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The *Malassezia Sympodialis* Allergen Mala s 11 Induces Human Dendritic Cell Maturation, in Contrast to Its Human Homologue Manganese Superoxide Dismutase

Monica Vilhelmsson[a] Catharina Johansson[a] Gunilla Jacobsson-Ekman[a]
Reto Crameri[b] Arezou Zargari[a] Annika Scheynius[a]

[a]Karolinska Institutet, Department of Medicine, Clinical Allergy Research Unit, Stockholm, Sweden;
[b]Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

Key Words
Allergen · Dendritic cells · *Malassezia sympodialis* · Manganese superoxide dismutase

Abstract
Background: Recently, we identified a major *Malassezia sympodialis* allergen, Mala s 11, which displays a high degree of DNA sequence homology to human manganese superoxide dismutase (hMnSOD). In atopic eczema patients sensitized to *M. sympodialis*, hMnSOD can elicit eczematous reactions and positive skin prick tests, suggesting cross-reactivity to Mala s 11 based on molecular mimicry. The objective of the current study was to compare the influence of Mala s 11 and hMnSOD on human dendritic antigen-presenting cells. ***Methods:*** Monocyte-derived dendritic cells (MDDCs) from healthy blood donors were co-cultured with recombinant Mala s 11 (rMala s 11), recombinant hMnSOD (rhMnSOD), lipopolysaccharide or cultured in medium alone. Phenotypic changes were analysed using flow cytometry and allogeneic lymphocyte proliferation assays. Cytokine release into culture supernatants was investigated using cytometric bead array. ***Results:*** Whereas rhMnSOD did not affect the MDDC phenotype, rMala s 11 up-regulated the maturation marker CD83, the co-stimulatory molecules CD40, CD80, CD86 and HLA-DR to a similar extent as lipopolysaccharide. Furthermore, rMala s 11, but not rhMnSOD, induced significantly higher levels of TNF-α, IL-6, IL-8, IL-10 and IL-12p70 in the culture supernatants at 24 h in comparison with MDDCs cultured in medium alone. Finally, MDDCs pre-incubated with rMala s 11 induced a significantly higher proliferation of allogeneic CD14-depleted peripheral blood monocytes than MDDCs pre-incubated with rhMnSOD. ***Conclusion:*** Our results suggest that Mala s 11, but not hMnSOD, affects the immune response of healthy individuals through dendritic cell maturation and cytokine release. This indicates that dendritic cells possess the ability to distinguish between Mala s 11 and its human homologue MnSOD.

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Introduction

The chronic inflammatory skin disease atopic eczema (AE) has increased in prevalence in industrialized countries for the past 3 decades [1]. Although the pathogenesis is not fully understood, a genetic predisposition in combination with environmental factors such as microbes are

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Correspondence to: Dr. Monica Vilhelmsson
Department of Medicine, Clinical Allergy Research Unit
Karolinska University Hospital Solna, L2:04
SE–171 76 Stockholm (Sweden)
Tel. +46 8 517 766 96, Fax +46 8 33 57 24, E-Mail monica.vilhelmsson@ki.se

suggested to contribute to the symptoms [1]. IgE and T cell reactivity to the opportunistic yeast *Malassezia* has been observed in patients with AE, indicating that it is of importance as a trigger factor in the pathogenesis of AE [2, 3]. This lipophilic yeast is part of the normal human cutaneous flora [3]. Eleven different species have been identified [4], and *Malassezia sympodialis* is one of the most frequently isolated from the skin of AE patients and healthy controls [3, 5]. At present, 10 allergens from *M. sympodialis*, designated Mala s 1 and Mala s 5–13, have been identified [6].

The Mala s 11 allergen (22.4 kDa) is a major allergen with an IgE-binding frequency of 75% in sera from AE patients sensitized to *M. sympodialis* [7]. Interestingly, Mala s 11 has a high degree of sequence homology to manganese superoxide dismutase (MnSOD) from *Homo sapiens* (50%) and *Aspergillus fumigatus* (56%) [7]. It has been observed that MnSOD derived from *H. sapiens* is able to bind IgE in sera from asthmatic patients allergic to *A. fumigatus* [8]. In addition, a study comparing the crystal structures of human MnSOD (hMnSOD) to that of MnSOD derived from *A. fumigatus* showed patches of identical amino acids displayed to the surface of both enzymes, potentially constituting cross-reactive IgE-binding epitopes [8]. Humoral and cell-mediated cross-reactivity between these enzymes has been demonstrated [9, 10]. Recent data showing IgE antibodies to hMnSOD along with positive skin prick and atopy patch tests in AE patients sensitized to *M. sympodialis* suggest that cross-reactivity also occurs between Mala s 11 and hMnSOD in AE [11]. In this context, endogenous hMnSOD may maintain or even initiate the immune response through molecular mimicry as indicated by the ability of the human enzyme to induce eczematous reactions when used in atopy patch tests [11].

The most potent antigen-presenting cell for T lymphocyte stimulation is the dendritic cell (DC) [12]. Cutaneous DCs are key mediators in the pathogenic mechanisms of AE [13]. Immature DCs circulate the peripheral tissue and have the ability to take up antigens from the surroundings. After antigen uptake, immature DCs develop into a mature state. Upon maturation, DCs lose their ability to take up antigens but improve their capacity to present antigens by up-regulation of co-stimulatory and adhesion molecules along with MHC molecules. At the same time they are continuously on patrol to discriminate between self and non-self [14]. Depending on phenotype and cytokine release, DCs then have the ability to induce either tolerance or immunity [15]. In the present study, we therefore hypothesized that Mala s 11 and hMnSOD will differently affect DCs generated from healthy individuals. Our results show that recombinant Mala s 11 (rMala s 11), but not recombinant hMnSOD (rhMnSOD), induces DC maturation and the release of inflammatory cytokines. Furthermore, rMala s 11 enhances the capacity of DCs to induce the proliferation of allogeneic lymphocytes to a significantly higher extent than hMnSOD. These data suggest that DCs in healthy individuals can discriminate between Mala s 11 and hMnSOD, the highly homologous self antigen.

Material and Methods

Recombinant Proteins

cDNAs coding for Mala s 11 and hMnSOD proteins were amplified by PCR, subcloned into the expression vectors pET-17b (Novagen, San Diego, Calif., USA) and pQE30 (Qiagen, Hilden, Germany), respectively, verified by sequencing, and used to produce highly pure hexahistidine-tagged recombinant proteins as previously described [9]. The protein concentrations were determined by measuring the absorbance at 280 nm. The endotoxin concentrations in the used batches of rMala s 11 and rhMnSOD were 0.9 and 5 ng/mg of protein, respectively, as determined by the Limulus amoebocyte lysate assay according to the manufacturer's instructions (Charles River Endosafe Inc., Charleston, S.C., USA). This corresponds to endotoxin concentrations of 2.7 pg/ml (rMala s 11) and 15 pg/ml (rhMnSOD) in our monocyte-derived DC (MDDC) cultures. This level of endotoxin did not affect the release of cytokines or the expression of co-stimulatory or maturation markers by MDDCs, as demonstrated by control experiments in which 15 pg/ml of lipopolysaccharide (LPS; L8274, *Escherichia coli* serotype 026-B6; Sigma, Steinheim, Germany) was added to the MDDCs.

Generation of MDDCs from Peripheral Blood

Peripheral blood cell concentrates (buffy coats) were obtained from healthy blood donors from the Karolinska University Hospital Blood Bank, with approval from the local ethics committee. Plasma from each donor was analysed for total IgE with UniCAP1000® (Pharmacia Diagnostics, Uppsala, Sweden), IgE reactivity to 11 common airborne allergens with Phadiatop® (Pharmacia Diagnostics) and IgE against *M. sympodialis* (ATCC strain No. 42132) with ImmunoCAP m70 (Pharmacia Diagnostics). Only Phadiatop and ImmunoCAP m70 negative donors (IgE <0.35 kU/l) with normal total IgE levels (<122 kU/l) were included in the study. Immature MDDCs (iMDDCs) were generated from monocytes as previously described [16, 17]. In short, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). Monocytes were purified from PBMCs using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions and diluted to 4×10^5 cells/ml in complete culture medium; RPMIc [RPMI 1640 medium supplemented with 25 μg/ml gentamicin, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (GIBCO™; Invitrogen, Carlsbad, Calif., USA), 10% (v/v)

heat-inactivated fetal calf serum (Hyclone, Logan, Utah, USA) and 50 μM 2-β-mercaptoethanol (Sigma Chemical, St. Louis, Mo., USA)], with the addition of 10 ng/ml granulocyte macrophage colony-stimulating factor (Biosource, Camarillo, Calif., USA) and 800 U/ml of recombinant human IL-4 (Nordic Biosite, Täby, Sweden). The cells were cultured in 75- or 175-cm^2 culture flasks (Falcon; Becton Dickinson, Franklin Lakes, N.J., USA) at 37°C in a humidified incubator with 6% CO_2 in air. After 3 days, half the volume of RPMIc was replaced with freshly made RPMIc with the addition of 20 ng/ml granulocyte-macrophage colony-stimulating factor and 1,600 U/ml recombinant human IL-4. On day 6, the MDDCs were harvested by gentle resuspension and they exhibited a typical immature phenotype as demonstrated by flow cytometry [16, 17]. Only samples where at least 86% of the MDDCs expressed CD1a and a maximum of 1.4 and 13% expressed CD14 and CD83, respectively, were used in the experiments. The median viability of the MDDCs was 99% (range 94–100%, n = 9), as determined by trypan blue exclusion.

Culturing of MDDCs with rMala s 11 and rhMnSOD

On day 6 of culture, iMDDCs (4×10^5 cells/ml) were incubated in 5 ml RPMIc in 25-cm^2 culture flasks (Falcon) at 37°C in a humid condition with 6% CO_2 in air in the presence of 3 μg/ml of rMala s 11 or rhMnSOD. iMDDCs incubated with LPS (0.1 μg/ml) or cultured with RPMIc alone served as controls. Cells were harvested after 24 h and analysed for surface phenotype using flow cytometry or for allogeneic lymphocyte stimulation. Cell culture supernatants were collected after 4 and 24 h and stored at –20°C for later analysis of cytokine concentration.

Flow Cytometry

Approximately 10^5 cells were incubated for 30 min on ice with either FITC- or phycoerythrin-conjugated mouse monoclonal antibodies diluted in PBS. The following monoclonal antibodies were used: anti-CD1a phycoerythrin (T6-RD1; Beckman-Coulter, Fullerton, Calif., USA), anti-CD14 FITC (Leu-M3), anti-CD40 FITC (5C3), anti-CD80 FITC (L307.4), anti-CD83 FITC (HB15e), anti-CD86 FITC (2331 FUN-1) and anti-HLA-DR FITC (L243; Pharmingen/Becton Dickinson, San Jose, Calif., USA). Isotype-matched antibodies, mouse IgG_1 FITC or phycoerythrin conjugated, and mouse IgG_{2a} FITC conjugated (Becton Dickinson) were used as negative controls. Gates were set according to a characteristic forward scatter pattern [16, 17]. The flow cytometer was calibrated according to the manufacturer's instructions before each acquisition and a minimum of 10^4 cells were acquired on a FACSCalibur flow cytometer followed by analysis using the CellQuest software (Becton Dickinson). Only gated cells were considered when calculating the percentage of positively stained cells.

Cytokine Detection in Culture Supernatants

IL-8, IL-6, IL-10, IL-12p70, TNF-α and IL-1β were measured utilizing the cytometric bead array (Becton Dickinson) according to the manufacturer's protocol. The lower detection limit was 20 pg/ml for each cytokine measured.

Stimulation of Allogeneic Lymphocytes

For the lymphocyte proliferation assay, MDDCs were pre-incubated with rMala s 11 (3 μg/ml), rhMnSOD (3 μg/ml), LPS (0.1 μg/ml) or cultured with RPMIc alone for 24 h. Thereafter, MDDCs at different concentrations (10^2, 10^3 and 10^4 MDDCs per well) were co-cultured with 2×10^5 responder cells (frozen allogeneic CD14-depleted PBMCs obtained from a buffy coat) in 200 μl of RPMIc. The responder cells (2×10^5 per well) or MDDCs (10^4 cells per well) cultured in medium alone were used as a measure of the background proliferation. The cells were cultured in triplicates for 5 days at 37°C in 96-well round-bottomed plates (Falcon) in a humidified incubator with 6% CO_2 in air. One microcurie [^{3}H]thymidine (25 Ci/mmol; Amersham Pharmacia Biotech) per well was added for the last 18 h. [^{3}H]thymidine incorporation was determined by scintillation counting (Betaplate™; Wallac Oy, Turku, Finland). Results are expressed as stimulatory index calculated as counts per minute ($CD14^+$-depleted allogeneic PBMC co-cultured with 10^3 MDDCs pre-cultured with rMala s 11 or rhMnSOD) divided by counts per minute ($CD14^+$-depleted allogenic PBMC co-cultured with 10^3 MDDCs pre-cultured in medium alone).

Statistics

The expression of surface molecules and release of cytokines by MDDCs after incubation with rMala s 11, rhMnSOD, LPS or cultured in medium alone was compared using the non-parametric Friedman two-way analysis of variance (Statistica software; Statsoft Inc., Tulsa, Okla., USA) followed by Friedman multiple comparison tests [18]. Regarding the analysis of cytokine levels, in cases where the concentration of cytokine was below the limit of detection, the value of this limit (20 pg/ml) was used in the statistical calculations. The statistical difference between stimulatory index values for allogeneic PBMC stimulation with MDDCs pre-cultured with rMala s 11 or rhMnSOD was calculated with the Wilcoxon matched pairs test (Statistica software). $p < 0.05$ was considered statistically significant.

Results

Phenotype of MDDCs

The generated iMDDCs were co-cultured with rMala s 11, rhMnSOD, LPS or cultured in medium alone. After 24 h, the MDDCs stimulated with rMala s 11 displayed a mature phenotype with statistically significant higher expressions of CD40, CD80, CD83, CD86 and HLA-DR in comparison with MDDCs cultured in medium alone or co-cultured with rhMnSOD (fig. 1). The MDDCs stimulated with rMala s 11 had a phenotype similar to that of MDDCs stimulated with LPS (fig. 1). Co-culture with rhMnSOD did not induce any phenotypic change compared to the MDDCs cultured in medium alone (fig. 1).

Cytokine Release

To study the cytokine release from MDDCs co-cultured with rMala s 11, rhMnSOD, LPS or cultured in medium alone, cell culture supernatants were analysed at 4 and 24 h for TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 with cytometric bead array. Already after 4 h there was a

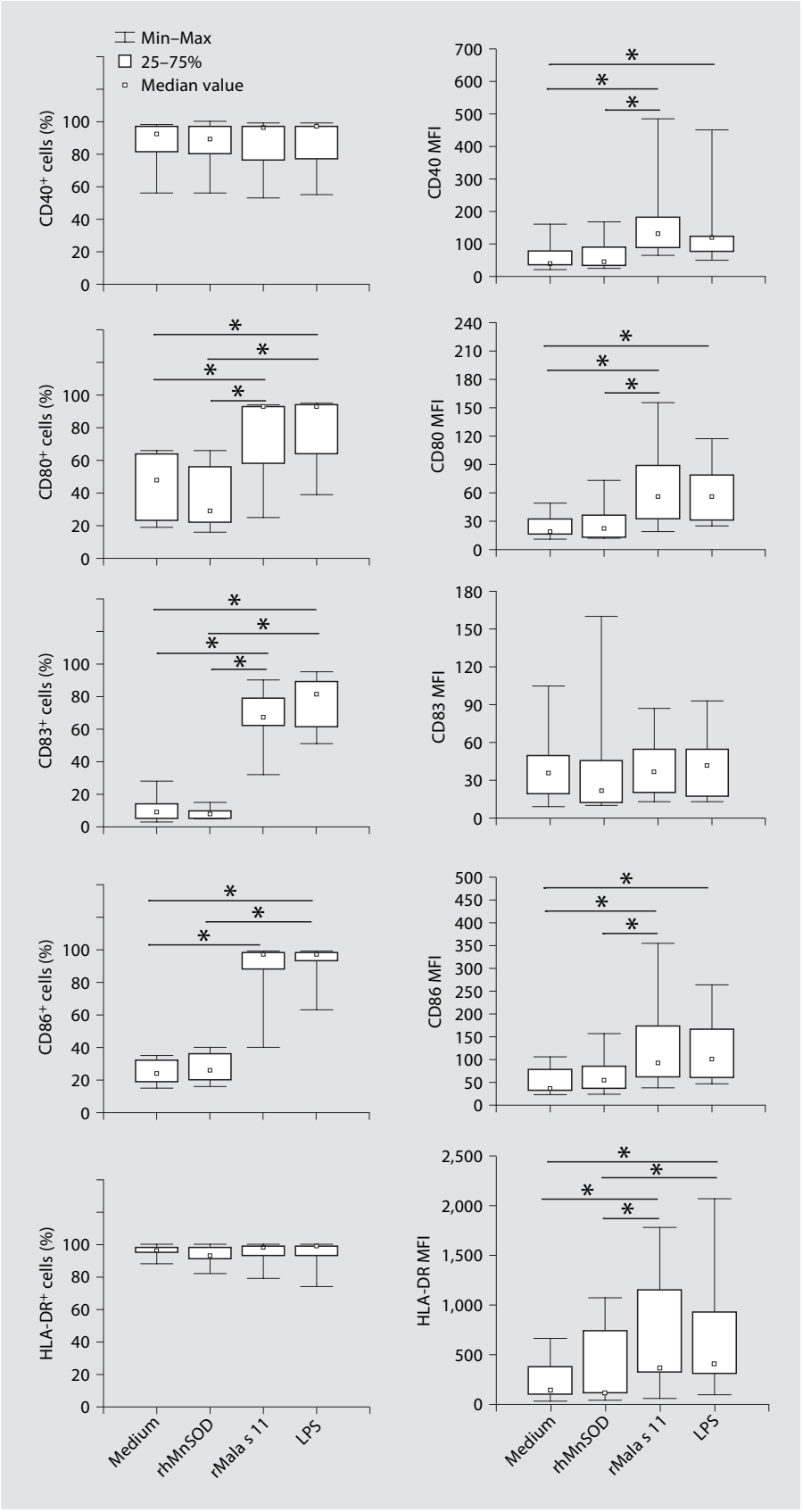


Fig. 1. Expression of CD40, CD80, CD83, CD86 and HLA-DR on MDDCs cultured in medium alone or co-cultured with rhMnSOD (3 μg/ml), rMala s 11 (3 μg/ml) or LPS (0.1 μg/ml) for 24 h. The results of flow cytometric analysis are presented as the percentage of positively stained cells and as mean fluorescence intensity (MFI). At least 10^4 cells per sample were analysed. Nine experiments using cells from different healthy blood donors were performed. * $p < 0.05$ as calculated with the Friedman two-way analysis of variance followed by Friedman multiple comparison tests.

Table 1. Cytokine concentrations at different time points in supernatants from MDDCs co-cultured with rMala s 11, rhMnSOD or cultured in medium alone

Cytokine	n	Medium	rhMnSOD (3 μg/ml)	rMala s 11 (3 μg/ml)
TNF-α				
4 h	7	<20	<20	347 (68–3,916)[a, b]
24 h	9	<20	<20	2,802 (167–29,875)[a, b]
IL-6				
4 h	7	<20	<20	465 (133–7,169)[a, b]
24 h	9	<20	<20 (<20–395)	16,945 (1,711–97,639)[a, b]
IL-8				
4 h	7	<20 (<20–851)	50 (<20–713)	2,206 (328–12,829)[a, b]
24 h	9	78 (32–539)	650 (80–2,829)	87,731 (13,388–230,607)[a, b]
IL-10				
4 h	7	<20	<20	<20
24 h	9	<20	<20	34 (<20–179)[a, b]
IL-12p70				
4 h	7	<20	<20	<20
24 h	9	<20	<20	330 (<20–1,522)[a, b]

Data are cytokine concentrations in picograms per millilitre of supernatants from MDDC cultures (4×10^5 cells/ml) as analysed with cytometric bead array. Results are presented as medians, with ranges in parentheses (all other medians had ranges below the detection limit of 20 pg/ml). n = Number of experiments, using cells from different healthy blood donors.

[a] $p < 0.05$: significantly higher than the corresponding value for MDDCs co-cultured in medium alone. [b] $p < 0.05$: significantly higher than the corresponding value for MDDCs co-cultured with rhMnSOD. p values are calculated with the Friedman two-way analysis of variance followed by Friedman multiple comparison tests.

significant increase with a further increase at 24 h in the levels of TNF-α, IL-6 and IL-8 in supernatants from MDDCs co-cultured with rMala s 11 in comparison with supernatants from MDDCs cultured in medium alone (table 1). Significantly higher levels of IL-10 and IL-12p70 were observed at 24 h in supernatants from MDDCs co-cultured with rMala s 11 in comparison with MDDCs cultured in medium alone. The cytokine levels of MDDCs co-cultured with rMala s 11 at both time points for all cytokines analysed were in the range with those of MDDCs co-cultured with LPS (data not shown). For MDDCs co-cultured with rhMnSOD, the cytokine concentrations were at the levels of MDDCs cultured in medium alone (table 1). The levels of IL-1β remained unaltered in all MDDC culture conditions (data not shown).

Stimulation of Allogeneic Lymphocytes

The lymphocyte stimulation capacity of MDDCs pre-cultured with rMala s 11, rhMnSOD or LPS or cultured in medium alone was studied in an allogeneic lymphocyte proliferation assay. As expected, iMDDCs (pre-cultured with medium alone) provided an MDDC/PBMC ratio-dependent proliferative response in allogeneic CD14-depleted PBMCs (data not shown). This proliferation was further enhanced by the exchange for MDDCs pre-cultured with rhMnSOD, rMala s 11 or LPS. However, the lymphocyte-stimulating capacity was significantly higher ($p < 0.05$) for MDDCs stimulated with rMala s 11 than with rhMnSOD (fig. 2). In 4 of 5 experiments performed, MDDCs pre-stimulated with rMala s 11 induced a proliferative response in range with that of MDDCs pre-stimulated with LPS. The maximum background proliferation for PBMCs alone (2×10^5 cells/well) and MDDCs alone (10^4 cells/well) never exceeded 1,416 ± 32 and 399 ± 78 cpm (mean ± SEM, n = 5), respectively.

Discussion

To gain further insight into the ability of DCs to discriminate between potentially harmful exogenous antigens and self proteins with a high sequence homology to environmental antigens, we compared the effects of the

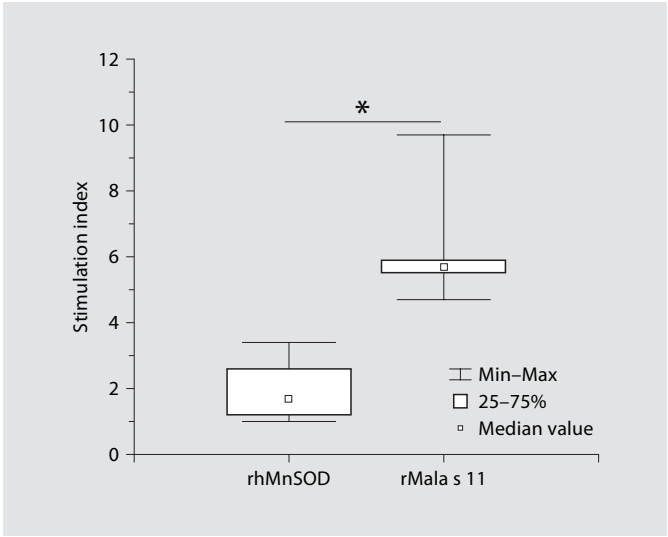


Fig. 2. rMala s 11 enhances the MDDC capacity to induce allogeneic lymphocyte proliferation to a significantly higher extent than rhMnSOD. $CD14^+$-depleted allogeneic PBMC (2×10^5 cells/well) were cultured for 5 days in the presence of 10^3 MDDCs pre-cultured for 24 h with rhMnSOD (3 μg/ml) or rMala s 11 (3 μg/ml) or cultured in medium alone, with the addition of 1 μCi [^{3}H]thymidine for the last 18 h. The data presented are from 5 experiments, with MDDC generated from 5 different healthy blood donors. The median value for $CD14^+$-depleted allogeneic PBMC co-cultured with MDDCs pre-cultured in medium alone was 3,735 cpm (range 1,036–6,261). The results are presented as stimulation index calculated as counts per minute ($CD14^+$-depleted allogeneic PBMC co-cultured with MDDCs pre-cultured with rhMnSOD or rMala s 11) divided by counts per minute ($CD14^+$-depleted allogeneic PBMC co-cultured with MDDCs pre-cultured in medium alone). * $p < 0.05$ as calculated with the Wilcoxon matched pairs test.

M. sympodialis allergen Mala s 11 on MDDCs with those of hMnSOD, a stress-inducible mitochondrial enzyme which catalyses the dismutation of radicals into oxygen and peroxide [19]. Its antioxidant activity protects the skin against the toxicity of oxygen-related radicals and the enzyme is up-regulated by UV exposure, mechanical trauma [20], and in the lesional skin of patients with AE [11]. The 2 proteins chosen are of particular interest, since sensitization to Mala s 11 has been observed in AE patients [7] and hMnSOD has been suggested to be self-reactive in these patients [11]. The results of the present study suggest that Mala s 11 can affect the immune system through DCs with the up-regulation of the maturation marker CD83, the co-stimulatory molecules CD40, CD80, CD86 and HLA-DR necessary for antigen stimulation to T cells. In contrast, hMnSOD did not induce markers of DC maturation. These phenotypic differences were also reflected functionally where MDDCs stimulated with Mala s 11 induced a significantly higher allogeneic lymphocyte proliferation than MDDCs co-cultured with hMnSOD. Thus, DCs of healthy individuals possess the ability to discriminate between the exogenous Mala s 11 protein and hMnSOD despite their high degree of sequence homology.

A mature MDDC phenotype is associated with the release of pro-inflammatory and migratory cytokines [14]. We found that Mala s 11, but not hMnSOD, stimulated the release of TNF-α. TNF-α is known to induce DC migration [21], which is why our results suggest that Mala s 11 might induce DC migration whereas hMnSOD does not. Furthermore, Mala s 11 could be of importance for the recruitment of inflammatory cells by inducing the production of the chemo-attractant cytokine IL-8 [22]. IL-6 promotes a Th2 differentiation of naive T lymphocytes [23], whereas IL-12 production from DCs induces a Th1 differentiation and IL-10 is classified as a Th2-skewing cytokine by inhibition of IL-12 production [24]. Mala s 11 could promote a Th2 profile by inducing the production of IL-6 from DCs. The observation that IL-10 and IL-12 are simultaneously released from MDDCs generated from 6 of the 9 donors investigated indicates that healthy individuals do not respond with a Th1- or Th2-biased differentiation mediated by IL-10 or IL-2 upon exposure to Mala s 11.

We have previously shown that *M. sympodialis* whole yeast cells induce the maturation of human MDDCs from healthy individuals in terms of up-regulated levels of CD80, CD83 and CD86 along with elevated levels of TNF-α [17] which is in agreement with our present data using Mala s 11 as a single component. In contrast, IL-10 and IL-12p70 levels released from MDDCs from healthy donors co-cultured with *M. sympodialis* remained unaltered [17]. The difference to the present study, where increased levels of these 2 cytokines were observed in response to Mala s 11, besides the use of whole yeast cells, could be due to a later time point (46 h) for the cytokine measurements in the previous study and a different method used for cytokine detection [17].

One possible route for the discrimination between microbial and self antigens by DCs was recently demonstrated to be mediated by the presence of toll-like receptor ligands, such as LPS, at antigen uptake [25]. In the present study, the ability of MDDCs to discriminate between Mala s 11 and hMnSOD cannot be explained by LPS contamination, since the LPS levels were comparably low (2.7 pg/ml for rMala s 11 and 15 pg/ml for rhMnSOD) and did

not affect the MDDC phenotype or the release of cytokines in control experiments.

It is likely that skin-homing DCs in vivo can come in contact with *M. sympodialis* as well as with its released components. The presence of *M. sympodialis*-specific IgG in the serum of healthy individuals supports this assumption [26] and most likely reflects the healthy immune response to natural exposure. In AE, the impaired skin barrier facilitates host microbe interactions and the higher pH associated with AE skin [27] stimulates the release of allergens from *M. sympodialis* [28]. The presence of specific IgG to Mala s 11 in healthy individuals has not been studied. Pre-existing specific IgG to Mala s 11 bound to Fc receptors on DCs could possibly explain a difference in response to Mala s 11 and hMnSOD in the in vivo situation. However, since the MDDCs used in this study were generated from $CD14^+$ monocytes cultured for 6 days in the absence of human serum, the presence of Mala s 11-specific IgG in our cell cultures can be virtually excluded.

In conclusion, our results show that Mala s 11 induces MDDC maturation and the release of inflammatory cytokines in healthy individuals whereas its endogenous homologue MnSOD lacks this ability. In future studies, it will be essential to compare the effects of Mala s 11 and hMnSOD on DCs from AE patients sensitized to both homologues. This would help elucidating the role of DC in the reactivity against self proteins observed in patients suffering from AE.

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