

Active transport of contact allergens in human monocyte-derived dendritic cells is mediated by multidrug resistance related proteins

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ABSTRACT

The multidrug resistance related proteins (MRPs) function as efflux transporters of a variety of large organic anions or their conjugates. In recent studies we demonstrated that antigen-presenting cells express a specific pattern of MRPs. MRP-mediated efflux activity of human monocyte-derived dendritic cells (moDCs) was analyzed using an *in vitro* transport assay. The efflux transport of radiolabeled contact allergens was inhibited using the specific MRP inhibitor indomethacin. Treatment with indomethacin increased intracellular concentration of [^3H] eugenol and [^3H] isoeugenol in moDCs. In addition by using MRP1 expressing inside-out membrane vesicles we revealed that the transport of eugenol is mediated by MRP1. Human DCs were employed to assess the sensitizing potential of contact allergens and alters their cytokine gene expression profile. Hence, to survey the functionality of indomethacin after stimulation with contact allergens IL-8 and TRIM16 regulation was measured by a DC-based *in vitro* assay. Incubation with isoeugenol after pre-treatment with indomethacin leads to increased IL-8 and TRIM16 gene expression. These results strongly support the functional role of MRPs in the active efflux of contact allergens also in antigen-presenting cells like moDCs, a novel mechanism which could possibly play a role in the pathogenesis of contact allergy.

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Introduction

Allergic contact dermatitis (ACD), a delayed hypersensitivity reaction to small molecular weight compounds (<1000 Da) called haptens including contact allergens such as metal salts and fragrances, is a T-cell driven common inflammatory skin disease orchestrated by cutaneous antigen-presenting cells [1,2]. So far, knowledge regarding the transport and *in vitro* detection of these small molecular weight compounds, particularly prohaptens, in skin cells is limited.

The phenomenon of multidrug resistance (MDR) is defined as the ability of a cell to show resistance to a wide variety of structurally and functionally unrelated molecules and is, in large part, the story of membrane efflux transporters [3]. One important superfamily of active membrane transporters is the family of ATP-binding cassette (ABC) transport proteins that use the energy of ATP hydrolysis to efflux substances across a membrane against a concentration gradient [4]. Thus, ABC transporters can actively transport many structurally different and functionally unrelated drugs and numerous other physiological substrates, such as steroid and inflammatory mediators [5–7] either directly or, in case of many natural substrates, conjugated to acidic ligands, such as glutathi-

one, glucuronate, or sulfate out of the cell [8]. Therefore, it is more and more understood that these efflux pumps have -besides conferring resistance of tumor cells to chemotherapeutic drugs- also crucial physiological roles [9]. Several studies have been performed to investigate the expression, regulation and substrate specificity of these transport proteins [10]. Recent studies revealed that ABC transporters not only have a protective function related to their capacity to extrude toxic compounds but suggest additional regulatory functions of these transport systems on immune cells [11,12]. The multidrug resistance related proteins (MRP, gene name: ATP-binding cassette C transporters, ABCC) are a subfamily of ABC transporters and consists of at least seven members [13]. Concerning the regulatory mechanisms of these transport proteins in skin cells it was demonstrated that IL-6 type cytokines significantly enhance the expression of different MRPs in epidermal keratinocytes. Moreover, upregulation of MRP expression was found in lesional skin taken from patients with inflammatory skin disorders like psoriasis and lichen planus [14].

Expression of several efflux proteins has been detected in various skin cells [15–17]. Gene expression analysis of normal human epidermal keratinocytes (NHEK) revealed constitutive expression of MRP1 (ABCC1) as well as MRP 3–7 (ABCC3–7) but was negative for MDR1 and MRP2 (ABCC2) [16]. Recent studies could show that antigen-presenting cells like monocytes, macrophages and also monocyte-derived dendritic cells (moDCs) constitutively express MRP1 as well as MRP3–6 [18]. Functional studies proved that efflux

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transport proteins such as MRPs mediate the active transport of steroid hormones and contact allergens in NHEK [19].

Therefore, we studied the role of MRP proteins in antigen-presenting cells and could provide evidence for a functional MRP-mediated efflux activity of moDCs, using *in vitro* transport and DC-activation assays.

Materials and methods

Monocyte-derived dendritic cells

Human peripheral blood monocytes were separated from purchased single donor buffy coats (Institut für Transfusionsmedizin, Universitätsklinikum Aachen, Germany) over a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) and CD2, CD7, CD19, CD56, CD16 and CD235a positive leukocytes were depleted using a negative monocyte isolation kit (Dyna[®] Monocyte Negative Isolation Kit, Invitrogen, Oslo, Norway). The remaining monocytes were suspended in complete medium consisting of RPMI-1640 (PAA laboratories, Pasching, Germany) enriched with 1.5% heat inactivated autologous plasma and 1.1 ml L-glutamine/100 ml. The cell suspension was plated at a density of 1×10^6 cells/ml. In order to induce DC differentiation, the culture medium was supplemented with 800 U/ml Gm-CSF and 1000 U/ml IL-4 (R&D – System, Bühlmann, Basel, Switzerland). The cells were incubated at 37 °C and 5% CO₂ for 3 days. 1600 U/ml GM-CSF and 1000 U/ml IL-4 were added to the culture medium one day 4.

Mo-DC based assay

The test compounds were individually prepared in complete medium. 6×10^6 moDCs were pre-incubated with the specific MRP inhibitor indomethacin (200 µM) for 30 min at 37 °C. Subsequently the moDCs were exposed to isoeugenol (200 µM) for 30 h. Untreated cells were used as control. RNA isolation of the treated cells was performed. The RNA samples were harvested and stored at –80 °C before quantitative reverse transcriptase-PCR analysis. PCR analysis was performed as described below using an Assay-on-Demand primer/probe set (Applied Biosystems), which is specific for the detection of IL-8 and TRIM16 mRNA expression. The relative mRNA levels were normalized to cyclophilin A.

RNA isolation and quantitative RT-PCR

Total RNA from moDCs was isolated using the RNeasy Mini kit (Qiagen, Hilden Germany) according to the manufacturer's instructions, including efficient on-column digestion of DNA with RNase-free DNaseI. RNA concentration was measured with the Nanodrop system (Nanodrop Technologies, Rockland, DE), and equal amounts of RNA were used for quantitative RT-PCR. Purified RNA was reverse transcribed with the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Weiterstadt, Germany) with random hexamers as primers. TaqMan experiments are carried out on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using Assay-on-Demand gene expression products for the detection of IL-8 (Hs00174103_m1) and TRIM16 (Hs00414879_m1) according to the manufacturer's recommendations. An Assay-on-Demand product for cyclophilin A (Hs99999904_m1) will be used as an internal reference to normalize the target transcripts.

Transport assay

1×10^6 /500 µl moDCs were seeded in 24-well plates. Cells were washed once with pre-warmed PBS and then pre-incubated with the inhibitor indomethacin (Sigma, Deisenhofen, Germany) solved

in DMSO in a concentration of 500 µM for 30 min and at 37 °C. Control cells were incubated without the inhibitor and treated with DMSO. Subsequently the cells were exposed to the contact allergens eugenol (2×10^{-7} mM/ml, 2 µCi/ml A) and isoeugenol (0.2 mM/ml, 2 µCi/ml B) in the presence or the absence of the inhibitor. After incubation for 15, 30 or 60 min at 37 °C, the radioactive medium was removed and cells were washed three times with PBS. Cells were lysed by adding 1 ml per well Optiphaser "Supermix" (Wallac, Turku, Finland) as scintillation fluid. The cell-associated radioactivity was determined by transferring 800 µl aliquots of the lysate in a flexible 24-well plate and counting radioactivity using a crosscontamination preventing tape and a Wallac 1450 MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland). The values are expressed as counts per minute (cpm). Median values of three independent experiments in triplicate were used for statistical data analysis.

Inside-out membrane assay

A vesicular transport assay for MRP1 (Solvo Biotechnology, Szeged, Hungary) was performed according to the manufacturer's instructions. Inside-out membrane vesicles were purified from Sf9 cells. Membrane vesicle suspension was incubated with the radiolabeled substrates LTC4 (10 µCi/ml A) as assay control and eugenol (10 µCi/ml B) in the presence or absence of indomethacin (1 mM) for 15 min at 37 °C. Subsequently 0.2 M ATP was added for 2 min. Membrane vesicles were harvested on a glass fiber filter and vesicle-associated radioactivity was determined in a Wallac 1450 MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland) as counts per minute (cpm).

The studies were approved by the ethical committee of the University Hospital, RWTH, Aachen and the study was conducted according to the Declaration of Helsinki Principles.

Results

In vitro transport assays were performed to characterize the functional role of MRP transport proteins in moDCs. In these studies the efflux transport of radiolabeled contact allergens eugenol (4-allyl-2-methoxyphenol) and isoeugenol was inhibited using the specific MRP inhibitor indomethacin (Fig. 1A) [20,21] and intracellular accumulation of these substrates was determined after 15, 30 and 60 min. Cells not treated with the inhibitor served as control. Treatment with indomethacin in a concentration of 500 µM inhibited MRP dependent efflux transport and therefore increased intracellular concentration of [³H] eugenol up to 170% (Fig. 1A) and of [³H] isoeugenol up to 182% (Fig. 1B) in moDCs in comparison to untreated cells.

To further define the role of MRPs and especially the role of MRP1 in transport of contact allergens, *in vitro* studies using MRP-1 inside-out-membrane vesicles were performed (Fig. 2). MRP1 inside-out-membrane vesicles were incubated with radiolabeled substrates [³H] leukotriene C4 (LTC4) and [³H] eugenol in the presence and in the absence of indomethacin (1 mM) and radioactivity within the vesicle was determined. LTC4 is a well characterized substrate of MRP1 [13,22] and therefore served as a positive control. In absence of the MRP inhibitor indomethacin a 2.8-fold higher intracellular concentration of [³H] LTC4 could be detected within the vesicle in comparison to vesicles pretreated with indomethacin (Fig. 2A). Influx of [³H] eugenol was even 9.4-fold higher in untreated vesicles compared to membrane vesicles pretreated with indomethacin (Fig. 2B).

Human DCs have been employed to assess the sensitizing potential of contact allergens and alters their cytokine gene expression profile. In particular marked IL-8 upregulation has been

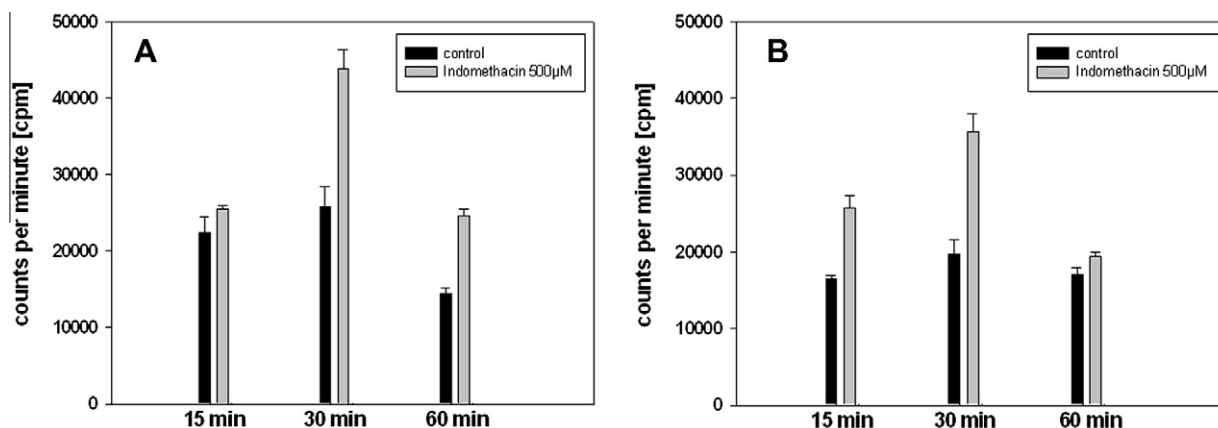


Fig. 1. Transport assays: the inhibitory effect of indomethacin on MRP-mediated efflux of contact allergens in moDCs. Cells were pre-incubated with the inhibitor indomethacin (500 μ M) for 30 min at 37 °C. Control cells were treated with DMSO. Subsequently the cells were exposed to the contact allergens eugenol (2×10^{-7} mM/ml, 2 μ Ci/ml A) and isoeugenol (0.2 mM/ml, 2 μ Ci/ml B) in the presence or the absence of the inhibitor. After incubation for 15, 30 or 60 min at 37 °C, the radioactive medium was removed and cells were washed. Cells were lysed by adding scintillation fluid. The cell-associated radioactivity was determined. The values are expressed as counts per minute (cpm). Median values of three independent experiments in triplicate were used for statistical data analysis.

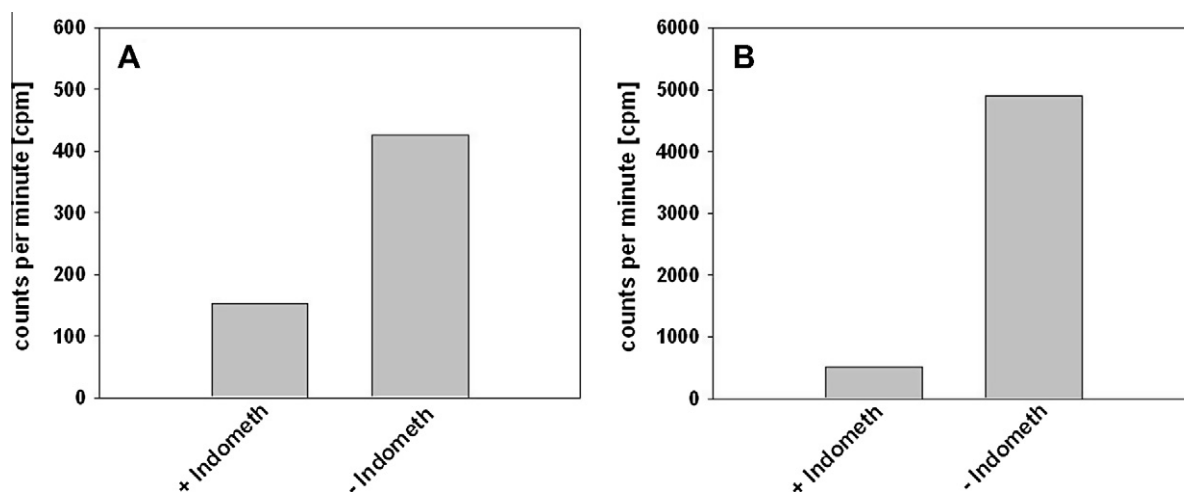


Fig. 2. Transport of eugenol is mediated by MRP1. A vesicular transport assay for MRP1 was performed. Membrane vesicle suspension was incubated with radiolabeled substrates LTC4 (10 μ Ci/ml A) and eugenol (10 μ Ci/ml B) in the presence or absence of indomethacin (1 mM) for 15 min at 37 °C. Subsequently 0.2 M ATP was added for 2 min. Membrane vesicles were harvested on a glass fiber filter and vesicle-associated radioactivity was determined as counts per minute (cpm).

shown to occur during DC exposure to contact allergens [23]. Hence, to survey the functionality of the specific MRP inhibitor indomethacin after stimulation with contact allergens IL-8 and TRIM16 regulation was measured by a DC-based *in vitro* assay (Fig. 3). MoDCs were pre-incubated with the specific MRP inhibitor indomethacin (200 μ M). Subsequently the moDCs were exposed to isoeugenol (200 μ M) for 30 h. Untreated cells were used as control. We detected a moderate upregulation of IL-8 (up to 4-fold) and TRIM16 (up to 6.8-fold) mRNA expression after incubation of immature dendritic cells with isoeugenol compared to untreated cells. After co-incubation of moDCs with indomethacin and isoeugenol IL-8 and TRIM16 mRNA expression was significantly enhanced (up to 8-fold and 19.7-fold, respectively) (Fig. 3).

Discussion

Fragrances are common cause of allergic contact dermatitis, a T-cell-mediated delayed hypersensitivity reaction to low molecular weight chemicals (haptens) with complex symptoms. Studies by Sieben et al. [24] showed that metabolic activation of eugenol

using cytochrome P450 enzymes may be essential for positive T cell responses to this fragrance. Previous investigations demonstrated that MRPs mediate the active transport of contact allergens in epidermal keratinocytes [19].

Human MRP1 was first identified in 1992 in human multidrug resistant lung cancer cells [25]. In the last decade it was discovered that MRP1 transport a wide range of organic anions, including glutathione conjugates like LTC4 as well as glucuronate and sulfate conjugates, such as oestrone 3-sulfate or E217 β G [7,13,26]. Previous studies revealed the expression of MRP1 in moDCs [18] which appears to be involved in DC migration toward lymph nodes through afferent lymphatic vessels [11,27]. Moreover, it was found that MRP1 transporter activity is required for DC differentiation because blocking of MRP1 transport activity reduced maturation associated membrane molecules like CD40, CD86 and in particular CD1a. Blocking of the MRP1 activity with the specific inhibitor MK571 leads to a phenotypic change in moDCs [18,22]. In this study we demonstrated, to our knowledge for the first time, a MRP-mediated active efflux of contact allergens in moDCs (Fig. 1).

Substrate specificity of MRP1 was previously characterized using inside-out vesicles from many cells types expressing the

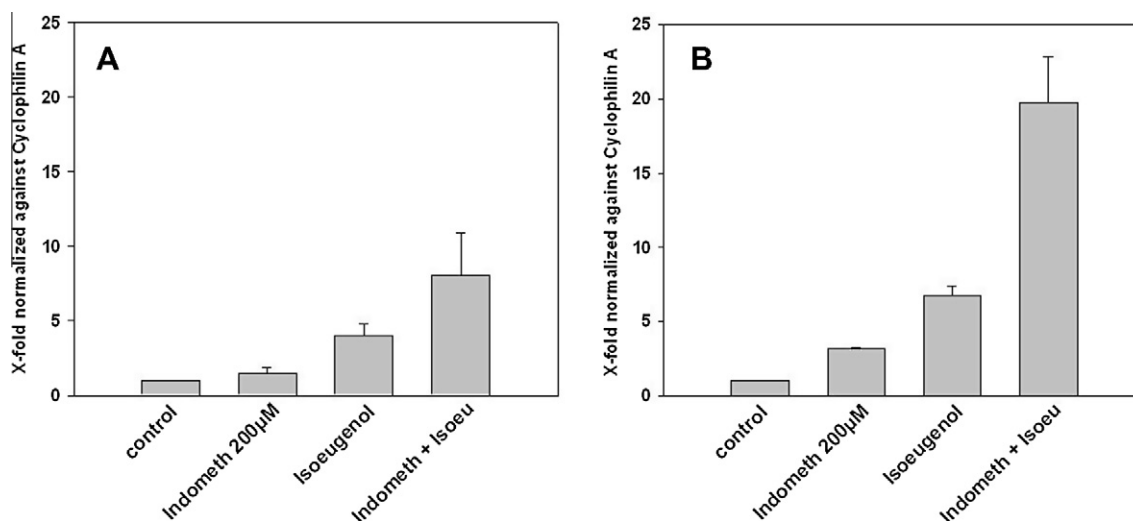


Fig. 3. MoDC-based *in vitro* assay IL-8 (A) and TRIM 16 (B) mRNA expression is upregulated after stimulation with isoeugenol and indomethacin: moDCs were pre-incubated with the specific MRP inhibitor indomethacin (200 μ M) for 30 min at 37 °C. Subsequently the moDCs were exposed to isoeugenol (200 μ M) for 30 h. Untreated cells were used as control. TaqMan real-time PCR analysis was performed. The relative mRNA levels were normalized to cyclophilin A. The median values of three independent experiments were used for statistical data analysis.

recombinant protein at a high level, including erythrocytes, hepatocytes, heart and mastocytoma cells [8]. In this study we identified a so far unknown substrate for MRP1 also by using MRP1 expressing inside-out membrane vesicles. We revealed that the transport of the contact allergen eugenol is mediated by MRP1 (Fig. 2) and that this transport can be inhibited by indomethacin.

Previous investigations showed that IL-8 upregulation in a moDC-based *in vitro* model identifies contact allergens [28–30]. Toebak et al. [31] demonstrated that exposure to contact allergens but not irritants results in increased IL-8 secretion and CD83 and CD86 upregulation. In line with these findings, we found relevant enhancement of IL-8 gene expression after incubation with the contact allergen isoeugenol after treatment with the MRP-specific inhibitor indomethacin (Fig. 3A). IL-8 expression is known to be associated with functional activation as marker of hapten-specific APC activation [28,32,33]. Recent publications depicted IL-8 as a promising biomarker for distinguishing between sensitizers and non-sensitizers [29,31,34].

TRIM16 is a gene encoding for the estrogen-responsive B box protein (EBBP) and a member of the Tripartite Motif (TRIM) protein family [35]. Besides, TRIM16 is known to be involved in keratinocytes differentiation [36] and retinoid metabolism [35]. Furthermore EBBP has recently shown to enhance the secretion of IL-1 β , a cytokine specifically synthesized by APC upon allergen stimulation [37]. TRIM16 is also upregulated in moDCs stimulated with isoeugenol after treatment with the inhibitor indomethacin (Fig. 3B). These results are in line with findings of Ott et al. showing increased TRIM16 mRNA expression in hapten-stimulated antigen-presenting cells [23].

Blocking of the efflux leads to an accumulation of the substrate in the cell. Accordingly to this assumption treatment of moDCs with isoeugenol after pre-incubation with indomethacin resulted in an enhancement of IL-8 and TRIM16 mRNA expression in comparison to incubation in the absence of the inhibitor. Therefore accumulation of contact allergens within the dendritic cells enhances activation of these cells measured by IL-8 or TRIM16 expression. This effect might be applied to improve the sensitivity of novel *in vitro* test systems which are currently used to predict the skin sensitizing capacity of a chemical and especially of weak contact allergens such as fragrances. Therefore our findings might be useful to improve *in vitro* assays for identification of skin sensi-

tizing chemicals which are currently under development in order to replace animal based assays such as the local lymphnode assay [28,33,38,39].

To investigate this feasibility, further experiments using different contact allergens and irritants are required.

In addition to previous studies revealing the expression of efflux transport-associated proteins in human antigen-presenting cells we now provide data which strongly supports the functional role of MRPs in the active efflux of contact allergens in human moDCs. This novel mechanism may play a role in the pathogenesis of contact allergy.

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