Aus der Klinik für Dermatologie, Venerologie und Allergologie der

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Microbial influences on allergic inflammation: Crosstalk of the C-type lectin receptors Dectin-1 and Dectin-2 and implications in allergy

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Table of Contents

Figures and Legends

Abbreviation list

AD: Atopic dermatitis

CLR: C-type lectin receptor

DAMP: Damage-associated molecular pattern

DC: Dendritic cells

Dectin-1: CLEC7A/ C type lectin domain family 7 member A

Dectin-2: CLEC6A/ C type lectin domain family 6 member A

DPBS: Dulbecco's Phosphate-Buffered Saline

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorting

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

hTrx: Human thioredoxin

IL-1β: Interleukin-1-beta

IL-4: Interleukin 4

IL-23: Interleukin 23

LPS: Lipolysaccharide

M1: Macrophage M1

M2: Macrophage M2

Mala s 13: Malasszia sympodialis 13

moDC: Monocyte-derived dendritic cells

NK cell: Natural killer cell

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B-cells

PAMP: Pathogen-associated molecular pattern

PBMC: Peripheral blood mononuclear cell

PRR: Pattern recognition receptor

WGP d: WGP dispersible

WGP s: WGP soluble

SYK: Tyrosine-protein kinase

RLR: Rig-like receptor

ROS: Reactive oxygen species

TH1: Type 1 T helper cell

TH17: Type 17 T helper cell

TLR: Toll- like receptor

ZYM: Zymosan Depleted

Introduction

Fungi of the *Malassezia* genus are found to be part of the normal flora of human skin. Currently there are 14 species of *Malassezia,* and most of them are lipid-dependent except *Malassezia pachydermatis*[1]. In healthy people, *Malassezia* species are harmless or cause mild skin disorders, however, they are also involved in some systemic infections in immunocompromised people[1]. In general, *Malassezia*-associated skin disorders, such as pityriasis versicolor, dandruff, seborrheic dermatitis (SD) and atopic dermatitis (AD, also called as atopic eczema) are chronic skin diseases. Because of the mild symptoms, low mortality, and complicated immune responses directed against yeasts, the progress of research in *Malassezia*-associated skin disorders is slow. Therefore, in the present study it was studied how the *Malassezia spp.* interact with the patient/host immune system with the perspective of the identification new target molecules for a better treatment of Malassezia-associated skin disorders.

Atopic dermatitis (AD)

Atopic dermatitis is a chronic inflammatory skin disease. Symptoms include intense itch, redness, swelling and thickening of the epidermis of the affected area. According to a study from *T.C. White et al.*, the prevalence of AD is about 15-30% in children and 2%-10% in adults[2]. So far, the precise cause of AD is still unknown, however, most of the AD patients show elevated serum levels of IgE. About 30% - 80% of AD patients develop clinical symptoms based on sensitizations via IgE [3]. Therefore, AD is highly associated with IgE-mediated food or dust mite or pollen allergy and is clinically associated with allergic rhinitis and asthma[4]. A previous study showed that *M. sympodialis* is the most commonly isolated fungus within AD patients. Almost half of

the AD patients have positive allergic reactions to *M. sympodialis* including patch test reactions leading to eczematous skin reactions in the so-called atopy patch test[5]. In a previous study from Hannover Medical School, Division of Immunodermatology and Allergy Research, it was shown that the T cells of a subgroup of AD patients can be stimulated by an allergen from *Malassezia* that belongs to the thioredoxin protein family (Mala s 13) and its homologous endogenous human thioredoxin (hTrx)[6]. Therefore, due to the studies above, *Malassezia* is thought to be a cause or a factor that exacerbates AD directly and indirectly inducing autoimmune phenomena due to antigenic mimicry.

Innate immunity

The innate immune system, also called non-specific immune system, is the first barrier that defends the host from microorganism infection. In humans, it includes the anatomical barriers, for example skin, and immune cells such as natural killer cells (NK cells), mast cells, dendritic cells (DCs), and macrophages. Pathogenic invasion is rapidly sensed by the innate immune system leading to acute inflammation. The innate immune system also plays important role in the acquired immune system activation[7].

Pattern Recognition Receptors (PRRs)

In innate immunity germline-encoded pattern recognition receptors are the most important sensors especially for pathogen detection. PRRs are mainly expressed in and on DCs and macrophages but also in and on monocytes and epithelial cells detecting pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) from microorganisms and damaged cells, respectively.

PAMPs/DAMPs comprise different types of microbial molecules including lipopolysaccharides (LPS), lipoproteins, RNA and DNA. Currently, four types of PRRs have been identified: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene-I-like receptors (RLRs), and NOD-like receptors (NLRs) (reviewed in ref. 8).

C-type Lectin Receptors (CLRs)

CLRs are members of the PRR superfamily and have lectin-like carbohydrate recognition domains (CRDs) which mainly recognize carbohydrate structures in pathogens. Fungal cell wall was reported to have multiple types of carbohydrates, such as mannose, β-glucans, and chitins. Furthermore, previous studies also showed that CLRs (Dectin-1/Dectin-2/Mincle), especially Dectin-1 and Dectin-2, can function as recognition receptor for *Malassezia* spp[29]. In addition, not only carbohydrates but also proteins are known to interact with CLRs. For example, CLEC9A has been shown to bind F-actin inside the cell under healthy conditions but gets exposed after cell death[9]. Another example is CLEC8A (also known as LOX-1 or SCARE-1). CLEC8A is a receptor for chaperone heat shock protein 70 (HSP70)[10]. Interestingly, CLEC8A-related proteins like Siglec, SREC-1 (SCARF-1) and FEEL-1 (SCARH-2) have also been reported to bind HSP70. The three transmembrane receptors mentioned above share homologous structures and react to low density lipoprotein (LDL) as a common ligand. Recently, Siglec-5 and Siglec-14 in the I-type lectin family have also been shown to bind HSP70[11].

Fungus recognition through the Dectin-1 receptor

β-glucan is highly expressed on the surface of fungi, and Dectin-1 is one of the most

important receptors for β-glucan. In fact, Dectin-1 was already proven to mediate several fungus recognitions *in vitro*[12], for example, *Pneumocystis carinii*[13], *Canidia albicans*[14], *Aspergillus fumigatus*[15], *Coccidioides immitis* and *Coccidioides posadasii*[16]. In addition to fungus recognition, Dectin-1 is also involved in antifungal defense including phagocytosis, cytokine secretion and Reactive oxygen species (ROS) production[16].

Dectin-1 signaling pathway

Gringhuis et al. demonstrated that Dectin-1 not only activates the Syk-dependent NF-kB subunit p65 and c-Rel but also RelB which is a noncanonical subunit[17]. From these results, it can be concluded that Dectin-1 may activate two different independent signaling pathways to induce immune responses when recognizing fungi[18].

Dectin-1 stimulators and blockers

Since Dectin-1 is a surface membrane receptor, there are several ways to activate or inhibit its function, such as the well-known Zymosan d, a glucan with repeating glucose units. In addition to Zymosan d, whole glucan particles (WGP) were also used in this study to modulate the expression of Dectin-1. WGP is one of the beta-glucans which has the ability to regulate the immune response. There are two types of WGP, namely WGPd (WGP Dispersible) and WGPs (WGP Soluble). WGPd is a component of the cell wall of *S. cerevisiae* which has the ability to activate the Dectin-1 receptor[19]. Different from the other Dectin-1 activator Zymosan, WGPd has no TLR-stimulating activity[20.] Therefore, WGPd can specifically induce Dectin-1. WGPs also binds to the Dectin-1 receptor. However, it does not activate but blocks the Dectin-1 downstream response.

Same as WGPd, WGPs is derived from the cell wall of *S. cerevisiae* and has no TLR-stimulating activity.

Dectin-2

Although Dectin-2 is also a member of the Dectin family, it only has 20-25% homology to Dectin-1[21]. A previous study showed that Dectin-2 has a high specificity for recognizing high mannose structures. Like Dectin-1, Dectin-2 binds to *Cryptococcus neoformans*, *Candida albicans* and *Trichophyton rubrum*[22]. Regarding to the signaling pathway, Dectin-2 was shown to signal with the FcRγ-chain through the immunoreceptor tyrosine-based activation motif (ITAM) and thus activates NF-Kb[23]. Another study also showed that Dectin-2 induces the adaptive immune system - especially Th17 cells - through the Syk pathway[24]. The function of the Dectin-2, however, remains largely unknown and more studies are needed to elucidate it.

Dectin-1/-2 expression on different immune cells and induction of cytokine secretion

Dectin-1 and Dectin-2 are known to be expressed on macrophages, DCs, monocytes and neutrophil cell lineages, and activate cytokine secretion, such as IL-23, IL-17, IL-1b and IL-12, after recognizing fungal antigen[25-27]. In the present study the connection between Dectin-1 and Dectin-2 was focused via examining surface expression and cytokine secretion of myeloid cells.

Major research questions addressed in this thesis:

Adolescent and adult patients commonly present clinically relevant colonization rates with Malassazia spp. mainly on inflamed skin regions in the head and neck regions. Malassezia spp. sometimes induces specific sensitizations with crossreactions to human antigens in these patients which may have clinical impact on the severity and chronicity of the skin disease.

In this thesis the following major research questions were addressed to better understand Malassezia spp. – host interactions in atopic dermatitis and perhaps more general: yeast – host interactions at interfaces of the human body:

1. To study the signaling pathway of Dectin-1 and Dectin-2 on moDCs/macrophages
2. To study the possible activators and blockers of Dectin-1/Dectin-2 on moDCs/macrophages

Materials and Methods

Table 1. Large Equipment

Large Equipment	Company
BD FACS Canto ll	BD Biosciences
Cell Culture Hood Centrifuge ROTINA 42OK	NUAIRE HETTICH
CO2 Incubators ELx50	Heraeus BioTek
Freezer-20°C	LIEBHERR
Freezer-80°C Centrifuge ROTINA /RP	SANYO HETTICH
Centrifuge 5424 R	eppendorf
DNA Engine	MJ Research

Table 2. Small Devices

Device	Company
Analytical Balance	sartorius
Centrifuge 5424 R Microsystems CMS	eppendorf Leica
Minitype centerfuge	Biozym

Device	Company
Nano Drop2000	Thermo Fisher Scientific
Polymax 1040 Vortex.Genie2	Heidolph
Pipettes 0.5-10µl	eppendorf
Pipettes 2-20 µl, 20-200 µl, 100-1000 µl	eppendorf
Top-Mixer Water Bath	BIOBLOCK SCIENTIFIC Grant

Table 3. Consumables

Product	Company
Cell culture plates (6-well, 12well, 24 well)	CELLSTAR
Reaction tubes (15, 50ml) MACS LS Columns	SARSTEDT Miltenyi
Serological pipette (5ml, 10ml, 25ml)	SARSTEDT
Sterican needles 16.5G Syringe 50 ml	BD Microlance BD Plastipak
Nickel Coated Plates, Clear, 96-Well	Thermo Fisher Scientific
TC-Platte 96 well Tubes 1.5ml	SARSTEDT eppendorf

Tubes 50 ml Pipette tip (10ul, 200ul, 1000ul)	SARSTEDT Biosphere

Table 4. Chemicals and Reagents

Product	Company
anti-h Dectin-1 anti-h Dectin-2	R&D Systems R&D Systems
Aqua ad iniectabilia	Ampuwa®
Furfurman	InivivoGen
GM-CSF	R&D Systems
hTrx	kind gift from Prof. R. Crameri, SIAF, Switzerland
Human IL-4 (for macrophage M2)	R&D Systems
Human Serum Type AB, male	PAN™ Biotech
IFN-g (for macrophage M1)	R&D Systems
LPS (for macrophage M1) Lysis Solution RL	SIGMA analytic jena
MACS Buffer	MACS Miltenyi Biotec
Pancoll	PAN™ Biotech

Product	Company
PBS	PAN™ Biotech
Primer IL-23 Primer GAPDH	QIAGEN QIAGEN
Primer SYBR Green	ROCHE
Piceatannol	MERCK
Tween® 20	ROTH
WGP Dispersible WGP Soluble	InivivoGen InivivoGen
Zymosan d	InivivoGen

Isolation of human moDCs (monocyte-differentiated dendritic cells)

The blood samples were received from the German Blood Donation Center in Springe and were anonymous. All blood samples were checked for specific IgE antibodies in the plasma with a validated screening test detecting the eight most common specific IgE against aeroallergens in Central Europe (Sx1, ImmunoCAP, ThermoFisher Phadia). Four 50 ml conical tubes were filled with 15 ml of Pancoll (PAN Biotech). 80 ml of PBS were mixed with 40 ml of buffy coat blood, and 30 ml of the diluted blood were added onto the Pancoll slowly. This step was done very slowly to prevent mixing of blood and Pancoll. The conical tubes were centrifugated at 800 g for 15 min at room temperature (RT), and the PBMC layer was transferred to another 50 ml conical tube (Fig. 1).

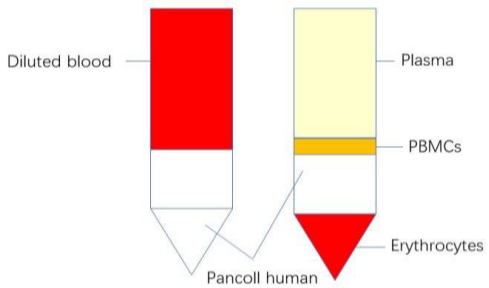


Figure 1. Schematic diagram of PBMC isolation

Then PBS was added to the new conical tube to a total volume of 45 ml. Next, the tubes were centrifugated at 300 g for 15 mins at 4°C. After centrifugation, the supernatant was removed completely, and the cells were washed 2 more times with 45 ml PBS by centrifuging at 150 g for 15 min at 4°C, the supernatant was discarded and cells were counted. Therefore, 1 ml of PBS was added to the cell pellet to resuspend the cells, and then 9 ml more of PBS was added to further dilute cells (final volume: 10 ml). 190 µl of Türk's solution were mixed with 10 µl of cell suspension, and 10 µl of cell mixture were applied to a cell counting chamber (Neubauer improved) counted under the microscope.

Isolation of monocytes

After counting, $1x10^8$ cells were transferred to a 15ml conical tube and were centrifuged at 300x g for 10 min at 4°C. At the same time, the monocyte Isolation Kit II, MACS buffer and LS columns were prepared (50 ml MACS buffer: AutoMACS rinsing solution + 500 µl human serum type AB). After centrifugation, the supernatant was removed completely, 300 µl of the MACS buffer was added for every $1x10^8$ cells. Next, Monocyte Isolation kit ll (MACS miltenyi biotec) was used according to manufacturer´s instructions as follows:

100 µl FcR blocking reagent (MACS miltenyi biotec) and 100 µl of biotin-antibody cocktail were added for every $1x10^8$ cells, and incubated for 10 min at 4-8°C. Next, 300 µl MACS buffer and 200 µl anti-Biotin microbeads (MACS miltenyi biotec) were applied to the cell-reagent mixture and were further incubated for 15 min at 4-8°C. After incubation, 300 µl more of MACS buffer were added. The tubes were then centrifuged at 300 g for 10 min at 4°C. After centrifugation, the supernatant was removed completely, and then 500 µl of MACS-serum mixture was added to each tube. The MACS-serum mixture and cell suspension prepared last step were transferred to the LS column and centrifuged. The filtered cells were then collected in a 15 ml conical tube and rinsed with 3000 µl MACS-serum mixture for three times. Centrifuge cell suspension at 300 g for 15 min at 4°C.

Preparation of cell culture medium

Before culturing the MoDCs, the cell culture medium was prepared (MoMed, 45.9 ml RPMI base medium, 600 µl of 1M HEPES, 500 µl of 200 mM L-glutamine, 500 µl Penicillin, 2.5 ml FCS, 5 µl of IL-4 (10 ng/ml), 5 µl of GM-CSF (10ng/ml)). To culture the moDCs, 1000 µl of MoDC medium was added to the cells which were collected in last step. To count the cell number, 190 µl Türk's solution and 10 µl cell suspension were mixed carefully, and then 10 µl of the mixture was transferred to the Neubauer improved Cell Counting Chamber and counted under the microscope. For every 5 x 10^6 cells, 5 ml of MoDC medium was added and cultured in 6-well culture plate. The medium was changed every 2-3 days according to the following step: 5 ml of cell suspension was taken from each well to a 15 ml conical tube. The cell suspension was then centrifuged at 300 g for 15 min at 4°C. After centrifugation, 2.5 ml of the

supernatant was removed, and 2.5 ml new MoDC medium was added and mixed well. The MoDCs were then cultured in the incubator supplied with 5% CO_2 at 37°C for 7 days.

On day 7, all moDCs were collected from the whole 6-well plate and transferred to a 50 ml conical tube, change medium one time and calculate the cell number, 5 x 10^5 moDCs were then cultured in 24-well plate.

Table 5. Stimuli for experiments with moDCs

No.	Project	Stimulus, conc.	Receptor	Duration
1	Dectin	Zymosan depleted 100 µg/ml (hot alkali treated carbohydrate)	Dectin-1	24 h
2	Dectin	Malassezia s13 2.5 µg/ml	Test	24 h
3	Dectin/Furfuman	WGP dispersible 20 µg/ml (1.3/ 1.6-β-glucan)	Dectin-1	48/24 h
4	Dectin/Furfuman	Human Thioredoxin 2.5 µg/ml	Test	48/24 h
5	Dectin/Furfuman	Furfurman 1, 5, 10 µg/ml	Dectin-2	24 h

Table 6. Blockers for experiments with moDCs

No.	Blockers, conc.	Receptor	Duration
1	WGP soluble 1 ng/ml	Dectin-1	24 h
3	Piceatannol 15 µg/ml	Syk	24 h

Time points for the treatment with stimuli or blockers

Cells were first treated with different blockers (WGPs/Piceatannol) for 1 h and then treated with different stimuli (Zymd/WGPd/hTrx/Mala s13) for another 24 h at 37°C. For Furfurman treatment, cells were incubated with different stimuli (WGPd/hTrx) for 48 h, and then re-stimulated with Furfurman for another 24 h at 37°C.

Preparation of supernatants/moDCs

After being treated for 24 h, cells were transferred from 24-well plate to a 1.5 ml safe-lock tube and centrifuged at 900 g for 5 min at 4°C. After centrifugation, the supernatant was collected to a new 1.5 ml safe-lock tube. The supernatant was then frozen at -80°C for later ELISA detection. The remaining cell pellet from last step was mixed with 100 µl of buffer blocker IF and then seeded in 96-well plate (5 x 10^5 cells/well) for later antibody hybridization. All reagents and cells should be kept on ice.

Immunostaining of Dectin-1/-2

1×10^6 moDCs were seeded into 100µl block buffer in a 96-well plate. Phycoerythrin (PE)-labelled Dectin-1 or Dectin-2 antibody (10 µl/10^6 cells) were added to the well, at the same time, 0.5 µl/well of Fixable Viability Dye eFluor™ 660 was added and incubated for 1 h at 2-8°C (protected from light). After incubation, the cells were washed 3 times with 200 µl/well flow cytometry staining buffer, and then the cells were centrifuged at 1300 rpm for 3 min at 4°C to remove the wash buffer. The wash steps were repeated for 3 times and after washed, 200 µl/well of 1% fixing solution

was added to the cells. The cells were then placed on ice and proceeded for flow cytometry assay.

Treatment of moDCs with Furfurman

The moDCs were prepared for RNA extraction and ELISA. First the moDCs were stimulated with hTrx or WGPd for 48 h, and then moDCs were further treated with Furfurman at two different time points:

a. 4-6 h: the MoDCs were re-stimulated with Furfurman for 4-6 h in the incubator, after treatment, the cells were collected and centrifuged at 300x g for 10 min at 4°C and supernatant was discarded. The cells were then washed with 500 µl PBS for 2 times. 400 µl RL buffer was added for every 5 x 10^5 cells, the suspension was mixed well by vortexing and the cells were frozen at -80°C for later RNA extraction.
b. 24 h: the MoDCs were re-stimulated with Furfurman for 24 h in the incubator, after treatment, the cells were collected and centrifuged at 300x g for 15 min at 4°C. After centrifugation, the supernatant was collected and further frozen at -80°C for later ELISA experiment.

Culture of primary macrophages and cell lines

Cell culture of macrophages

The macrophages were a kind gift from Dr. rer. nat. Susanne Mommert from the Division of Immunodermatology and Allergy Research, Dpt Dermatology and Allergy, Hannover Medical School. For macrophage M1 polarization, macrophages were cultured for 7 days and on the day 7, 500 µl medium was added for every 5 x 10^5 cells

in 12-well plate. Then two groups were labelled, one is polarization group which treated with 50 ng/ml Lipopolysaccharides (LPS) and 10 ng/ml Interferon-γ (IFN-γ). The other group is control group which has no treatment. Macrophages were treated for 24 h in a 37°C and 5% CO_2 incubator. For macrophage M2 polarization, the only difference is treating cells with 20 ng/ml of IL-4 instead of LPS and IFN-γ, other steps are the same as M1 polarization.

Stimuli and blockers for the treatment of macrophages

After being polarized for 24 hours, macrophages were further treated with following reagents:

a. Stimulus: WGP dispersible (WGPd), 20 µg/ml
b. Blocker: WGP soluble (WGPs), 1 ng/ml

In this study, cells were first treated with the blocker for 1 h, and then treated with the stimulus for another 24 h in a 37°C and 5% CO_2 incubator. After stimulating for 24 h, Dectin-1 or Dectin-2 antibody (R&D system, Human Dectin-PE, 10 µl/10^6 cells) and 0.5 µl Fixable Viability Dye eFluor™ 660 were added to macrophage M1 or M2 and incubated for 1 h at 2-8°C (protect from light). After incubation, the cells were washed 3 times with 200 µl/well flow cytometry staining buffer, and then the cells were centrifuged at 1300 rpm for 3 min at 4°C to remove the wash buffer. The washing steps were repeated for 3 times and after washed, 200 µl/well of 0.02% EDTA fixing solution was added to the cells. The cells were then placed on ice and proceeded for flow cytometry assay.

Cell culture of U937 and THP-1 cell lines

U937 is a human monocyte cell line isolated from lymphoma, and under certain stimulation U937 cells have been reported to differentiate into mature macrophages. THP-1 is another monocyte cell line isolated from an acute leukemia patient and used as a model to study monocytes and macrophages. The THP-1 and U937 cell lines were thawed from liquid nitrogen, seeded, and medium (RPMI medium supplied with 10% FCS and 1% Pen/Strep) was changed every 2-3 days.

THP-1 cell lines: macrophage differentiation

Prior to differentiation, 1 x 10^6 cells were seeded in 6-well plate one day before. On the day of differentiation, 100 ng/ml of PMA were treated for 24 h or 48 h in a 37°C and 5% CO_2 incubator.

Stimuli and blockers for both cell lines

a. Stimulus WGP dispersible (WGP d), 20 µg/ml
b. Blocker: WGP soluble (WGPs), 1 ng/ml

In this study, the blocker was first treated for 1 h, and then stimulus was treated for another 24 h in a 37°C and 5% CO_2 incubator. After stimulating for 24 h, 2 x 10^4 cells/well (THP-1 or U937 cells) were collected and seeded in a 96-well plate. Dectin-1 or Dectin-2 antibody (R&D system, Human Dectin-PE, 10 µl/10^6 cells) and 0.5 µl of Fixable Viability Dye eFluor™ 660 were added and incubated for 1 h at 2-8°C (protect from light). After incubation, the plate was washed 3 times with flow cytometry staining buffer as mentioned before.

Enzyme-Linked Immunosorbent Assay (ELISA)
ELISA for IL-23

ELISA was performed with human IL-23 ELISA Kit (eBIoscience, #88-7327-88). The capture antibody was prepared by adding 48 µl of antibody in 12 ml of 1X coating buffer. 100 µl of capture antibody was then added to 96-well plate and incubated at 2-8°C for overnight. On day 2, the plate was washed for 3 times with wash buffer (BioTek #ELx50, 250µl/well). After washing, 100 µl of blocker/1X ELISA/ELISPOT was added to each well and incubated for 1 h at room temperature. The plate was washed again for 3-5 times with wash buffer. Next, the moDC supernatants (from Dectin and Furfurman-treated cells) were thawed and diluted to different concentrations by adding 1X ELISA/ELISPOT. After diluting the samples according to the dilution list, add 100 µL of diluted samples to each well.

Table 7. Sample dilution list for IL-23 ELISA experiments

Chemicals	Dilution factor	Sample (µl/well)	1x ELISA ELISPOT (µl/well)	Total Volume (µl/well)
Zymosan d	1:5	20	80	100
Mala s13	N/A	100	0	100
WGPd	1:10	10	90	100
hTrx	1:2	40	80	100

Prepare standards (human IL-23 recombinant protein, 1 µg/ml, 8 points):

4 µl of standard was added and mixed to 2 ml of sample diluent A to make first point of standard (2000 pg/ml) in a 15 ml conical tubes. Serial dilutions were made as following concentration: 2000, 1000, 500, 250, 125, 62.5 and 31.25 pg/ml. Next, the samples and the standards were incubated at room temperature for 2 h or at 4°C for overnight. The plates were then washed for 3-5 times with wash buffer. After washing,

100 µl of detection antibody which was diluted in 1X ELISA/ELISPOT was added to each well and incubated for 1 h at room temperature. To prepare detection antibody (anti-human IL-23 Biotin, 250X), 48 µl of detection antibody was added to 12 ml of 1X ELISA/ELISPOT diluent. The plates were washed again for 3-5 times with wash buffer. Next, 100 µl of Avidin-HRP was added and incubated for another 30 min at room temperature. To prepare Avidin-HRP (250X), 48 µl of Avidin-HRP was added to 12 ml of 1X ELISA/ELISPOT diluent. The plates were further washed for 3-5 times with wash buffer and then 100 µl of 1X Tetramethylbenzidine (TMB) solution was added and incubated at room temperature for 15 min. Lastly, 50 µl of stop solution (H_2SO_4, 2N) was added to each well. Signal was detected at 450nm wave length with a FLUO star OPTIMA device.

ELISA for IL-1β

ELISA was performed with human IL-1β ELISA Kit (R&D system). The capture antibody was prepared (4 µg/ml) by adding 83 µl of antibody in 10 ml of PBS. 100 µl of capture antibody was then added to 96-well plate and incubated at 2-8°C for overnight. On day 2, the plate was washed for 3 times with wash buffer (BioTek 250µl/well). After washing, 300 µl of blocker was prepared with PBS + 1% BSA, added to each well and incubated for 1 h at room temperature. The plate was washed again for 3-5 times with wash buffer. Next, the moDC cell culture supernatants were thawed and diluted to different concentration by adding PBS + 1% BSA. After diluting the samples according to the dilution list, add 100 µL of diluted samples to each well.

Table 8. Sample dilution list for IL-1β ELISA experiments

Chemicals	Dilution factor	Sample (µl/well)	1x ELISA ELISPOT (µl/well)	Total Volume (µl/well)
Zymosan d	1:5	20	80	100
Mala s13	N/A	100	0	100
WGPd	1:10	10	90	100
hTrx	1:2	40	80	100

Prepare standards (human IL-1β recombinant protein, 1 µg/ml, 7 points):

4.5 µl of standard was added and mixed to 2 ml of PBS + 1% BSA to make first point of standard (250 pg/ml) in a 15 ml conical tubes. Serial dilutions were made as following concentration: 250, 125, 62.5, 31.25, 15.6, and 7.8 pg/ml. Next, the samples and the standards were incubated at room temperature for 2 h or at 4°C overnight. The plates were then washed for 3-5 times with wash buffer. After washing, 100 µl of detection antibody which was diluted in PBS + 1% BSA was added to each well and incubated for 2 h at room temperature. To prepare detection antibody (anti-human IL-1β, 200 ng/ml), 167 µl of detection antibody was added to 10 ml of PBS + 1% BSA. The plates were again washed for 3-5 times with wash buffer. Next, 100 µl of Sterptavidin-HRP was added to each well and incubated for another 20 mins at room temperature. To prepare Sterptavidin-HRP (40X), 256 µl of Sterptavidin-HRP was diluted to 10 ml of PBS + 1% BSA. The plates were further washed for 3-5 times with wash buffer. 100 µl of 1:1 mixture of color reagent A and color reagent B was then added to each well. Lastly, 50 µl of stop solution (H_2SO_4, 2N) was added to each well. Signal was detected at 450nm wave length with a FLUO star OPTIMA device.

Flow Cytometry

For all FACS experiments, a BD FACSCanto ll was used for flow cytometric experiments and data were analyzed with BD FACSDiva 8.0.1 software.

RNA Isolation, cDNA generation and real-time quantitative PCR (qPCR)

The qPCR was performed with microRNA kit (analytikjena #innuPREP microRNA Kit). Cell lysis was prepared in advance and frozen at -80°C. Right before performing qPCR, the samples were thawed at room temperature and re-suspended completely. To remove genomic DNA (gDNA), cell supernatant prepared from last step was applied to filter D with receiver tube and centrifuged at 12000 rpm for 2 min at 4°C. After centrifugation, spin filter D was removed and 400 µl SRB was added to the receiver tube. The spin filter R was then transferred to a new receiver tube, sample was applied to the spin filter R and centrifuged at 12000 rpm for 2 mins at 4°C. After centrifugation, the receiver tube was discarded and the membrane was washed with 500 µl of HS and centrifuged again at 12000 rpm for 1 min at 4°C, repeat once more with 750 µl of HS. The sample was centrifuged again at 12000 rpm for 3 min at 4°C to remove the ethanol and dry the membrane. The spin filter R was combined with elution tube to elute DNA, 50 µl RNase-free water was added and incubated for 1 min at room temperature, and then the tube was centrifuge at 8000 rpm for 1 min at 4°C. To quantify the RNA concentration, 0.5-1 µl of sample was transferred to Nanodrop machine measured by Nanodrop software. After quantification, cDNA was generated by RT. Note: before cDNA generation, the following materials need to be prepared: gDNA Wipeout Buffer, 7x for 2 µl and Template RNA (up to 1 µg) and RNase-free water, the total volume is 14 µl. The samples were then Incubated for 2 min at 42°C to remove the gDNA and

then placed immediately on ice. 1 µl of Quantiscript Reverse Transcriptase and 4 µl of Quantiscript RT Buffer were mixed and added the entire genomic DNA from last step (14 µl), to a total volume of 20 µl. The sample was incubated for 15 min at 42°C and then incubated for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase. 2 µl of sample cDNA, 1 µl of Primer (IL-23/GAPDH), 5 µl of SYBR Green/Polymerase MasterMix and 1 µl of RNase-free water were mixed and added to a 96-well PCR plate. After adding all samples, the plate was centrifuged at 1300 rpm for 1 min at 4°C. Apply samples to LightCycler 480 Software. The program for running qPCR was:

(1) Pre-incubation: 95°C, 5 min

(2) Amplification: 45 cycles (95°C for 10 sec, 55°C for 10 sec, 72°C for 10 sec)

(3) Melting curve: 95°C for 5 sec, and then 65°C for 1 min

(4) Cooling: cool down the reaction to 4°C

The primer used for quantification of IL-23 transcript was Hs_IL23A_1_SG QuantiTect Primer (QIAGEN, #QT00204078), and for the housekeeping gene GAPDH Hs_GAPDH_2_SG QuantiTect Primer (QIAGEN, #QT01192646).

Statistics

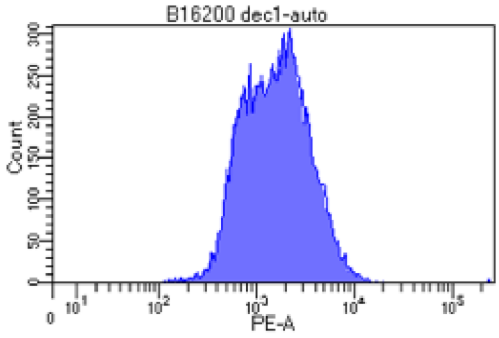
Statistical analysis was performed by using GraphPad Prism 6. Comparison between two groups was made by two-tailed unpaired Student's t-test. For comparison of three or more than two groups, one-way analysis of variance (ANOVA) was applied. Regarding the boxplot, the Box resembles the 25% and 75% percentiles and that the whiskers represent the minimum and maximum values. Statistical significance level (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$) is indicated in the figure legend.

Results

Investigation of cytokine secretion by moDCs

First, the differential expression of Dectin-1 and Dectin-2 on MoDCs was studied. As shown in Fig. 2, both Dectin-1 and Dectin-2 displayed robust expression on human moDC, while expression strength varied among patients.

A



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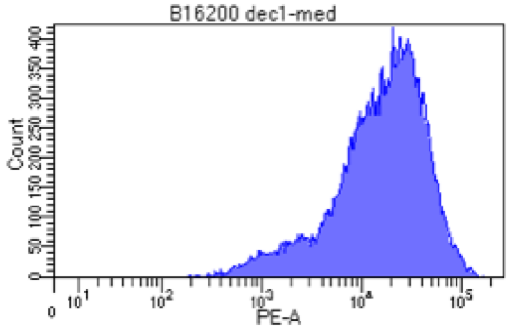

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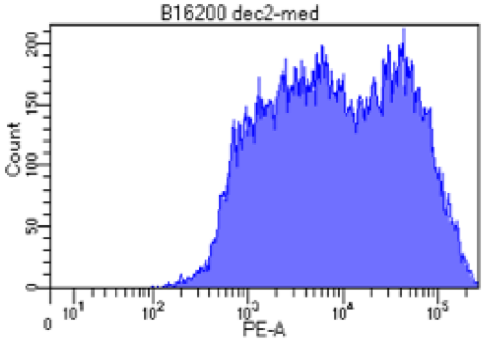


Figure 2. Expression of Dectin- 1 and Dectin-2 on human moDC. (A) no antibody staining. (B) staining with PE-labelled Dectin-1, and (C) with PE-labelled Dectin-2 antibody. Exemplary results of three experiments are depicted.

Next, cytokine secretion was studied after Dectin-1 and -2 stimulation. IL-1β secretion was increased after treatment with WGPd, a 1.3/ 1.6-β-glucan preparation. This up-regulation was decreased after pre-incubation with WGPs, a known Dectin-1 blocker (Fig. 3).

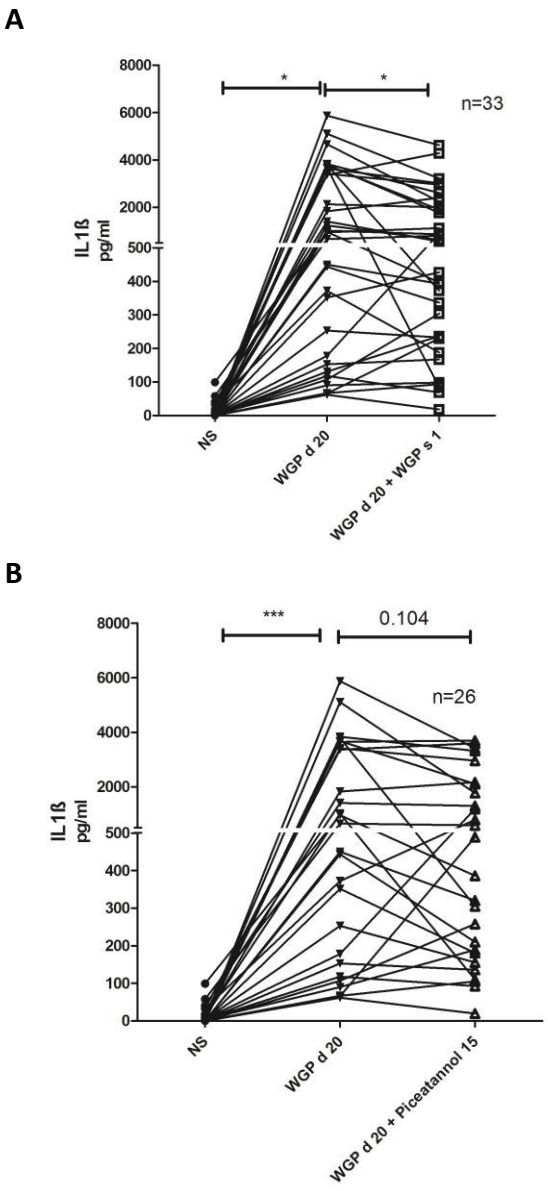
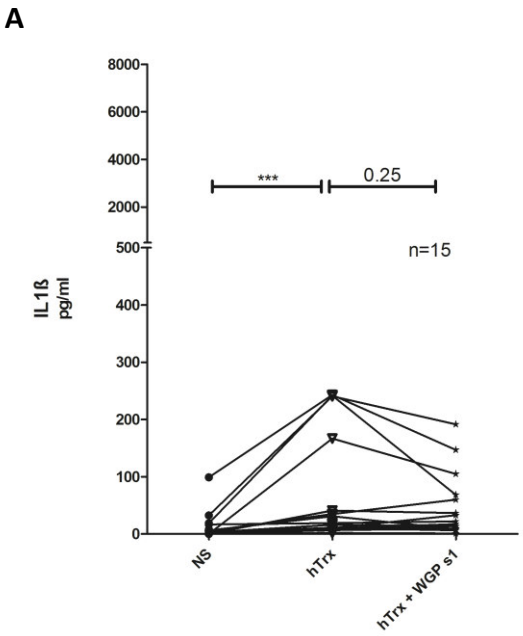


Figure 3. IL-1β secretion from human moDCs after stimulation with the Dectin-1 ligand WGPd stimulation resp. blockade with the Dectin-1 blocker WGPs and with the Syk blocker Piceatannol. (A) IL-1β secretion detected by ELISA after stimulation of moDCs with 20 µg/ml WGPd and 1 ng/ml WGPs. (B) IL-1β secretion after stimulation of moDCs with 20 µg/ml WGPd and 15 µg/ml Piceatannol. ***, $p < 0.001$, *, $p < 0.05$

However, IL-1β levels did not change when treated moDCs were with Piceatannol (3, 3', 4, 5'-trans-trihydroxystilbene), a natural analogue compound of resveratrol. Interestingly, when moDCs were treated with hTrx, the secretion of IL-1β could be inhibited by Piceatannol (Fig. 4), which means that hTrx might affect IL-1β production through the Syk signaling pathway.



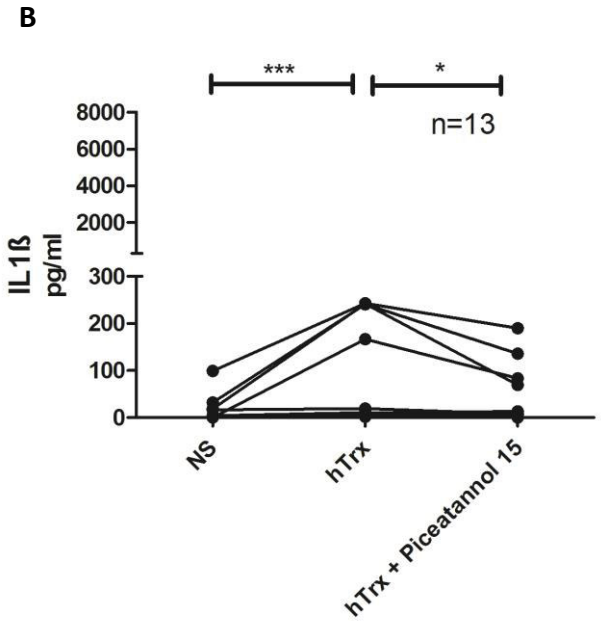
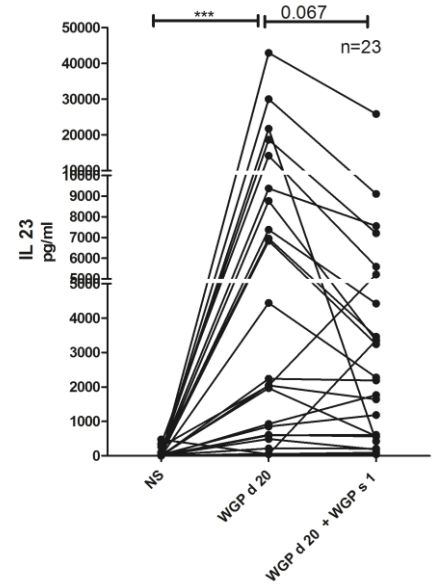


Figure 4. IL-1β secretion from human moDCs after stimulation hTrx resp. blockade with the Syk blocker Piceatannol. (A) IL-1β secretion detected by ELISA after treatment of moDCs with 2.5 µg/ml hTrx and 1 ng/ml WGPs. (B) IL-1β secretion detected by ELISA after treatment of moDCs with 2.5 µg/ml hTrx and 15 µg/ml Piceatannol. ***, $p < 0.001$; *, $p < 0.05$.

Different from IL-1β, IL-23 was increased from moDCs after treatment with WGPd and was blocked by Piceatannol (Fig. 5B) and WGPs by trend (Fig. 5A).

A



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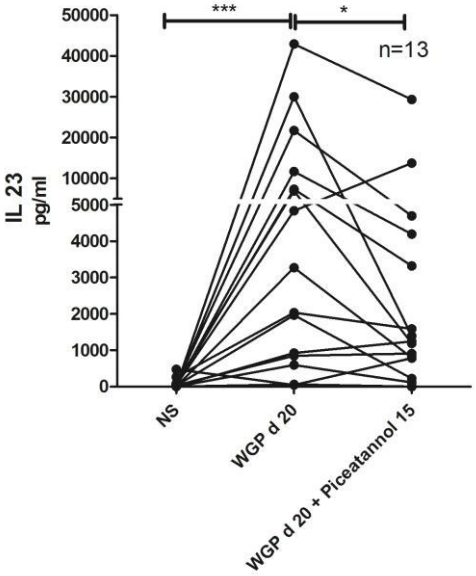


Figure 5. IL-23 secretion from human moDCs after stimulation with Dectin-1 ligand WGPd resp. blockade with the Dectin-1 blocker WGPs or the Syk blocker Piceatannol. (A) IL-23 secretion detected by ELISA after stimulation with 20 µg/ml WGPd and 1 ng/ml WGPs. (B) IL-23 secretion after stimulation with 20 µg/ml WGPd and 15 µg/ml Piceatannol. ***, $p < 0.001$; *, $p < 0.05$.

This indicates that WGPd may induce IL-23 production through Syk signaling pathway. When moDCs were treated with hTrx the IL-23 secretion could be inhibited by both WGPs (Fig. 6A) and Piceatannol (Fig. 6B), which means that hTrx might also affect IL-23 production through the Syk signaling pathway.

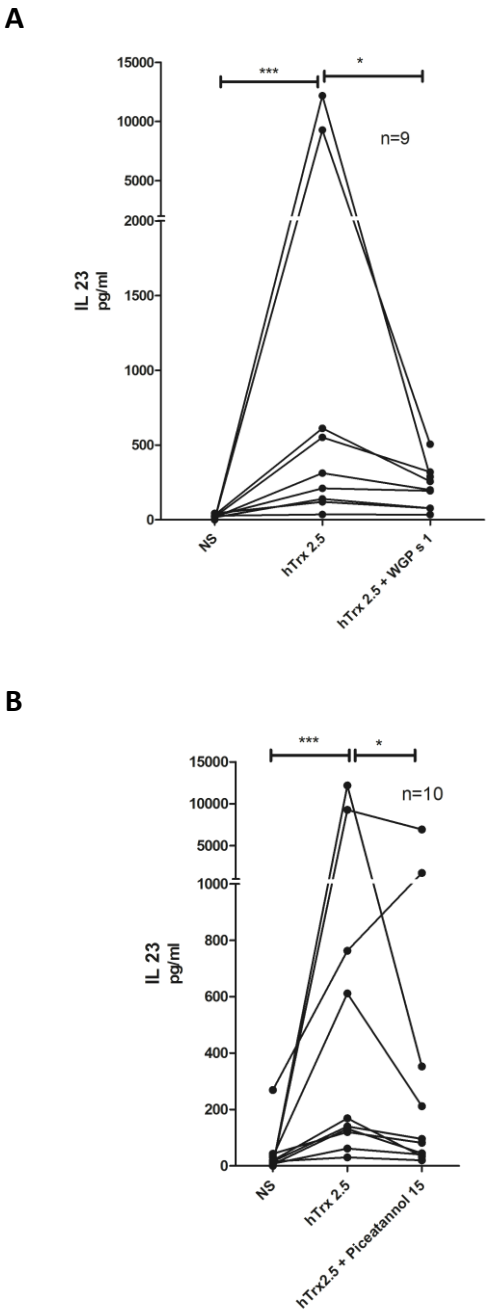


Figure 6. IL-23 secretion from human moDCs after stimulation with Dectin-1 ligand hTrx resp. blockade with the Dectin-1 blocker WGPs or the Syk blocker Piceatannol.

(A) IL-23 secretion after stimulation of moDCs with 2.5 µg/ml hTrx and 1 ng/ml WGPs. (B) IL-23 secretion after stimulation of moDCs with 2.5 µg/ml hTrx and 15 µg/ml Piceatannol. ***, $p < 0.005$; *, $p < 0.05$.

Investigation of Dectin-1 and Dectin-2 expression on moDCs

To further study the relationship between Dectin-1 and Dectin-2, moDCs were treated with two stimuli: Zymosan d and WGPd. Both stimuli are Dectin-1 specific ligands. After treatment of moDCs with Zymosan d and WGPd, Dectin-1 expression decreased indicating that an internalization process was induced (Fig. 7A, 7C). In contrast, the expression of Dectin-2 increased by trend after Zymosan d and WGPd treatment (Fig. 7B, 7D).

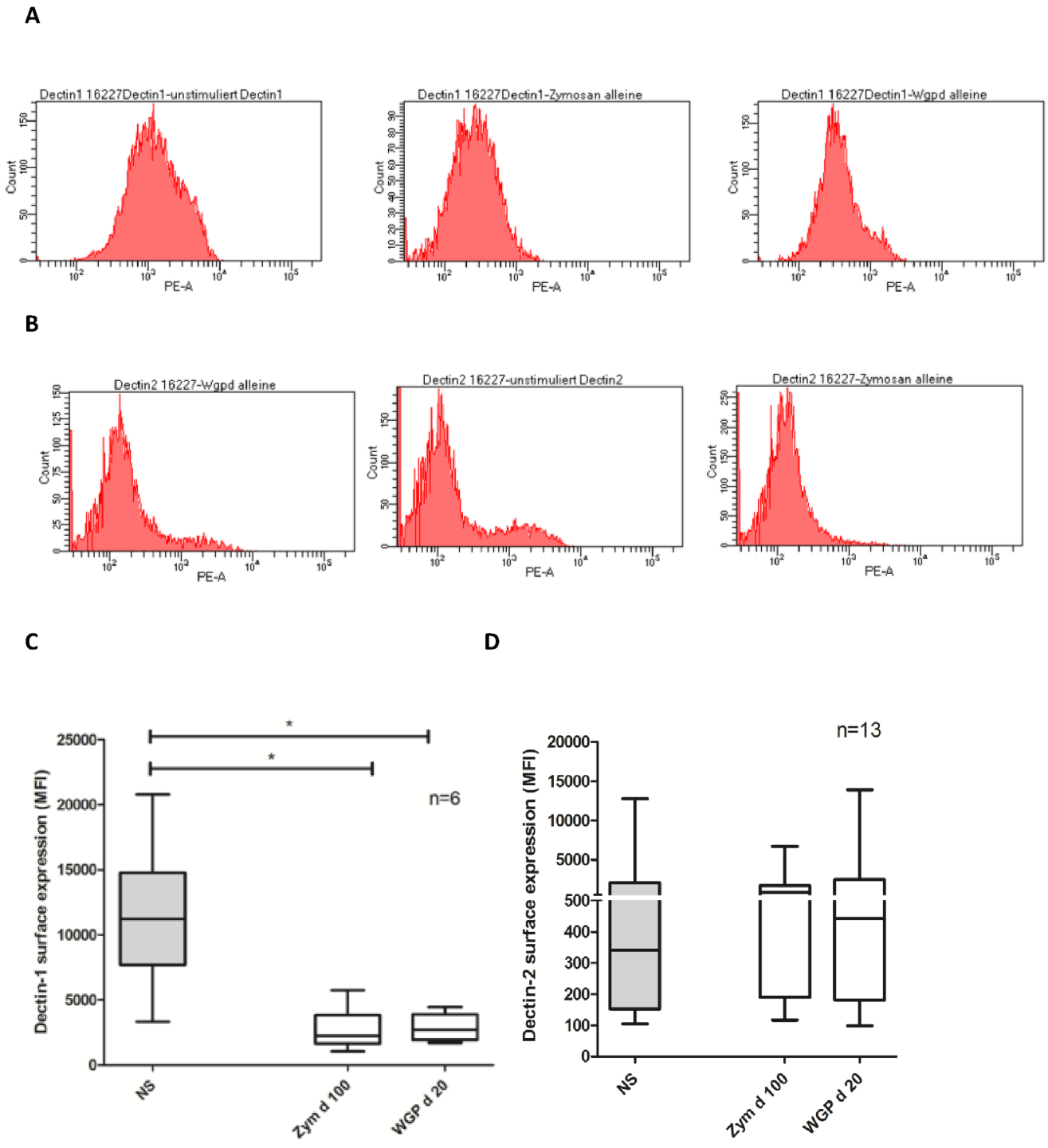


Figure 7. Dectin-1 and Dectin-2 expression on human moDC detected by flow cytometry after stimulation of the cells with Zymosan d or WGPd. (A)(C) Dectin-1 and (B)(D) Dectin-2 expression on moDCs when treated with the Dectin-1 stimuli Zymosan d (100 µg/ml) or 20 µg/ml WGPd. *, $p < 0.05$.

Interestingly, neither the internalization of Dectin-1 nor the upregulation of Dectin-2 could be significantly blocked by WGPs (Fig. 8).

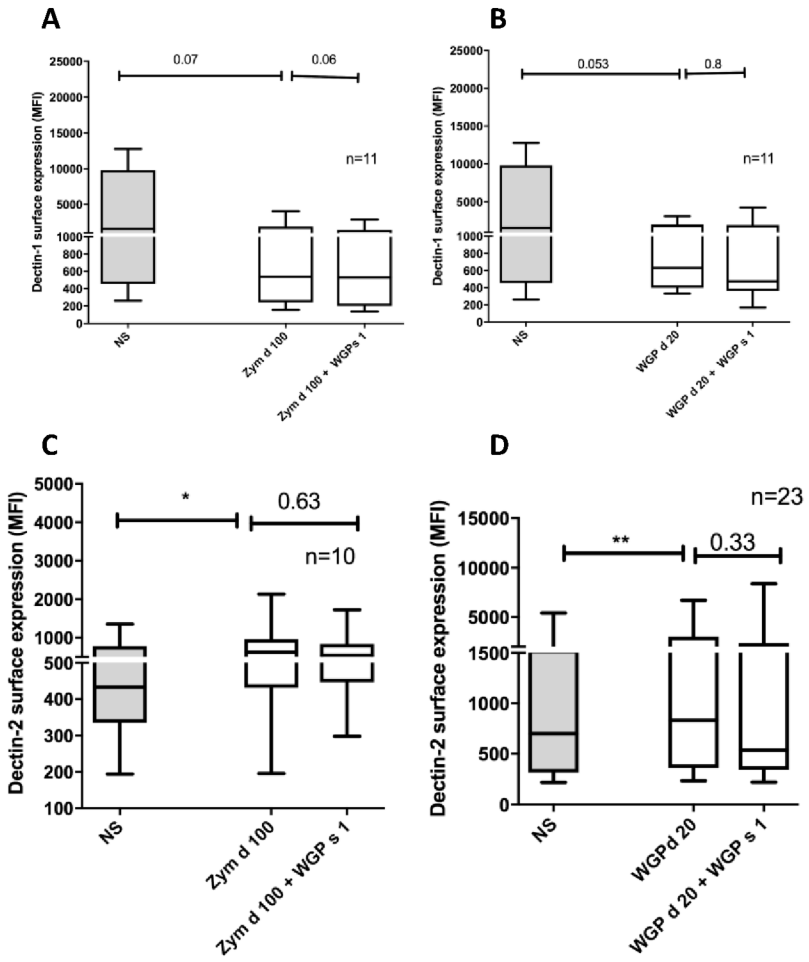
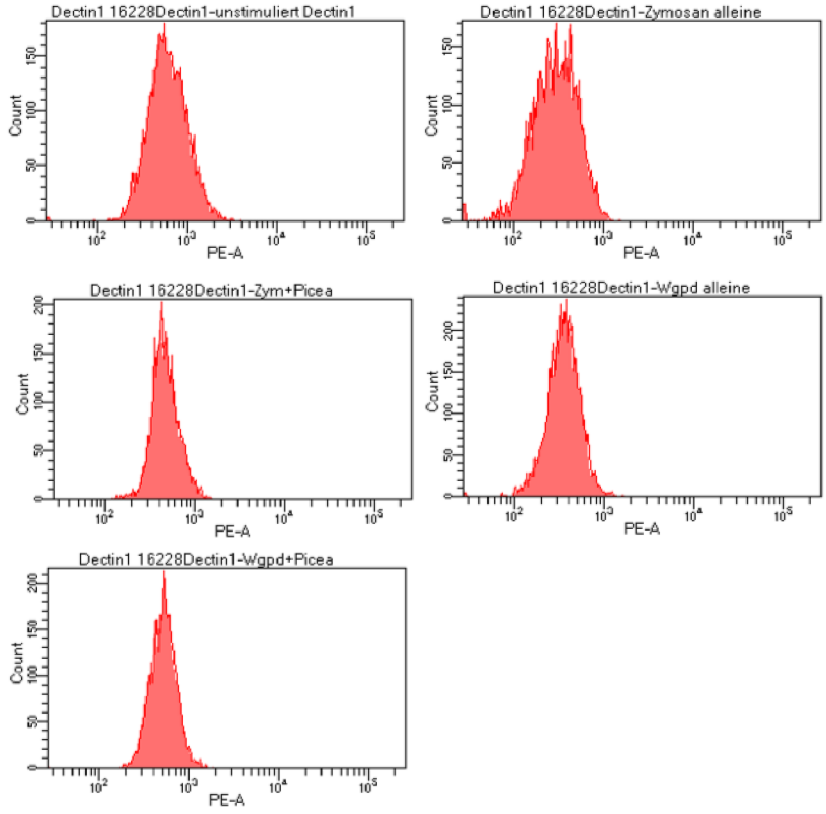


Figure 8. Dectin-1/-2 expression on human moDCs treated with different Dectin-1 stimuli and blockers. (A) Dectin-1 expression on moDCs treated with 100 µg/ml Zymosan d and 1 ng/ml WGPs. (B) Dectin-1 expression on moDCs treated with 20 µg/ml WGPd and 1 ng/ml WGPs. (C) Dectin-2 expression on moDCs treated with 100 µg/ml Zymosan d and 1 ng/ml WGPs. (D) Dectin-2 expression on moDCs treated with 20 µg/ml WGPd and 1 ng/ml WGPs. NS (Grey bar): non-stimulated, white bar: moDC treated with stimuli and blockers. **, $p < 0.01$; *, $p < 0.05$.

Previous studies showed that both Dectin-1 and Dectin-2 activate the Syk signaling

pathway. Here it was studied if blocking of Syk had effects on Dectin-1 internalization and/or Dectin-2 upregulation. Interestingly, Dectin-1 internalization but not Dectin-2 upregulation could be blocked by the Syk blocker Piceatannol (Fig. 9).

A



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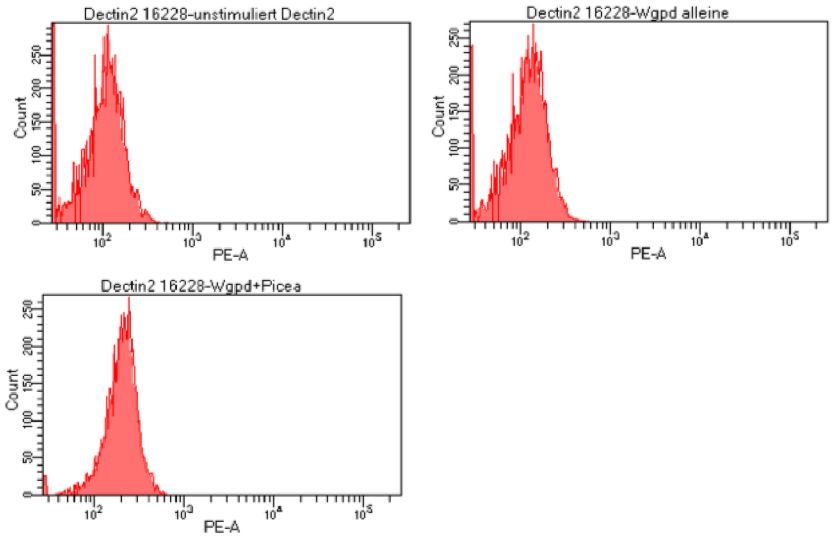


C **D**

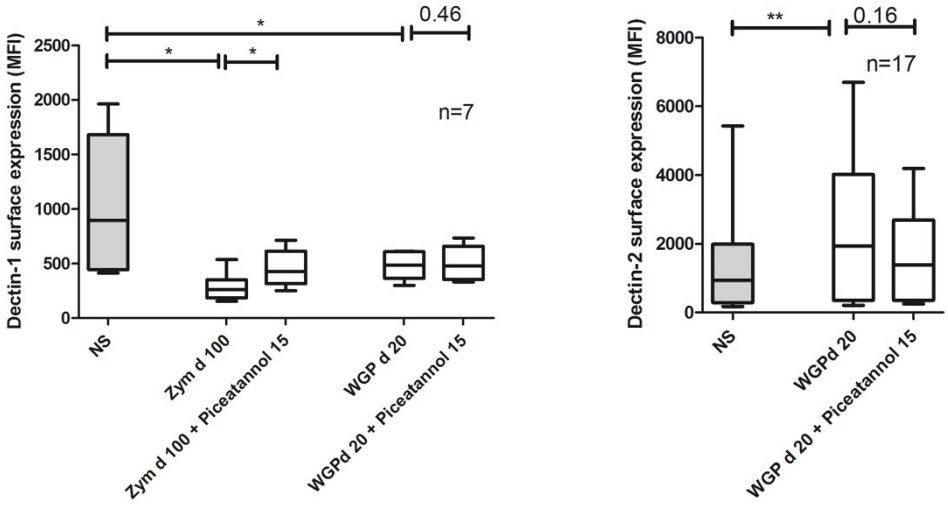


Figure 9. Dectin-1/-2 expression on human moDCs treated with different stimuli and Syk inhibitor. (A)(C) Dectin-1 expression on moDCs treated with different concentration (indicated as value, µg/ml) of stimuli Zymosan d, WGPd and Syk inhibitor Piceatannol. (B)(D) Dectin-2 expression on moDCs treated with stimuli WGPd and Syk inhibitor Piceatannol. NS (Grey bar): non-stimulated, white bar: moDC treated with stimuli and blockers. **, $p < 0.01$; *, $p < 0.05$.

As mentioned above hTrx is a known Dectin-1 specific ligand, however, its role on Dectin-2 expression remained unclear. Here it was shown that Dectin-2 expression was also up-regulated on moDCs after treating these cells with hTrx (Fig. 10A). This effect could be blocked by Piceatannol (Fig. 10B). To date, it is the first time showing that hTrx affects not only Dectin-1 but also Dectin-2 expression on moDCs.

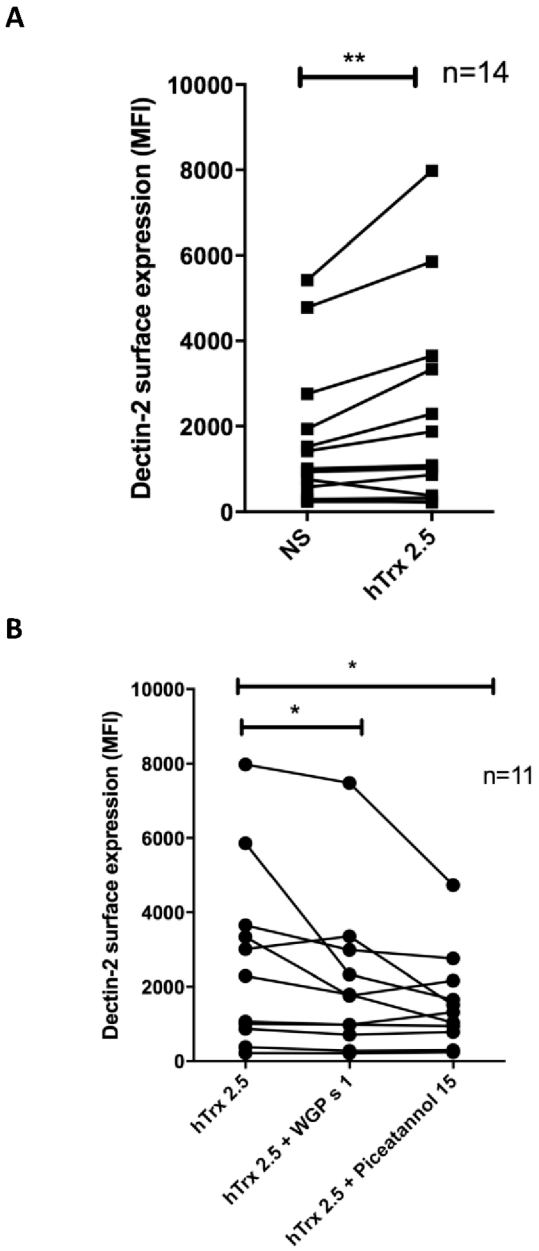


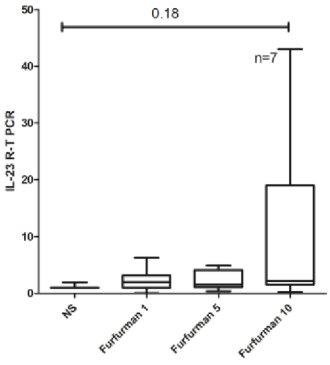
Figure 10. Dectin-2 expression on human moDCs treated with hTrx and different blockers. (A) Dectin-2 expression detected by flow cytometry on moDCs treated with 2.5 µg/ml hTrx. (B) Dectin-2 expression detected flow cytometry on moDCs treated with 1 ng/ml WGPs and 15 µg/ml Piceatannol. MFI: Mean Fluorescence Intensity. ***, $p < 0.005$; *, $p < 0.05$.

Investigation of a signaling pathway between Dectin-1 and Dectin-2

To further study the signaling pathway between Dectin-1 and -2, moDCs were first

treated with the Dectin-1 stimuli Zymosan d, WGPd or hTrx for 48 h to induce higher Dectin-2 levels. The cells were then treated with Furfurman, a specific Dectin-2 ligand, for another 24 h to investigate if this substance can further augment the Dectin-1-induced response. First it was examined if the IL-23 mRNA expression level changed after treatment with this series of stimuli and blockers. In summary, no clear change was observable on mRNA level by qPCR (Fig. 11, Fig. 12).

A



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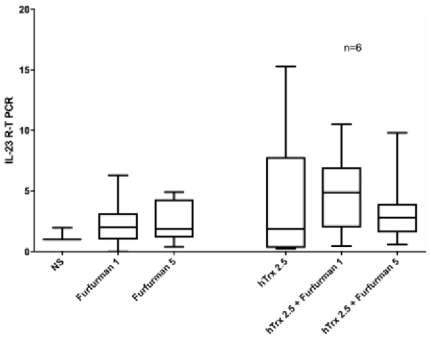

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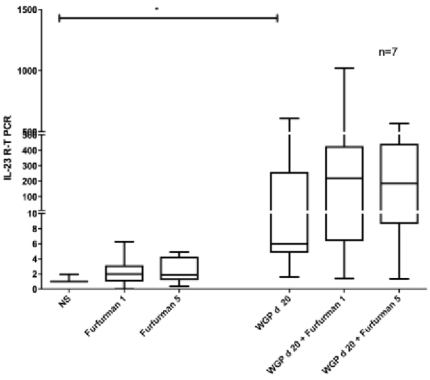


Figure 11. IL-23 mRNA expression detected by qPCR in human moDCs treated first with hTrx or WGPd, respectively, and subsequently with Furfurman. (A) IL-23 mRNA expression in moDCs treated with different concentrations of Furfurman (indicated as value for 1 resp. 5 µg/ml). (B) IL-23 mRNA expression in moDCs treated with hTrx for 48 h first and subsequently with different concentrations of Furfurman for another 24 h. (C) IL-23 mRNA expression in moDCs treated with WGPd for 48 h first and subsequently with different concentrations of Furfurman for another 24 h. *, $p < 0.05$.

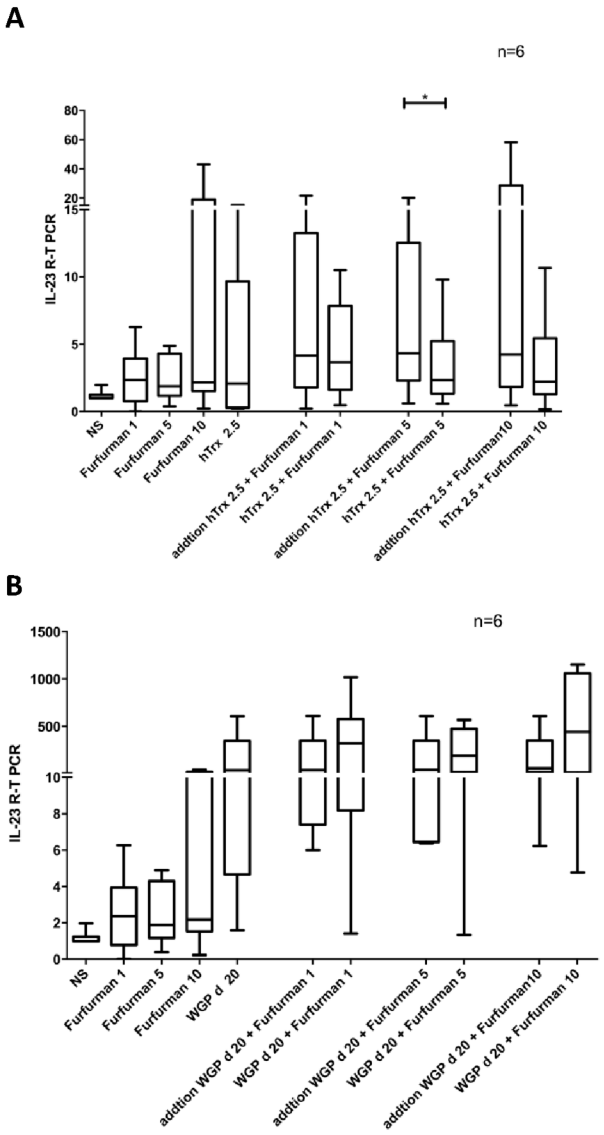
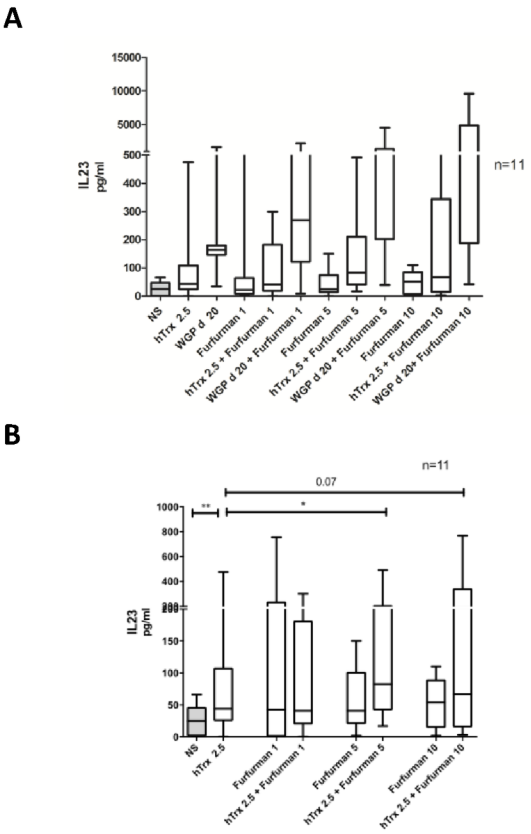


Figure 12. Synergistic effect of WGPd and Furfurman on IL-23 mRNA expression. (A) IL-23 mRNA expression detected by qPCR after stimulation with hTrx only (2.5 µg/ml)

or Furfurman treatment after 48 h of initial hTrx stimulation. (B) IL-23 mRNA expression detected by qPCR after stimulation with WGPd only (20 μg/ml) or Furfurman treatment after 48 h of initial WGPd stimulation. In groups labelled with "addition", Furfurman was applied in parallel with hTrx (A) or WGPd (B). *, $p < 0.05$.

However, a significant difference in IL-23 protein expression was observed by ELISA. As shown in Fig. 13, IL-23 expression was up-regulated when moDCs were treated with Furfurman only, and this up-regulation even increased more if Furfurman treatment was preceded by WGPd or hTrx treatment.



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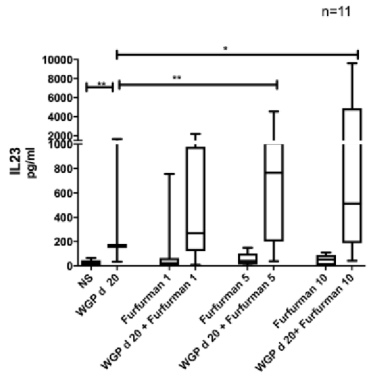


Figure 13. IL-23 protein secretion from human moDCs treated first with hTrx or WGPd, respectively, and subsequently with Furfurman. (A) IL-23 protein secretion from moDs detected by ELISA after first treated with different concentrations of (B) hTrx or (C) WGPd for 48 h, and subsequently with different concentrations of Furfurman for another 24 h (indicated as value for 1 resp. 5 µg/ml). The variation between samples were large, and therefore, the data from (A) are depicted again separately into (B) and (C) with either only hTrx or WGPd and Furfurman to enhance visibility. NS (Grey bar): non-stimulated, white bar: moDC treated with stimuli. **, $p < 0.01$; *, $p < 0.05$.

However, only WGPd but not hTrx had a synergistic effect with Furfurman on IL-23 secretion (Fig. 14). In summary, the data above showed that the Dectin-2 ligand could further augment the Dectin-1-induced response, which demonstrates the interconnection between Dectin-1 and -2 functions.

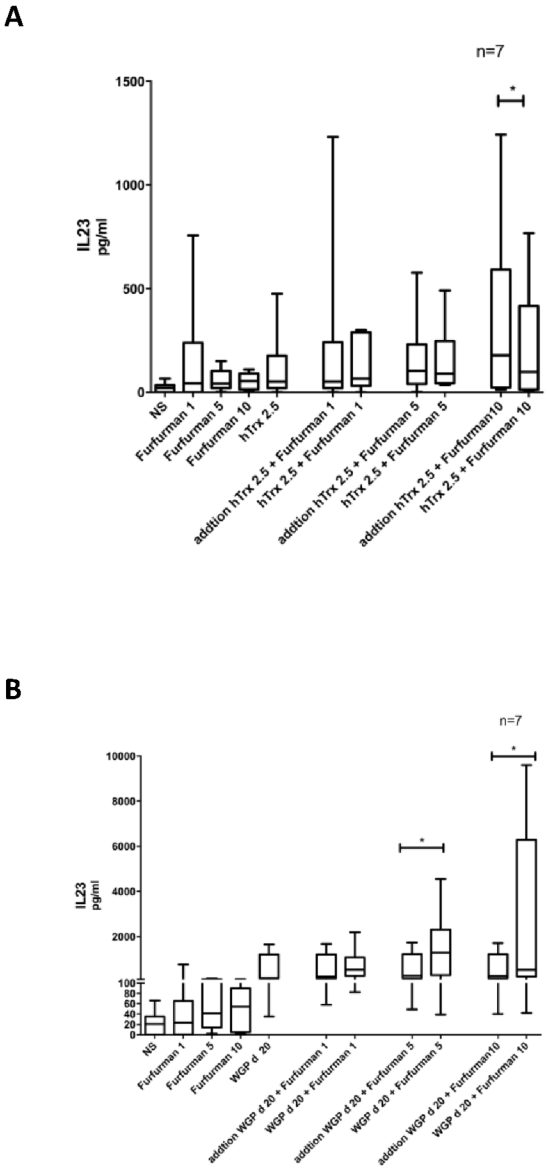


Figure 14. Synergistic effect of WGPd and Furfurman on IL-23 protein expression. (A) IL-23 protein secretion detected by ELISA after stimulation of human moDCs with hTrx only (2.5 µg/ml) or Furfurman treatment after 48 h of initial hTrx stimulation. (B) IL-23 protein secretion detected by ELISA after stimulation of human moDCs with WGPd only (20 µg/ml) or Furfurman treatment after 48 h of initial WGPd stimulation. In groups labelled with "addition", Furfurman was applied in parallel with hTrx (A) or WGPd (B). *, $p < 0.05$.

Regarding the *in vitro* differentiation of monocytes, it is known that Dectin-1 and Dectin-2 expression increases during the differentiation of monocytes to macrophage M1 or M2 polarization[28, 29]. Dectin-1 and Dectin-2 expression increased by trend after M1 polarization, but in M2 polarization, only Dectin-1 expression increased by trend (Fig. 15).

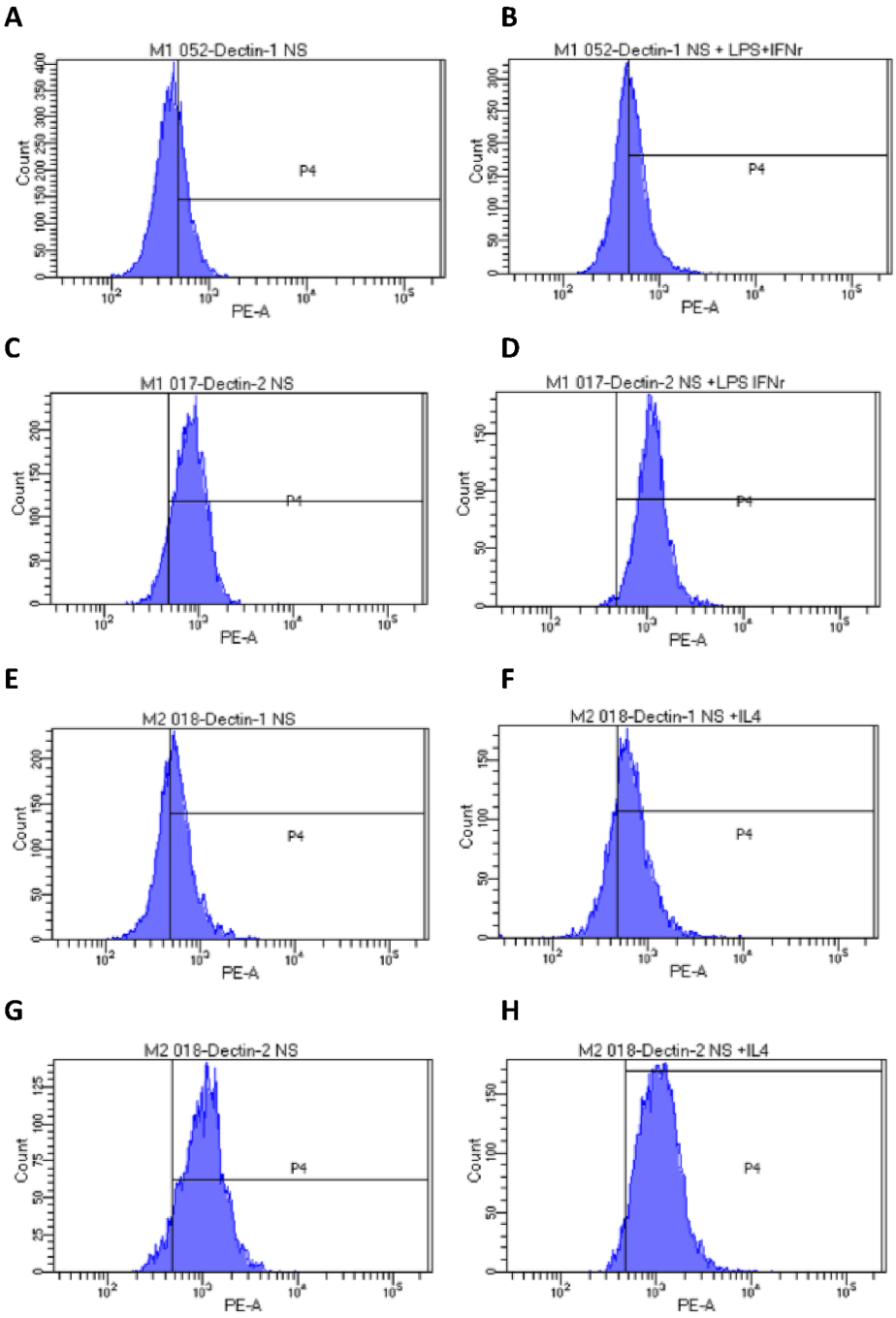
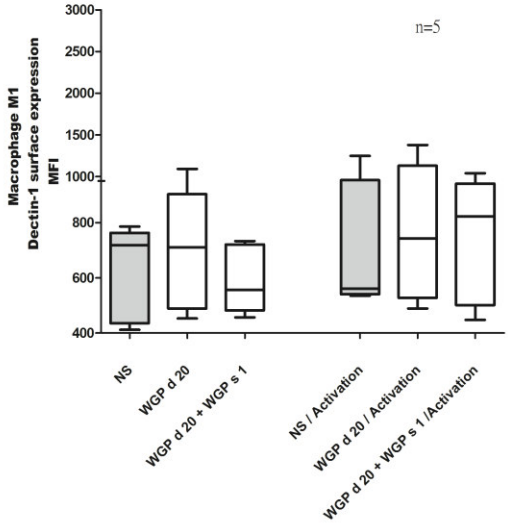


Figure 15. Dectin-1/-2 expression on human macrophages detected by flow cytometry before and after M1 and M2 polarization. (A) Dectin-1 expression on

monocytes before their polarization to M1 macrophage. (B) Dectin-1 expression on polarized M1 macrophages. (C) Dectin-2 expression on monocytes. (D) Dectin-2 on polarized M1 macrophages. (E) Dectin-1 expression before polarizing to M2 macrophage. (F) Dectin-1 expression after polarizing to M2 macrophage. (G) Dectin-2 expression before polarizing to M2 macrophage. (H) Dectin-2 expression after polarizing to M2 macrophage.

Next M1 and M2 macrophages were treated with the stimulus WGPd and the corresponding blocker WGPs. Both Dectin-1 and Dectin-2 expression were increased by trend after WGPd treatment (M1, Fig. 16 and M2, Fig. 17).

A



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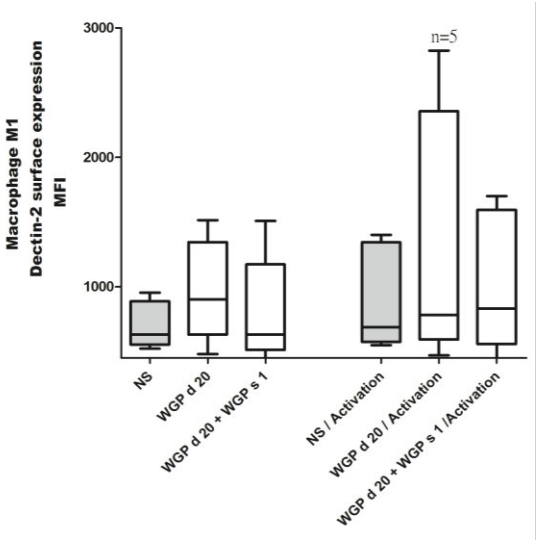
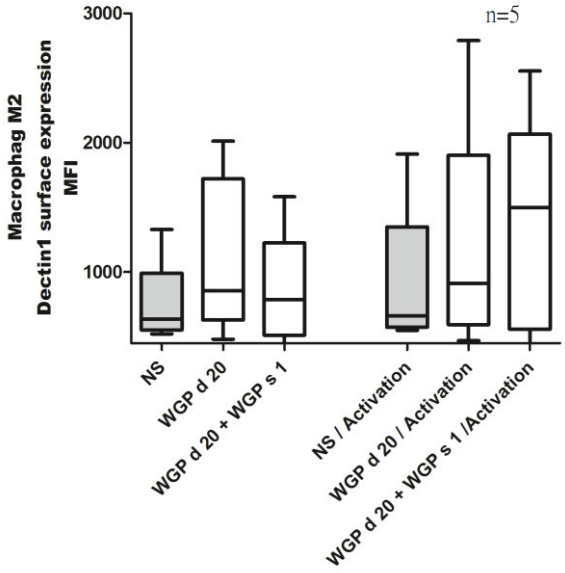


Figure 16. Dectin-1 (A) and Dectin-2 (B) expression on human macrophages treated with 20 µg/ml WGPd and 1 ng/ml WGPs. The Dectin expression was assayed by FACS analysis in macrophages before and after M1 polarization. NS (Grey bar): non-stimulated, white bar: human macrophage treated with stimuli. MFI: Mean Fluorescence Intensity.

A



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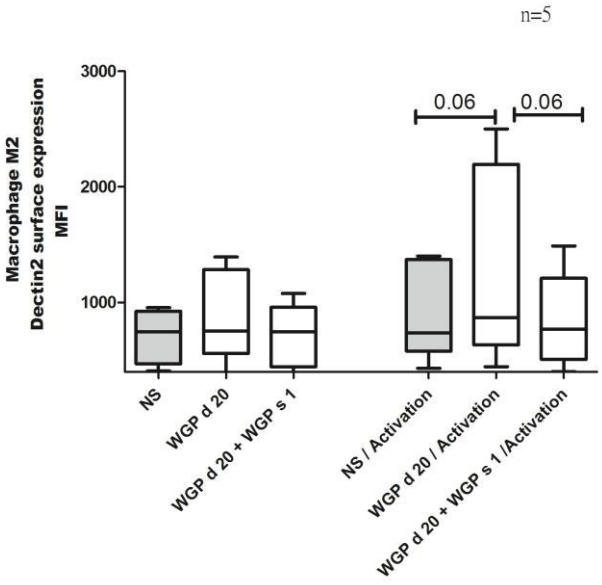

Figure 17. Dectin-1 (A) and Dectin-2 (B) expression on human macrophages treated with 20 µg/ml WGPd and 1 ng/ml WGPs. The Dectin expression was assayed by FACS analysis in macrophages before and after M2 polarization. NS (Grey bar): non-

stimulated, white bar: human macrophage treated with stimuli. MFI: Mean Fluorescence Intensity.

This increase was down-regulated by trend after treatment of the cells with WGPs. Surprisingly, when M2 macrophages were treated with hTrx, the expression of Dectin-1 was highly up-regulated (Fig. 18).

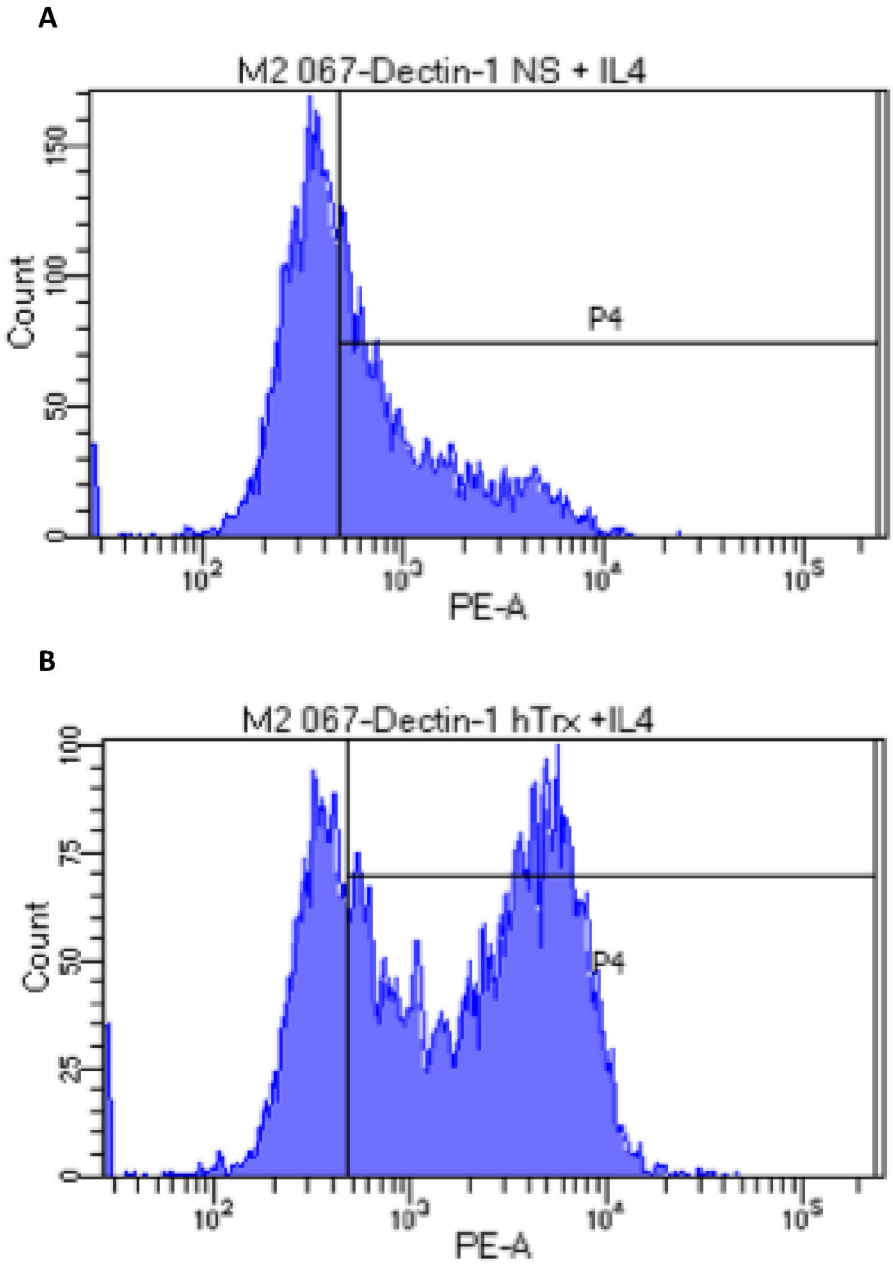

Figure 18. Dectin-1 expression was highly up-regulated by hTrx stimulation on M2 macrophages. (A) Dectin-1 expression on M2 macrophages without hTrx. (B) Dectin-

1 expression on M2 macrophages with 2.5 μg/ml hTrx treatment. The Dectin expression was assayed by FACS.

Discussion

Immune responses caused by fungi, including commensals, may play a role in inflammatory skin disorders[30]. AD is a common chronic skin disease, and symptomatic anti-inflammatory therapy is currently the major treatment option[31].

Malassezia spp. are part of the normal human skin flora but are also capable of inducing several human skin diseases[34]. In this study hTrx, a homologous protein to *Malassezia* sympodialis allergen Mala s13[35] was used to study the immune response of human myeloid cells. hTrx bears DAMP functions and is secreted upon stress from human cells. Interestingly, hTrx stimulation upregulated the IL-1β and IL-23 secretion from moDCs, and this up-regulation could be blocked by the Syk blocker Piceatannol, a natural anti-oxidant compound isolated from blueberry and red wine. Previous studies showed that Dectin-1, -2 and -3 induce ROS, IL-23 and IL-1β production through Syk and NF-kB signaling pathway. The data of this study demonstrate in addition that cytokine secretion can be regulated by hTrx and Piceatannol.

Recently, a monoclonal antibody named Dupilumab was approved as a new therapy option for AD. Dupilumab is an anti-IL-4 receptor alpha chain antibody which has the ability to suppress the TH_2 pathway. The therapeutic effect of Dupilumab was observed in patients with both intrinsic and extrinsic AD. The strong potential of this antibody may rely in the fact, that the IL-4 receptor alpha chain is part of the IL-4 receptor and as well the IL-13 receptor[32]. Although there is already drug for AD treatment, with more and more mechanism studies, new therapeutic target will for sure be discovered. The main idea of this study is to further understand the underlying

mechanism of Dectin-1/Dectin-2 and to develop a new target therapy for better treatment[33].

Previous studies in the skin research group of Hannover Medical School had revealed that hTrx is sensed by moDC via the C-type lectin receptor Dectin-1. In those experiments, a monoclonal antibody was applied to block the Dectin-1 receptor, resulting in reduced IL-1β and IL-23 levels[36]. In this work, it was intended to verify this effect by further blocking agents, namely WGPs and Piceatannol which is a complex shown to have several anti-proliferative effects and to inhibit Syk kinase, to study the cytokine secretion. Here, stimulation with WGPd led to secretion of IL-23 and IL-1β. While this effect could partially be blocked by WGPs, the latter also had a decreasing effect on hTrx cytokine induction (Fig. 3-6), underlining the binding capability of Dectin-1. It can therefore be concluded that blocking of Dectin-1 and Syk kinase may lead to reduced cytokine secretion. It was also observed that Piceatannol had better inhibitory effect than WGPs on IL-23 compared to IL-1β secretion, and the data in this study also indicate that the secretion of IL-1β might not come from Syk signaling pathway (Fig. 3B). Therefore, it can be concluded that IL-23 may be induced by signaling pathways different from those leading to IL-1β upregulation in response to Dectin receptor stimulation.

IL-23 gene transcription is believed to be directly induced after Dectin-1 stimulation, while IL-1β exists in a pre-form in the cytoplasm of the cell and needs to be enzymatically cleaved into its active form by caspase3. Caspase3 is activated via a complex called the inflammasome which can be formed upon Dectin-1 stimulation[37]. With regard to surface expression of C-type lectin receptors the well-described

internalization of Dectin-1 after specific stimulation could be confirmed here. Furthermore, a slight upregulation of Dectin-2 became apparent in parallel. Previous studies in the skin research group of Hannover Medical School demonstrated that hTrx is a specific ligand for Dectin-1 and hTrx-mediated Dectin-1 expression can be blocked by Dectin-1 blocker[32]. More precise, it was found that after treatment with specific Dectin-1 ligands and also hTrx, the expression of Dectin-2 increased on moDCs. Here it was demonstrated that in the case of hTrx, this effect could be blocked by a Dectin-1 blocker or a Syk blocker. Thus, these data show that there exists a relation between Dectin-1 and Dectin-2, and Dectin-2 expression can be up-regulated through Dectin-1 stimulation.

It was confirmed here that hTrx is a ligand for Dectin-1. With their C-type carbohydrate recognition domains (CRDs), CLRs are thought to recognize carbohydrate such as mannose and galactose[36,38,39]. Here it was shown that the protein ligand hTrx is recognized by Dectin-1 and induces downstream signaling pathway.

Due to the observed relation between Dectin-1 and -2, a specific Dectin-2 ligand (Furfurman), was used and a compound from the cell wall of *Malassezia* spp.[40], on Dectin-1-stimulated moDCs to study if the Dectin-1-mediated enhanced Dectin-2 surface expression leads to increased cytokine secretion. ELISA data indeed showed that combined WGPd and Furfurman treatment induces more IL-23 secretion than single treatments. However, the mRNA expression of IL-23 investigated by qPCR did not lead to statistically significant differences, which could on the one hand be due to in the small number of experiments. On the other hand, the process from mRNA to

protein is a complex biological reaction, including post-transcriptional, post-translational modification and protein degradation. For example, some mRNAs are relatively stable so even a small amount of mRNA can lead to large amount of protein. There might also exist a negative feedback regulation between IL-23 mRNA and protein via Dectin-1/-2 signaling pathway. Nevertheless, our data suggest an enhancement of the inflammatory processes within one cell that is stimulated via Dectin-1 if also Dectin-2 ligands are around. To our knowledge, this is the first report of a crosstalk between Dectin-1 and Dectin-2.

In the present study, we mostly focused on human moDCs, since moDCs highly express Dectin-1 and Dectin-2, and the related Syk signaling pathway. There are many pathways other than Syk pathway, for example those involving NF-kB, and this also needs to be studied well in the future.

Dectin-1 and Dectin-2 are highly expressed on moDCs, and Dectin 1 expression level is, however, higher than Dectin-2. Since Dectin-1 but not Dectin-2 was shown to be involved in an active internalization process future work should investigate the spatial expression pattern of both molecules to better understand the biology and availability of both receptors under different inflammatory conditions.

Polarized macrophages also play an important role in chronic skin diseases. Therefore Dectin-1/-2 expression was studied on macrophages here as well[41,42]. The data show that both Dectin-1/-2 were expressed strongly after macrophage polarization

compared to unpolarized, unactivated myeloid precursors. This was especially true for M2 macrophages, which matched the results of previous research[43].

In the present study, the sample size is in general big enough, we still observed that the variation between samples is too high which is one of the limitations in this research. Different population of moDCs and macrophages might be able to explain this phenomenon. A sorting step before drug treatment to sort our specific macrophage population with specific surface antigen marker might significantly reduce the variation between group.

In the future a stronger focus on the relation between Dectin-1/-2 and the newly discovered Dectin-3 should be laid instead of studying single Dectin receptors only. The development of Dectin blocker for clinical applications and novel drugs targeting Syk signaling pathway related to Dectin-1 induced cell activation might be a promising therapeutic concept for many skin diseases.

Summary

Atopic dermatitis (AD) is a chronic inflammatory skin disease. Multifactorial trigger factors have been identified, including microbial antigens, derived from for example from Malassezia spp. The pattern recognition receptors Dectin-1 and Dectin-2 are two of the most important receptors in fungus recognition and they are known to recognize also Malassezia. The research group of the Divison of Immunodermatology and Allergy Research in the MHH contributed to the finding that a human homologous protein to a Malassezia allergen, namely thioredoxin (hTrx), can lead to immunological IgE and T cell mediated autoreactivity in a subgroup of AD patients during the past years. They recently described that the cross-reactivity between Malassezia and hTrx also involves signals mediated via Dectin-1. In this thesis, this antigenic recognition was further studied and ELISA, flow cytometric and RT-PCR analysis were applied to get deeper insights into the connection between Dectin-1/-2 and hTrx. The results show that hTrx stimulates human monocytes via Dectin-1 in a Syk-dependent manner. Further, it was shown here that Dectin-2 expression can be strongly up-regulated via Dectin-1 stimulation, resulting in an increased responsiveness towards Dectin-2 ligands and subsequent secretion of pro-inflammatory cytokines. To date, this is the first study describing a cross-talk between Dectin-1 and Dectin-2. The findings of this thesis contribute to a better understanding of host – yeast interactions at interphases (skin, gut) of the human body. They may stimulate further translational studies in atopic dermatitis associated with a high frequency of clinically relevant colonization rates with yeasts (mainly Malassazia spp.) leading to crossreactions and autoimmune phenomena with an impact on the severity and chronicity of cutaneous symptoms in this skin disease.

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Abstract:

Human thioredoxin (hTrx), which can be secreted from cells upon stress, functions in allergic skin inflammation as a T cell antigen due to homology and cross-reactivity with the fungal allergen Mala s13 of the skin-colonizing yeast Malassezia sympodialis. Recent studies have shown that cell wall polysaccharides of Malassezia are detected by the immune system via the C-type lectin receptors Dectin-1 and Dectin-2, which are expressed on myeloid cells. Therefore, this study aimed to investigate a putative interaction between Dectin-1, Dectin-2 and the allergens Mala s13 and hTrx. Stimulation of human monocyte-derived dendritic cells or macrophages with Mala s13 or hTrx resulted in remarkable secretion of IL-1β and IL-23. Blocking experiments suggest that hTrx induces IL-23 by Dectin-1 binding and IL-1β by binding to either Dectin-1 or Dectin-2. Regarding Mala s13, Dectin-1 appears to be involved in IL-1β signaling. Interference of Syk kinase function was performed to investigate downstream signaling, which led to diminished hTrx responses. In our experiments, we observed rapid internalization of Mala s13 and hTrx upon cell contact and we were able to confirm direct interaction with Dectin-1 as well as Dectin-2 applying a fusion protein screening platform. We hypothesize that this cytokine response may result in a Th2/Th17-polarizing milieu, which may play a key role during the allergic sensitization in the skin, where allergen presentation to T cells is accompanied by microbial colonization and skin inflammation.

Muster der Erklärung nach §2 Abs. 2 Nr. 7 + 8

Ich erkläre, dass ich die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel

Microbial influences on allergic inflammation: Crosstalk of the C-type lectin receptors Dectin-1 and Dectin-2 and implications in allergy

im Institut/Krankenhaus / in der Klinik Dermatologie, Venerologie und Allergologie der Medizinischen Hochschule Hannover unter Betreuung von *Prof. Dr. med. T. A. Werfel* mit der Unterstützung von *Dr. rer. nat. L. M. Rösner* ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

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Ergebnisse der Dissertation wurden/werden in folgendem Publikationsorgan „**Scientific Reports**“ im Jahr 2019 veröffentlicht.

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(Unterschrift)