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Up-Regulation of Drug Resistance-Related Vaults During Dendritic Cell Development

Anouk B. Schroeijers,* Anneke W. Reurs,* George L. Scheffer,* Anita G. M. Stam,* Mariska C. de Jong,* Thomas Rustemeyer,* Erik A. C. Wiemer,‡ Tanja D. de Gruijl,† and Rik J. Scheper2*

P-glycoprotein (Pgp) and vaults are associated with multidrug resistance in tumor cells, but their physiological functions are not yet clear. Pgp, the prototypical transmembrane transporter molecule, may also facilitate the migration of skin dendritic cells (DC). Vaults—ribonucleoprotein cell organelles, frequently overexpressed in Pgp-negative drug-resistant tumor cells—have also been associated with intracellular transport processes. Given the pivotal role of DC in dealing with exposure to potentially harmful substances, the present study was set out to examine the expression of Pgp and vaults during differentiation and maturation of DC. DC were obtained from different sources, including blood-derived monocytes, CD34+ mononuclear cells, and chronic myeloid leukemia cells. Whereas flow cytometric and immunocytochemical analyses showed slightly augmented levels of Pgp, up-regulation of vault expression during DC culturing was strong, readily confirmed by Western blotting, and independent of the source of DC. In further exploring the functional significance of vault expression, it was found that supplementing DC cultures with polyclonal or mAbs against the major vault protein led to lower viabilities of LPS- or TNF-α-matured monocytes-DC. Moreover, expression of critical differentiation, maturation, and costimulatory molecules, including CD1a and CD83, was reduced and their capacity to induce Ag-specific T cell proliferative and IFN-γ release responses was impaired. These data point to a role for vaults in both DC survival and functioning as APC. The Journal of Immunology, 2002, 168: 1572–1578.

Dendritic cells (DC)3 are recognized as major players in the regulation of immune responses by directing both their vigor and quality. The ability of DC to activate naïve T cells depends on their maturation state. Immature DC are mainly distributed in tissues interfacing with the external environment where they can capture and process Ags with high efficiency. After Ag internalization, DC leave the peripheral tissues to reach the draining lymphoid organs. During this migration, DC undergo maturation, involving augmented surface expression of MHC, chemokine receptors and costimulatory molecules, and acquire the ability to prime T cells (1).

It was only recently that P-glycoprotein (Pgp), a molecule well known for its ability to transport a broad spectrum of xenobiotics out of cells and thereby induce multidrug resistance (MDR) (2), was identified as yet another molecule that might play a role in this migratory process (3). Since the putative contribution of Pgp and, possibly, other mechanisms defined earlier in drug resistance studies, to DC functioning is still unknown, we set out to further explore this issue. Notably, immune responsiveness might well benefit from such mechanisms. To preserve their sentinel function in immune responses, DC should not readily suffer untoward damage from exposure to potentially toxic materials, as often derived from microbial and environmental sources. Hence, the expression of Pgp and a newly discovered cell organelle, the vault, was studied during in vitro DC development from various peripheral blood-derived precursor cells (4, 5). Vaults are evolutionarily highly conserved, large ribonucleoprotein particles (6). The particles represent multimeric RNA-protein complexes with one predominant component, the major vault protein (MVP). Although the cellular role of vaults has remained elusive, several findings support the view that vaults have a transport function by acting as a carrier, mediating bi-directional nucleo-cytoplasmic exchange as well as vesicular transport of compounds, including cytostatic drugs (7–14). The present results demonstrate that not only Pgp but also vaults are expressed and up-regulated during the differentiation and maturation of DC from various sources. Furthermore, endocytosis of anti-MVP Abs leads to reduced viabilities and interference with DC maturation and Ag-presenting capacity, thus pointing to a critical role of vaults in DC functioning.

Materials and Methods

Monocyte (MO)-derived DC culture

Human peripheral blood mononuclear cells isolated by adherence to plastic were (either upon cryopreservation or directly) cultured in IMDM (10 × 10⁶ cells/2.5 ml) containing 25 mM HEPES (BioWhittaker, Verviers, Belgium), 10% FCS (Integro, Zaandam, The Netherlands), 2 mM l-glutamine (Life Technologies, Paisley, U.K.), 50 IE/ml sodium-penicillin G, 50 µg/ml streptomycin sulfate, and 50 µM 2-ME (Merck, Darmstadt, Germany) (complete medium) supplemented with 100 ng/ml rGM-CSF (Luecomax, Sandor, The Netherlands) and 1000 U/ml rIL-4 (CLB, Amsterdam, The Netherlands). After 6 days of culture, a semiadherent cell population was obtained which displayed a veiled DC morphology. These immature DC were subsequently cultured for 1 day in the absence or presence of either LPS (25 ng/ml, Escherichia coli; Difco, Detroit, MI) or rTNF-α (50

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5 Abbreviations used in this paper: DC, dendritic cell; CML, chronic myeloid leukemia; DFI, dye fluorescence index; DNR, daunorubicin; MDR, multidrug resistance; MFI, mean fluorescence index; MO, monocyte; MVP, major vault protein; Pgp, P-glycoprotein; VER, verapamil.
CD34-derived DC culture

DC were generated from CD34+ cells isolated from peripheral blood of G-CSF-mobilized patients. Cells bearing CD34 Ag were isolated from mononuclear fractions through positive selection by mini-MACS using the CD34+ progenitor cell isolation kit according to the manufacturer’s recommendations (Miltenyi Biotec, Bergisch Gladbach, Germany). CD34+ progenitors were cryopreserved or seeded for expansion at 10^5 cells/ml in complete medium in the presence of rGM-CSF (100 ng/ml) and rTNF-α (2.5 ng/ml) for 14 days before harvesting. Adherent cells were harvested using 0.5 mM EDTA/PBS.

Chronic myeloid leukemia (CML)-derived DC culture

PBMC from CML patients were either upon cryopreservation or directly seeded in a 2 x 10^6 cells/ml concentration and cultured in complete medium supplemented with rGM-CSF (100 ng/ml), rTNF-α (2.5 ng/ml), and rIL-4 (1000 U/ml) for 10 days. These immature CML-derived DC were cultured in the presence of Pgp inhibitors and the mean fluorescence of DNR retention in the presence of Pgp inhibitors and the mean fluorescence of DNR retention in the absence of Pgp inhibitors (dye fluorescence index (DFI)).

Dye efflux studies

For double-labeling experiments, cytospin preparations of immature and mature MO-DC were fixed and permeabilized with 2% formaldehyde in acetone for 10 min, or, in the case of the mAbs MVP-9, MVP-37, and LMR5, mAbs against MVP, respectively, irrelevant control mouse and rat mAbs produced in our laboratory (16, 17). These Abs were dialedyzed before use (Slide-Alzer Cassette; Pierce, Rockford, IL).

Immunoblot

Immunoblot experiments were performed with mouse (LRP-56 and MVP-9) and rat (LMR5) mAbs against MVP, respectively, irrelevant control mouse and rat mAbs produced in our laboratory (16, 17). These Abs were dialedyzed before use (Slide-Alzer Cassette; Pierce, Rockford, IL).
of MO-DC was monitored by phenotyping the cells at the different stages in culture. Characteristically, expression of the MO marker CD14 expression was lost, whereas the DC differentiation marker CD1a was up-regulated (Fig. 1A). Expression of other DC differentiation markers (CD80, CD86, and HLA-DR) was also up-regulated (data not shown), and there was de novo expression of the DC maturation marker CD83 (Fig. 1A). Immunolabeling of permeabilized cells with mAb directed against intracellularly situated epitopes of Pgp (mAb JSB-1) and MVP (mAb LRP-56 and MVP-9) revealed neither of the proteins in the CD14^+ MO. Immature MO-DC displayed weak expression of Pgp (MFI, 1.6), which was slightly increased in fully matured MO-DC (MFI, 1.8). Clear MVP expression was found in immature MO-DC (MFI, 1.9), which was further increased in fully matured MO-DC (MFI, 4.3; Fig. 1A).

Immunocytochemical analysis of cytospin preparations confirmed these findings, i.e., low, but distinctly augmented levels of Pgp and robust up-regulation of the MVP protein during the differentiation and maturation of MO-DC (Fig. 1B). With two anti-Pgp mAbs, JSB-1 and C494, we observed cytoplasmic immunoreactivity, sometimes in mature MO-DC associated with the plasma membrane. Three different anti-MVP mAbs, i.e., MVP-9, MVP-37, and LRP-56 (shown in Fig. 1B) displayed the characteristic coarsely granular cytoplasmic staining of MVP, which was significantly increased in fully mature MO-DC as compared with immature MO-DC. In addition, both immature and mature MO-DC showed parallel overexpression of MVP and a minor vault component, the 193-kDa minor vault protein (p193), indicating that whole vault particles are up-regulated during MO-DC differentiation and maturation (15).

To further establish overexpression of Pgp and MVP molecules in mature MO-DC, postnuclear supernatants were prepared and immunoblotted by using anti-Pgp mAb C494 and anti-MVP mAb MVP-37. Although a protein band of ~170 kDa was readily detectable in the control postnuclear supernatant of Pgp-positive SW-1573/2R160 tumor cells, no Pgp could be identified in mature DC, indicating that the expression level of Pgp in mature MO-DC remains below the level of detection by Western blotting (data not shown). High levels of MVP in mature MO-DC were confirmed by Western blotting; a clear band of ~110 kDa was detected, corresponding to the molecular mass of MVP (Fig. 1C).

In vitro, DC-like cells can be generated from a number of sources by culturing hemopoietic precursor cells in the presence of a variety of cytokines and medium supplements (4, 5). To elaborate on the correlation between differentiation stage and expression of Pgp and vaults that was found in MO-DC, we further evaluated their expression in CD34-derived DC and CML-derived DC. Immunocytochemical analyses showed that also in these cell types cellular differentiation/maturation, as reflected by up-regulated expression of CD1a and CD83, was accompanied by increased expression of Pgp and MVP (Table I). Given its prominence in DC propagated from three different sources under different culture conditions, up-regulation of Pgp and vaults seems to reflect a common developmentally regulated event. Thus, in parallel to functional changes accompanying the differentiation from precursor cells and subsequent activation, as a response to danger-signaling agents present in the microenvironment (such as TNF-α and LPS), DC may rapidly up-regulate Pgp and vault expression.

MO-DC do not exhibit Pgp-mediated transmembrane transport
In chemotherapeutic treatment strategies, the induction or up-regulation of transmembrane transporter molecules, such as Pgp, in cancerous cells represents a major obstacle. In this view one might speculate that expression of the same MDR-related molecules by

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

**A.** Flow cytometric evaluation of MO, immature MO-DC, and LPS-matured MO-DC using anti-CD14, CD1a, and CD83 for immunophenotypical analysis and the mAbs JSB-1 and LRP-56 for Pgp and MVP detection, respectively. **B.** Immunocytochemical detection of Pgp (JSB-1), MVP (LRP-56), and the minor vault protein p193 (p193-4) in cytospin preparations of MO, immature MO-DC, and mature (LPS) MO-DC. **C.** Western blot analysis of MVP (using mAb MVP-37) levels in postnuclear supernatants of control tumor cell lines, MO, and mature (LPS) MO-DC.
DC, known to literally scavenge their environment for potentially toxic materials, fulfills an evolutionary conserved defense mechanism that may also protect DC during chemotherapy. Therefore, we evaluated the functional activity of Pgp in fully mature MO-DC by investigating the efflux of the Pgp-transported fluorescent cytotoxic drug DNR in the absence and presence of established Pgp inhibitors, PSC833 and VER (Fig. 2A). Pgp-positive control SW-1573/2R160 tumor cells transported the substrate DNR into the surrounding medium, showing no retention as reflected by a decrease in mean fluorescence intensity down to the level of unloaded cells. This efflux was inhibited by PSC833 (DFI, 2.0) as well as VER (DFI, 1.8). Pgp-negative control SW-1573 tumor cells did not efflux DNR mediated by Pgp as was shown by full retention, which was not further affected by the Pgp antagonists. Also, in mature MO-DC, neither PSC833 nor VER affected the mean fluorescence intensity level (DFI, 1.0).

To verify whether, as suggested by these data, Pgp might not be expressed on the outer plasma cell membrane, we performed flow cytometric analysis on viable, mature DC using four different anti-Pgp mAbs (MRK16, UIC2, 4.17, and 4E3) directed against extra-cellular epitopes. Indeed, no immunoreactivity with surface Pgp was found on mature DC (Fig. 2B). Thus, although Pgp could be detected by immunocytochemical and flow cytometric analyses using permeabilized MO-DC (Fig. 1, A and B), no surface-exposed Pgp could be detected nor could Pgp-dependent trans-plasma membrane transport activity be shown. These results confirm those reported earlier by Randolph et al. (3), Chao et al. (27), and Laupeze et al. (28) in showing that DC cultured from MO do not exhibit Pgp-mediated trans-plasma membrane transport, but extend those data in revealing distinct intracytoplasmic Pgp expression. Importantly, in the former study extracellular epitopes of Pgp could be detected in epidermal skin-derived DC, as collected from human skin explant culture media, and were found to be functionally associated with their migratory capacity. This suggests that Pgp can be differentially expressed in DC depending on environmental conditions. Intracellular Pgp expression as well as inactive forms of Pgp have also been observed in tumor cells and in lymphocytes (29–31). Intracellularly, MO-DC-associated Pgp might still exert unknown functions in the vacuolar system, whereas on the cell surface Pgp might be expressed as a truncated product (32) and/or require functional activation through conformational changes under specific environmental conditions (33). Whether Pgp overexpression in DC may facilitate the release of proinflammatory mediators, like recently reported for another transmembrane transporter molecule, i.e., multidrug resistance protein (MRP1), is still unknown (34).

**Subcellular localization of MVP in MO-DC**

The presence and function of vaults in DC have not been investigated before. To find clues as to a possible role for vaults in the functional and phenotypical development of DC, the subcellular localization of the MVP protein was studied in cytospin preparations of MO-derived immature and mature DC with the anti-MVP

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**Table I. Up-regulation of Pgp and MVP expression during DC development from different sources in vitro**

<table>
<thead>
<tr>
<th>DC Markers</th>
<th>MDR-Related Molecules</th>
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<tr>
<td>CD1a</td>
<td>CD83</td>
</tr>
<tr>
<td>Immature DC</td>
<td>±/+</td>
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<tr>
<td>Mature DC</td>
<td>–/+</td>
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<tr>
<td>CD34⁺ cells</td>
<td>–/–</td>
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<tr>
<td>CD34⁺/DC</td>
<td>+/+</td>
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<tr>
<td>CML cells</td>
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<td>Immature CML/DC</td>
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<td>Mature CML/DC</td>
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<tr>
<td>MDR control cell line</td>
<td>+/–</td>
</tr>
<tr>
<td>SW-1573/2R160</td>
<td>ND</td>
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<tr>
<td>GLC4/ADR</td>
<td>ND</td>
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*Data are derived from immunostaining of cytospin preparations and are representative for at least three different experimental samples each. Staining intensities were scored according to the following scale: –, negative; ±, weak; +, positive; +++, strongly positive. ND, not done.*

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**FIGURE 2.** MO-DC do not exhibit Pgp-mediated transmembrane transport. _A_. No effect of PSC833 and VER on efflux of DNR from LPS-matured MO-DC. SW-1573 (Pgp negative) and SW-1573/2R160 (Pgp positive) were included as control cell lines. Each graph shows the flow profiles of the measured fluorescence (on a log scale) in DNR-loaded cells (filled histograms) and unloaded cells (open histograms). Representative results of five independent analyses are shown. _B_. Lack of surface epitope detection of Pgp by the mAbs MRK16, UIC2, 4.17, and 4E3 on viable mature (LPS) MO-DC compared with SW-1573/2R160-positive control cells. Each graph shows the flow profile of staining (on a log scale) conducted with specific mAbs (filled histograms) and isotypic control mAb (open histograms). Representative results of two independent analyses are shown.
mouse mAb LRP-56 and MVP-9 using confocal laser scan microscopy (Fig. 3). Similar to what was seen by conventional immunocytochemistry, a typical cytoplasmic, granular MVP staining was observed in immature DC and an even stronger MVP staining in mature DC. No distinct colocalization could be observed of the MVP protein with MHCII or MHCI, distributed along the pathways for presentation of exogenous and endogenous protein Ag, respectively (Fig. 3A). Thus, no direct association with Ag presenting routes was revealed. Furthermore, the MVP protein did not colocalize with anti-TGN-46, specific for the trans-Golgi network (Fig. 3A). Clear partial colocalization was found for MVP and CD63, a lysosomal marker (Fig. 3B). Most likely, in the lysosomal degradative compartment the MVP protein, along with many other proteins, is ultimately destroyed.

**Inhibitory effects of anti-MVP Abs on MO-DC maturation and function**

Next, during both differentiation and LPS-induced maturation, MO-DC were cultured in the absence or continuous presence of anti-MVP polyclonal or an oligoclonal mix of three different anti-MVP mAbs or nonspecific control sera. Staining of the internalized Abs was conducted on cytospin preparations, applying only biotinylated secondary step reagent and the streptavidin-biotin-peroxidase complex. In all MO-DC cultured with Abs, cytoplasmic immunostaining was observed (Fig. 4A), revealing uptake of the administered Abs, irrespective of their specificity, through their strong endocytic capacity. Strikingly, the internalization of the anti-MVP polyclonal serum had a distinct effect on the phenotypic characteristics of MO-DC. When examining cytospin preparations, typically CD1a, CD86, CD83, and CD54 increased upon maturation. In contrast, MO-DC cultured with polyclonal anti-MVP showed reduced expression of each these markers in seven individual experiments as compared with the control cultures (Fig. 4B). Along with this reduced expression of distinctive surface molecules, the addition of polyclonal anti-MVP during MO-DC culture led to a reduced viability of the mature MO-DC population when compared with cultures with control serum (Fig. 4C; paired t test, \( p = 0.007, n = 7 \)). MO-DC cultured with the anti-MVP mAb mixture also remained low in their expression of DC markers, but the slight decrease of viability under this condition did not reach statistical significance (Fig. 4C; \( p = 0.310, n = 4 \)). Culturing MO-DC in the presence of any of the individual, single-epitope confined, mouse anti-MVP mAb had no detectable effects on expression of costimulatory membrane molecules or viabilities (data not shown).

After subsequent experiments with dextran-FITC were unsuccessful in revealing clear effects on intracellular uptake of exogenously administered Ag (data not shown), we explored the Ag-presenting capacities of mature MO-DC, generated in the presence of anti-MVP or control polyclonal Abs, respectively, anti-MVP, and control oligoclonal mAb mixes. When the respective DC were pulsed with influenza virus Ag and used to stimulate autologous T cells, highly significant suppression of resulting T cell responses was observed. This suppression was observed for both polyclonal and monoclonal anti-MVP Abs and revealed by both proliferation (paired t test, \( p < 0.001 \) for anti MVP polyclonal Abs (\( n = 7 \)) and \( p = 0.003 (n = 4) \) for anti-MVP monoclonal mixture; Fig. 4D) and IFN-\( \gamma \) release assays (paired t test, \( p = 0.001 \) for anti-MVP polyclonal and \( p = 0.005 \) for anti-MVP monoclonal mixture; Fig. 4E). Thus, in the absence of gross effects on Ag uptake as reflected by

**FIGURE 3.** Subcellular localization of MVP in MO-DC. **A,** Separate subcellular distribution of MVP/vaults (g, green) and TGN, MHCII, or MHCI (r, red) in immature and mature (LPS) MO-DC. **B,** Partial colocalization of MVP/vaults (g) and the lysosomal marker CD63 (r) in LPS-matured MO-DC, indicated by arrows.
intracellular dextran-FITC staining, the latter results support the view that intracellular targeting of vault particles with Abs binding the major vault protein interferes with appropriate handling and presentation of exogenously administered Ag. Interestingly, the lack of significant effects with the individual mAbs argues against interference with functionally relevant epitopes. Most likely, therefore, this blockade results from events secondary to this binding, e.g., from aggregation of vault particles.

Concluding remarks

Taken together, we propose that vaults act as components in the cascade of events regulating DC effector function. Since their discovery, the occurrence and abundance of vaults in eukaryotic cells have argued for an important general function. From studies in the slime mold Dictyostelium discoideum, it is known that disruption of MVP results in a phenotype incapable of growing under nutritional stress (35). From studies in tumor cell lines, vaults have been implied in drug resistance (12, 15, 16, 36, 37). The present findings first demonstrate that, even more so than Pgp, vault particles are increased during the differentiation of DC and that their numbers are further increased upon maturation. An attractive hypothesis is that this reflects a fundamental response to stress signals and that in mature DC vaults may serve as protection of their integrity during migration to the draining lymph node. Whether this involves a nucleo-cytoplasmic shuttle of signal transduction (7, 14) or association with microtubular functions (9, 11) remains to be seen. The present findings point to a role of vaults in DC functioning, more specifically in their capacity to induce Ag-specific T cell proliferative and IFN-γ release responses. Obviously, future studies should also clarify whether vault particles might also play roles in other DC functions, e.g., migratory behavior and CTL activation. To further address all of these questions, we are currently developing MVP gene knockout mice.

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