinant human GM-CSF (Leucomax; Novartis Pharma) for 5–6 days. iDC were matured by adding 33% monocyte-conditioned medium (MCM) and 50 ng/ml TNF-α (Strathmann Biotec) to the cultures for 2 days. iDC and mature DC were isolated for phenotypic and functional analysis. MDR protein antagonists were added to some cultures. Antagonists were added to the cultures at days 0, 3, and 6. For MRP1, MK571 (25 μM each time; Refs. 11 and 21) or probenecid (0.5 mM each time; Ref. 23) was used. When analyzing the DC from different cultures, appropriate controls were included to check for vehicle influences on DC characteristics (i.e., PBS, which was used to dilute MK571 and probenecid, and ethanol which was used to dissolve PSC833).

CD34⁺ progenitor MUTZ-3 cell line-derived Langerhans-like DC

LC were cultured from the human acute myeloid leukemia cell line MUTZ-3 as described before with minor changes (24). In brief, MUTZ-3 progenitor cells were cultured in MEM-α (MEM; Invitrogen Life Technologies) containing 20% FCS, 100 IU/ml sodium-penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 μM 2-ME, and 100 U/ml rIL-4 (CLB), and 100 ng/ml recombinant human GM-CSF (Leucomax; Novartis Pharma) for 5–6 days. iDC were matured by adding 33% monocyte-conditioned medium (MCM) and 50 ng/ml TNF-α (Strathmann Biotec) to the cultures for 2 days. iDC and mature DC were isolated for phenotypic and functional analysis. MDR protein antagonists were added to some cultures. Antagonists were added to the cultures at days 0, 3, and 6. For MRP1, MK571 (25 μM each time; Refs. 11 and 21) or probenecid (0.3 mM each time; Ref. 22) were used, and for P-gp, PSC833 (2 μM each time; Ref. 23) was used. When analyzing the DC from different cultures, appropriate controls were included to check for vehicle influences on DC characteristics (i.e., PBS, which was used to dilute MK571 and probenecid, and ethanol which was used to dissolve PSC833).

Western blotting

Cell pellets were lysed in ice-cold lysis buffer (100 mM Tris/HCl (pH 7.6); 0.5% Nonidet P-40; 5 mM 1,4-dithio-o-threitol; protease inhibitor mixture “Complete” (Roche Diagnostics)), sonicated, and stored at −80 °C until further use. Protein concentration was determined with a Bio-Rad protein assay. Proteins were fractionated on an 8% polyacrylamide gel and subsequently transferred to nitrocellulose filter by electoblotting. For MRP1 Western blot, filters were left in blocking buffer (PBS containing 1% BSA, 1% milk powder and 0.05% Tween 20) overnight at 4°C. Filters were incubated with the mAbs MRPr1 (0.5 μg/ml) in blocking buffer for 2 h. For P-gp, filters were blocked in blocking buffer for 1 h at 37°C. To check for vehicle influences with the antagonist PSC833 (25) dissolved in methanol-PBS (1:1), MK571 (25 μg/ml) for 2 h. For P-gp, filters were blocked in blocking buffer for 1 h at 37°C, followed by overnight incubation with JSB-1 mAb (2.5 μg/ml) in blocking buffer at 4°C. Immunoreactivity of the mAbs was visualized with goat-anti-mouse (P-gp) or rabbit-anti-mouse (MRPr1) and left to efflux for 1 h at 37°C. Finally, cells were washed twice in ice-cold PBS, taken up in 300 μl of green dye solution and kept on ice until fluorescence was analyzed on a FACS-star flow cytometer (BD Biosciences) equipped with CellQuest analysis software. Rhodamine 123 and calcine-AM were measured in the FL1 channel. P-gp and MRPr1 activities are expressed as ratios by dividing the median drug fluorescence in the presence of inhibitor by the median drug fluorescence in the absence of inhibitor, after subtraction of the median fluorescence of unloaded control cells (29).

Immunocytochemistry

Cyto centrifuge preparations (cytospins) were air-dried overnight and fixed in 100% acetone for 10 min. Slides were incubated with mAbs diluted in PBS containing 1% (w/v) BSA (1% BSA/PBS) for 90 min; JSB-1 (1.25 μg/ml; Ref. 26) or C219 (4 μg/ml; Alexis) for P-gp; MRPr1 (5 μg/ml; Ref. 27) for MRPr1, anti-CD1a-FITC (1/25; BD Pharmingen) or Okt-6 (28) for CD1a. Ab binding was detected using biotinylated rabbit-anti-rat Igs (1/100; DakoCytomation) for JSB-1, C219, anti-CD1a-FITC or Okt-6 and streptavidin conjugated to HRP (1/500; DakoCytomation), followed by development with 0.02% (w/v) 3-amino-9-ethylcarbazole and 0.02% (w/v) H₂O₂ in 0.1 M sodium acetate (pH 5.0). Slides were counterstained with hematoxylin and mounted. For negative controls, slides were stained with isotype-matched rat or mouse Abs in each experiment. In between different steps, slides were washed three times in PBS.

For MRPr1/CD1a fluorescent double staining, aceto-fixed cytospins were incubated with MRPr1 (5 μg/ml) for 90 min. Subsequently cytospins were incubated with biotinylated rabbit-anti-rat Ig (1/100; DakoCytomation), followed by incubation with Streptavidin-Cy3 (1/200; Jackson ImmunoResearch Laboratories) and 1% normal rat serum (DakoCytomation). Coverslips were incubated with anti-CD1a-FITC (1/25; BD Pharmingen) and with HRP-conjugated rabbit-anti-mouse Ig (1/500; DakoCytomation). Finally, fluorescein-labeled tyramine (FT 1/1000 in PBS, homemade) + 0.01% H₂O₂ was added for 10 min to increase the signal for CD1a. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) diluted in Vectashield mounting medium (Vector Laboratories) (0.8 μg/ml). All Abs and conjugates were diluted in 1% BSA/PBS and cytospins were washed in PBS three times in between steps. For background-staining controls, single staining was performed in which the anti-CD1a-FITC Ab replaced the MRPr1 Ab to check for binding of the rabbit-anti-rat Ig to the mouse CD1a Ab and vice versa.
Flow cytometric immunophenotypical analyses

DC cultured in the presence or absence of specific MDR protein antagonists were immunophenotyped using the following FITC- and/or PE-conjugated mAbs: anti-CD1a (1/25), anti-CD54 (1/25), anti-CD86 (1/25), anti-CD40 (1/10) (BD Pharmingen), anti-CD14 (1/25), anti-HLA-DR (1/25) (BD Biosciences), anti-CD83 (1/10) (Immunotech), and anti-CD1c (1/25) (Imgen). In short, 2.5–5 × 10^6 cells were washed in PBS supplemented with 0.1% BSA and 0.02% NaN3 and incubated with specific or corresponding control mAbs for 30 min at 4°C. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuest analysis software and the results were expressed as mean fluorescence or the percentage of positive cells. The mean fluorescence index was determined by dividing the mean fluorescence of the specific Ab by the mean fluorescence of the corresponding control Ab.

Mixed lymphocyte reaction

Immature and mature LC were cultured as described above, either in the presence or absence of the MRPI antagonist MK571 (25 μM). A total of 1 × 10^3 up to 3 × 10^3 LC were cocultured with 1 × 10^3 donor-derived PBL for 3–4 days in 96-well plates in IMDM containing 10% human pooled serum, 100 IU/ml sodium-penicillin, 100 μg/ml streptomycin, 2 mM t-glutamine, 50 μM 2-ME. At days 3–4, 2.5 μCi/ml [3H]thymidine (6.7 Ci/mmol; MP Biomedicals) was added per well for 16 h. Ninety-six-well plates were harvested onto glass fiber filters (Packard Instrument) using a Skatron cell harvester (Skatron Instruments), whereafter, [3H]thymidine incorporation was counted and quantified using a Topcount NXT Microbeta counter (Packard Instrument). Responses are shown as mean counts per minute from triplicate wells.

Cytokine production (IL-12/IL-10)

Intestinal DC were cultured from donor monocytes in the absence or the presence of 25 μM MK571 as described above and were tested for the production of IL-10 and (p70) IL-12 as described previously (30). IL-10 (IL-10 ELISA kit; CLB) and IL-12 p70 (31) concentration were determined by ELISA.

Inside-out plasma membrane vesicle ^3^H-labeled estradiol-β-glucuronide (E2G) uptake assay

Inside-out vesicles were prepared from plasma membranes of MRPI-expressing GLC-4/ADR cells as described previously (32) with slight modifications. Cells were collected by centrifugation (275 × g, 5 min) and washed twice in ice-cold PBS (pH 7.4). The cell suspension (10^6 cells/ml) was incubated in a buffer containing 100 mM KCl, 5 mM MgCl2, 1 mM PMSF, and 50 mM HEPES/KOH (pH 7.4) for 60 min at 0°C. Hereafter, cells were disrupted by sonication at 20% of the maximum power of an MSE sonicator (Soniprep 150) for three bursts of 15 s each. The suspension was centrifuged at 275 × g for 10 min. The supernatant was layered on top of a 46% sucrose cushion (KCl/HEPES buffer) and centrifuged at 100,000 × g for 60 min. The interface was removed and washed in the buffer described above. The final membrane preparations were stored at −80°C at a protein concentration of 4 mg/ml. The ratio between inside-out vesicles and outside-in vesicles within the preparations was determined as previously described (33).

A rapid filtration method (34) was used to measure the transport of E2G into isolated inside-out plasma membrane vesicles. Vesicles were incubated in a buffer containing 100 mM KCl/50 mM HEPES/KOH (pH 7.4) at 37°C (0.15 mg/ml protein), in the presence of 10 mM MgCl2, 2 mM ATP, and 25–100 nM E2G (specific activity, 40.5 Ci/mmol; PerkinElmer Life Science) with or without the addition of various concentrations of sulfadiazine (Fluka) and GM1 (Matreya) or 1 mM probenecid (positive control), 60 μM vitamin C (negative control) or 1% v/v DMISO (vehicle control). The final reaction volume was 50 μl. The transport was stopped after 2 min by addition of 2 ml of ice-cold KCl/HEPES buffer. Hereafter, the mixture was rapidly filtrated through OE67 membrane filters (Schleicher & Schuell). The filters were washed twice with 2 ml of KCl/HEPES buffer. The radioactivity associated with the filters was measured by liquid scintillation counting.

Statistical analysis

Statistical analysis of the data was performed using the unpaired two-tailed Student t test. Differences were considered statistically significant when p < 0.05.

Results

Expression of P-gp and MRPI during DC development

Intestinal-like DC were cultured from blood monocytes with IL-4 and GM-CSF (19). Expression of the classic ABC transporter proteins P-gp and MRPI in monocytes and immature and mature intestinal DC was studied by immunocytochemistry and Western blot analysis. Besides weak P-gp expression, we found a pronounced up-regulation of the 190-kDa MRPI protein during differentiation from monocytes to iDC both by cytopsin staining and Western blot analysis (Fig. 1A and B), confirming flow cytometric data from Laupéze et al. (35). Upon maturation, MRPI levels remained high (or were further up-regulated) (Fig. 1B). Besides abundant cytoplasmatic staining, distinct membranous staining could be observed for this transporter (Fig. 1A).

Transmembrane transporter activities of P-gp and MRPI in intestinal DC

The activity of the transmembrane transporters P-gp and MRPI was studied by dye efflux experiments with typical substrates and inhibitors of the proteins. Control experiments were performed with tumor cell lines selected for overexpression of either transporter and the respective parental cell lines (Fig. 2). These P-gp- or MRPI-overexpressing tumor cells (SW1573-2R160 and -2R120, respectively) could extruded their fluorescent dye in absence of a specific transporter inhibitor, and the addition of the specific inhibitors completely blocked dye efflux. The parental cell line, which does not express P-gp or MRPI, was not able to efflux rhodamine 123 (P-gp) or calcein-AM (MRPI). When testing immature and mature intestinal DC, the results reflected the staining data of P-gp and MRPI as observed by immunocytochemistry. No significant P-gp transmembrane transporter activity could be detected in iDC or mDC (Fig. 2). However, in line with previously reported findings (35), and the above shown plasma membrane expression of MRPI on intestinal DC (Fig. 1A), MRPI transmembrane activity was detected on iDC (Fig. 2) as dye efflux could be blocked by the addition of the MRPI inhibitor MK571. Despite high levels of MRPI on mature intestinal DC, only marginal functional membrane transport activity was detected. Taken together, the presence of functionally active MRPI on DC could be indicative of a role for this transporter in DC physiology.

![FIGURE 1](https://www.jimmunol.org/)

**FIGURE 1.** Expression of P-gp and MRPI on intestinal DC. A, Immunocytochemistry and, B, Western blot analysis of P-gp (Jsb-1) and MRPI (MRP-1) on monocytes, immature and mature intestinal DC. MDR tumor control cell lines used: SW1573/2R160 for P-gp and GLC-4/ADR for MRPI. β-actin control staining is shown for equal loading. The data presented in A and B are from different healthy individuals.
Effects of MDR protein inhibitors on DC differentiation

To explore possible roles of P-gp and MRP1 in DC differentiation, MDR antagonists, at nontoxic dosages, were added during interstitial DC culture. Resulting iDC were studied by flow cytometric analysis for expression of typical DC markers.

The MRP1 antagonist MK571 induced significant changes in DC morphology and phenotype. First, the addition of this MRP1 inhibitor markedly altered DC morphology as seen by lowered side scatter (SSC) values (Fig. 3A). Second, when analyzing marker surface expression, MK571 treatment had suppressive effects on CD86, CD40, CD54, and HLA-DR levels, and even more strongly reduced the number of CD1a- and CD1c-positive cells on average with 50% as compared with control cultures (CD1a; 14.5 ± 4.8% compared with 29.5 ± 7.8%, respectively (p = 0.037)) (CD1c; 34.9 ± 13.6% compared with 59.3 ± 15%, respectively (p = 0.023)) (Fig. 3, B and C). Equivalent results were obtained when DC were cultured with another

FIGURE 2. Functional transmembrane activity of MRP1, but not P-gp, on interstitial DC. Immature and mature interstitial DC and non-MDR and MDR tumor cells were incubated with the fluorescent P-gp substrate rhodamine 123 (top row) or the fluorescent MRP1 substrate calcein-AM (bottom row) in the presence of specific inhibitors (PSC833 for P-gp and MK571 for MRP1) and were allowed to efflux these compounds either in the absence (bold line) or the presence (broken line) of the respective inhibitor. Dotted lines represent unloaded cells. Ratios representing transporter activity are depicted in the top right corners of the graphs. Data shown are representative of four experiments.

FIGURE 3. Inhibition of MRP1 activity blocks interstitial DC differentiation. A, Side scatter (SSC)/forward scatter (FSC) plots (upper row) and cytospin preparations stained for CD1a (in low and high magnification) of immature interstitial DC cultured in the absence of MDR blocker (control), the P-gp blocker PSC833 (2 μM), or the MRP1 blocker MK571 (25 μM). B, Expression of the DC markers DC-SIGN, CD1c, and CD1a on interstitial DC cultured in the absence or presence of PSC833 or MK571. Depicted are the mean fluorescence intensities relative to control immature interstitial DC (n = 3). Values of p are indicated where significant. C, Histogram plots of DC markers on immature interstitial DC cultured in the absence of blocker (top row) or in the presence of MK571 (bottom row). Open histogram, the isotype controls; filled histogram, the specific markers. In the top right corners, mean fluorescence intensity values are depicted. Blocking P-gp had no effect on DC marker expression (data not shown). D, IL-12p70 and IL-10 production by interstitial DC cultured in the absence (□) or the presence (●) of the MRP1-blocker MK571, after CD40 ligation. IL-12/IL-10 ratios are depicted in the table beside the bars.
MRP1 antagonist, i.e., probenecid (data not shown). Blocking P-gp activity had no effect on DC differentiation. Neither MK571 nor PSC833 influenced DC-SIGN expression (Fig. 3B).

To verify the universal effects of MRP1 inhibition on DC differentiation from different precursor origins, similar experiments were performed using LC cultured from the acute myeloid leukemia-derived DC line MUTZ3, so-called MUTZ3-LC (referred to as LC in this study) (24). These cells are easy to obtain, lack donor variability, express very high levels of CD1a and Langerin (Fig. 4A), and were found to express LC-specific Birbeck granules (T. De Gruijl, A. Masterson, S. Santegoets, P. van der Sluis, S. Lougheed, D. Fluitsma, A. van den Eertwegh, H. Pinedo, and R. Scheper, unpublished data). Both immunocytochemistry (Fig. 4B) and Western blot analysis (data not shown) showed abundant MRP1 protein expression on immature and mature LC. Similar results as described for interstitial DC were found when MRP1 activity was blocked during LC differentiation. The MRP1-blocked LC showed a lower SSC compared with control counterparts (Fig. 4C) and had drastically impaired expression of various DC markers (Fig. 4C), of which the reduction in CD1a, CD1c, and Langerin expression was significant (Fig. 4D) (p = 0.01, p = 0.005, and p = 0.01, respectively). LC treated with MK571 also maintained a CD14-positive population and showed a stronger reduction in the levels of costimulatory molecules compared with interstitial DC.

**Effect of MRP1 inhibition on T cell stimulatory capacity of LC in mixed lymphocyte cultures**

The effect of blocking MRP1 activity during LC differentiation and maturation on their capacity to stimulate T cell proliferation was tested in a MLR. LC, matured with MCM and high concentrations of TNF-α, were cultured in the presence or absence of the MRP1 antagonist MK571 and were subsequently cocultured with PBLs in different DC to T cell ratios for 3–4 days. T cell proliferation was measured by overnight [3H]thymidine incorporation. As shown in Fig. 4E, LC matured with the MRP1 inhibitor were less capable of inducing T cell proliferation than control cells (representative of three experiments).

**FIGURE 4. Inhibition of MRP1 activity blocks LC differentiation.** A, Flow cytometric analysis of CD1a and Langerin expression on MUTZ3-derived LC with the percentage of Langerin⁺/CD1a⁺ cells indicated at the right. B, Immunocytochemistry for MRP1 expression (MRP-r1) on MUTZ3, immature and mature LC. C, Histogram plots of DC markers on immature LC cultured in the absence of blocker (top row) or in the presence of MK571 (bottom row). Open histogram, the isotype controls; filled histogram, the specific markers. In the top right corners, mean fluorescence intensity values are depicted. D, Percentage of CD1a⁺, CD1c⁺, and Langerin-positive cells in control- and MK571-treated LC, with corresponding p values. E, MLR with control and MK571-treated LC to test their capacity to stimulate allogeneic T cell proliferation. Error bars represent SD of triplicate samples within one experiment. Experiment shown is representative of three.

MRP1-inhibited interstitial DC produce lower levels of IL-12

To study whether inhibition of MRP1 activity during DC differentiation could also affect the ability of these DC to initiate Th1 or Th2 responses, we tested whether MK571 treatment during interstitial DC differentiation affected the secretion of IL-12 and/or IL-10. Control- or MRP1-inhibited immature interstitial DC were stimulated with irradiated CD40L-expressing J558 cells in the absence or the presence of IFN-γ to study the effects on IL-10 and IL-12 production, respectively. As shown in Fig. 3D, MRP1 inhibition during interstitial DC differentiation resulted in decreased IL-12 production upon CD40 ligation, while IL-10 production was hardly affected.

MRP1 and CD1a molecules show overlapping subcellular localization in LC

As a significant drop in CD1a surface expression was found in both interstitial DC and LC when MRP1 transporter activity was inhibited, we investigated whether this could relate to physical contact between the two molecules. Therefore, immunocytochemical double staining for MRP1 and CD1a was performed on cytoplasts of immature LC, to investigate the localization of both proteins. Expression of both CD1a and MRP1 was found on the plasma membrane as well as intracellularly in a vesicular-like pattern (Fig. 5A). The merged photograph, and the single-cell magnifications (Fig. 5A) show that there are regions of colocalization of MRP1 and CD1a (yellow staining) in LC, suggestive of a possible direct interaction between these two molecules.

We next examined whether there was a potential link between MRP1 activity and CD1a expression, in analogy to what is known for MHC class I and the ABC transporter TAP (36, 37). Ganglioside congeners, notably sulfatide and monosialoganglioside GM1, have been identified as major endogenous CD1a ligands (38). To verify whether sulfatide and GM1 can function as substrates for the MRP1 transporter, an inside-out vesicle transport assay was performed (32). Typically, compounds were studied for their capacity to interfere with accumulation of a radioactively...
labeled, high-affinity MRP1 substrate, e.g., E2G, into MRP1-expressing inside-out vesicles (see Material and Methods for details). Results of these experiments showed that both sulfatide and GM1 function as MRP1 substrates. The uptake of various concentrations of E2G into MRP1-containing vesicles was drastically inhibited by the addition of nanomolar concentrations of sulfatide, in a concentration-dependent manner (Fig. 5B). Sulfatide and GM1 inhibited the transport of E2G at markedly lower concentrations than the commonly used MRP1 substrate and inhibitor probenecid (12.5 nM vs 1 mM, respectively) (Fig. 5C). There was no inhibition of E2G uptake by the negative control vitamin C, which is not a substrate of MRP1, or the vehicle control DMSO, which was used to dissolve both sulfatide and GM1 (Fig. 5C).

The above-described observations lent support to the hypothesis that, in analogy with TAP dependence for MHC class I expression, CD1a surface expression might depend on MRP1-mediated GSL transport. If CD1a molecules require the presence of endogenous ligands for stable surface expression, blocking MRP1 activity during DC differentiation could interfere with the availability of these ligands. We tested whether exogenous addition of sulfatide could restore CD1a stability and consequently surface expression of CD1a in MRP1-blocked LC. Sulfatide was exogenously added during control and MK571-treated LC cultures to see whether this could restore CD1a expression on MRP1-blocked LC (Fig. 5D).

Further evidence against a role of endogenous GSL ligands in stable CD1a surface expression was obtained by blocking GSL synthesis during LC differentiation. For this, we made use of the GSL synthesis inhibitor Fumonisin B1 (FM1, 30 µg/ml) (25), which blocks the formation of ceramide and consequently the synthesis of gangliosides like GM1 and galactosylceramides like sulfatide (39). No effect on CD1a surface expression levels was found when FM1 was added during LC differentiation (Fig. 5E).

FIGURE 5. MRP1 and CD1a colocalize in LC, but MRP1 activity is not required for CD1a expression. A, Immunofluorescent double staining for CD1a (green) and MRP1 (red) on LC cytopsins. The yellow staining in the top right picture represents CD1a/MRP1 colocalization. In a–c, single-cell magnifications of A (top right) are shown. B, Different concentrations of the CD1a-ligand sulfatide were used to test whether this compound could compete with the radioactively labeled E2G-substrate E2G for MRP1-mediated transport into MRP1-expressing vesicles. E2G uptake without blockers was set at 100%. C, Graph representing the reduction in E2G (50 nM) uptake in MRP1-expressing vesicles in the presence of the CD1a ligands sulfatide and GM1, the MRP1-inhibitor probenecid (positive control), the negative control vitamin C or the vehicle control DMSO (1% v/v). D, Sulfatide (30 µM) was exogenously added during control and MK571-treated LC cultures to see whether this could restore CD1a expression on MRP1-blocked LC (n = 3). E, GSL synthesis was blocked by adding fumonisin B1 (FM1, 30 µg/ml) during LC differentiation to test whether this would affect LC differentiation and CD1a expression in particular (n = 2).

FIGURE 6. The leukotriene pathway is not essential for LC differentiation. MUTZ3 progenitor cells were differentiated into LC in the presence of the MRP1 inhibitor MK571 and 100 nM LTC4 or LTD4. Percentages of CD1a-, Langerin-, and CD1c-positive cells are depicted relative to control (untreated) LC (n = 3).
Leukotrienes cannot restore DC differentiation

Robbiani et al. (11) showed that the addition of LTC4 and LTD4 could restore DC migration in murine Mrp1-deficient cells. These leukotrienes were similarly tested during LC differentiation in combination with MRP1 inhibition. Fig. 6 shows that the addition of 100 nM LTC4 or LTD4 had no effect on LC differentiation. The small increases in CD1a, CD1c, and Langerin expression were not significant. Thus, unlike in DC migration, the effects seen by blocking MRP1 activity during DC differentiation appear independent of MRP1-mediated LTC4 transport.

Discussion

We report that DC require MRP1, but not P-gp, activity for differentiation. Blocking MRP1 activity with the LTD4-analog MK571 or with probenecid during DC differentiation resulted in phenotypic and morphological aberrations. When cultured in the presence of either of these MRP1 inhibitors, both interstitial-like DC cultured from blood-derived monocytes (interstitial DC) and LC cultured from MUTZ3 progenitor cells (LC) became less lobulated/dendritic, reflected by decreased side scatter values in flow cytometric analysis, and had altered expression of typical DC markers compared with control counterparts. MRP1 activity seemed to be important early in LC differentiation as in contrast to control cultures, MRP1-inhibited cultures still contained CD14-expressing precursor cells. In contrast, MRP1 activity in interstitial DC development seemed to be critical at a later stage, as in these culture numbers of CD14-expressing precursor cells remained low. Also changes in the expression levels of the interstitial DC marker DC-SIGN, which is an earlier differentiation marker, were not observed. However, it must be kept in mind that the CD34+ MUTZ3 progenitor cells, unlike monocytes, already express detectable amounts of MRP1 protein. Possibly, the presence of MRP1 protein expression at the progenitor level enhances the effects of MRP1 inhibition during LC differentiation, resulting in an earlier block in the differentiation process. In addition, the MUTZ3 cell line consist of a CD34+ progenitor population, that differentiates into CD14+ DC-precursor cells (24). Perhaps there already is an effect of MRP1 inhibition on the conversion of CD34+ cells into CD14+ cells that could explain the observed difference in CD14 expression between the interstitial DC and LC upon MRP1 inhibition. Both DC types showed lower expression of CD40, CD54, CD86, and HLA-DR and a significant reduction in the number of CD1a- and CD1c-expressing cells. These decreases were more pronounced in the LC cultures and in addition, Langerin expression was significantly affected in LC. Using a weak MRP1 antagonist, i.e., indomethacin, Laupéze et al. (35) noticed a significant reduction in CD40 expression levels on DC, but effects on other DC surface markers did not reach statistical significance. Besides morphological and phenotypic changes, MRP1 inhibition during interstitial and LC differentiation resulted in altered DC functionality reflected by reduced secretion of IL-12p70 by interstitial DC and a reduced capacity to stimulate allogeneic T cell proliferation by LC. The reduction in IL-12 production suggests that these cells might be poorer in inducing a Th1 response.

MRP1-mediated transport of LTC4 has been described to be important for both DC (11) and T lymphocyte migration (15). In DC migration, the addition of LTD2 could rescue the migratory capacity of murine Mrp1-deficient DC toward the CCR7 chemokine CCL19 (11). Spanbroek et al. (40) described that levels of the enzyme required for LTC4 production, i.e., 5-lipoxygenase (5-LO), are enhanced upon DC differentiation from CD34+ cord-blood derived precursors. These authors also reported that adding TGF-β1 to in vitro cultures of LC even further enhanced the expression levels of 5-LO and additionally increased the levels of LTβ4 and the expression levels of the DC markers CD1a, Langerin, CD80, and CD86. This could indicate that the leukotriene pathway might influence DC differentiation. We found that in contrast to DC migration, addition of the MRP1 substrate LTC4 or its derivative LTD2 could not restore the differentiation defects caused by blocking MRP1. Not withstanding, our results indicate that the role of MRP1 in DC differentiation is independent of MRP1-mediated LTC4 transport.

Although we identified the endogenous CD1a and CD1c ligands sulfatide and GM1 to be MRP1 substrates, we showed that the transport of these GSL is also not of importance in DC differentiation. First, the exogenous addition of sulfatide during MRP1-inhibited LC cultures could not restore any of the observed defects. Second, we did not find effects on the CD1a expression levels when inhibiting GSL synthesis during LC differentiation with Fumonisin B, as was reported earlier by Manolova et al. (25). A block in GSL transport is therefore unlikely to be responsible for the DC phenotype obtained by MRP1 inhibition. However, the finding that both sulfatide and GM1 are substrates of MRP1 might still be of distinct interest. Sulfatide has been linked to several processes like neuronal development (41), the modulation of blood coagulation (42), and tumor cell metastasis (43). As the MRP1 protein is expressed ubiquitously throughout the body and can be expressed on chemotherapy-resistant tumors, MRP1-mediated transport of sulfatide may well play a role in these processes.

Taken together, this report shows that different types of myeloid DC express the ABC-transporter MRP1. Immunocytochemical staining revealed that this expression was localized in the cytoplasm as well as at the plasma membrane, and drug transport experiments showed that MRP1, but not P-gp, was capable of actively extruding drugs particularly in iDC. Our observation that MRP1 activity is needed for adequate interstitial DC and LC differentiation, combined with prior reports on its requirement in DC migration, makes this transporter even more interesting as a potential target in DC-based immunotherapies. Increasing MRP1 expression on DC could perhaps result in more potent APC being used as a tool in combined chemoimmunotherapy treatments. In addition, these observations are important to consider when developing new anti-cancer therapies. The administration of MDR blockers affecting the activity of MDR-related ABC transporters, especially MRP1, might not only affect the sensitivity of tumor cells for certain chemotherapeutics, but could also have distinct immunosuppressive side effects by interfering with DC differentiation and migration. This could result in an increased susceptibility of cancer patients to microbial and viral infections and could frustrate antitumor immunity. In conclusion, DC seem to require MRP1 transporter activity for optimal differentiation. More extensive research, including in vivo mouse studies, is required to elucidate the biological substrate(s), the underlying mechanisms, and the possibilities for future chemoimmunotherapy strategies.

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Disclosures

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