Malassezia sympodialis thioredoxin–specific T cells are highly cross-reactive to human thioredoxin in atopic dermatitis

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Background: IgE-mediated cross-reactivity between fungal antigens and human proteins has been described in patients with atopic dermatitis (AD), but it remains to be elucidated whether there is also cross-reactivity at the T-cell level.
Objective: We sought to explore cross-reactivity at the T-cell level between the fungal thioredoxin (Mala s 13) of the skin-colonizing yeast *Malassezia sympodialis* and its homologous human thioredoxin (hTrx).
Methods: T-cell lines (TCLs) were generated in the presence of rMala s 13 from the peripheral blood and from skin biopsy specimens of positive patch test reactions of patients with AD sensitized to Mala s 13 and hTrx. Patients with AD not sensitized to *Malassezia* species, healthy subjects, and patients with psoriasis served as control subjects. Mala s 13–specific T-cell clones (TCCs) were generated from TCLs. TCCs were characterized by antigen specificity, phenotype, and cytokine secretion pattern. Human keratinocytes were stimulated with IFN-γ, TNF-α, and IL-4, and the release of hTrx was determined by means of ELISA.
Results: Mala s 13–specific TCLs and TCCs from the blood and skin of patients with AD sensitized to Mala s 13 and hTrx were fully cross-reactive with hTrx. Mala s 13– and hTrx–specific TCCs could not be generated from control subjects. The majority of cross-reactive TCCs were $CD4^+$ and coexpressed cutaneous lymphocyte antigen. In addition to T_H1 and T_H2 TCCs, we could also identify TCCs secreting IL-17 and IL-22. After stimulation with IFN-γ and TNF-α, keratinocytes released substantial amounts of thioredoxin.
Conclusion: In patients with AD sensitized to *Malassezia* species, cross-reactivity at the T-cell level to Mala s 13 and the homologous hTrx is detectable. hTrx autoreactive skin-homing T cells might be relevant for cutaneous inflammation in patients with AD. (J Allergy Clin Immunol 2011;128: 92-9.)

Key words: *T-cell autoreactivity, autoantigen, skin-homing T cells, T_H17, T_H22, atopic dermatitis, thioredoxin,* Malassezia sympodialis

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease that often begins in infancy. Despite intense research over the past few decades, the pathogenesis of AD is still not fully understood.[1] A number of exogenous and endogenous trigger factors, such as inhalant and food allergens, have been identified. Gene-environment interactions in genetically predisposed subjects also appear to play a central role.[1-4] Furthermore, there is evidence for a dysregulation of the cellular and humoral immune response and for defects of skin barrier function in patients with AD. Microorganisms, in particular *Staphylococcus aureus* and its proteins and the opportunistic skin-colonizing yeast *Malassezia sympodialis*, have been described as contributing to the disease pathogenesis as well.[3-6] Recently, autoreactivity to human proteins has been postulated as a pathomechanism for AD. Such autoreactivity to human proteins has been described for a repertoire of IgE-binding autoantigens that cross-react with environmental antigens, including fungal allergens.[7-10]

T lymphocytes dominate the cellular infiltrate in the skin of patients with AD, and the knowledge of antigen specificities of these cells is of particular importance for understanding the disease mechanisms. Cross-reactivity between inhalant and food allergens has been studied in detail at the T-cell level (eg, for Bet v 1 and Api g 1[11]), but cross-reactivity of T cells between a human protein and a fungal protein has not yet been described in detail.

In the present investigation we provide evidence for cross-reactive T cells between *M sympodialis* thioredoxin (Mala s 13) and its human counterpart (human thioredoxin [hTrx]). Thioredoxins are small redox proteins found in all living cells in the cytoplasm. Of note, thioredoxins have recently been identified as a new panallergen family member able to elicit IgE-mediated hypersensitivity reactions in patients with AD.[12] Thioredoxins from the mold *Aspergillus fumigatus* are the causative agents identified in the majority of *Aspergillus* species–related human diseases.[9,12] Thioredoxins from *M sympodialis* were isolated from fungal cDNA libraries displayed on the phage surface and found to elicit IgE-mediated hypersensitivity reactions in patients with AD.[13] Recently, IgE-mediated cross-reactivity between

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Abbreviations used
AD: Atopic dermatitis
APT: Atopy patch test
bTCC: Blood-derived T-cell clone
CLA: Cutaneous lymphocyte antigen
hTrx: Human thioredoxin
MnSOD: Manganese superoxide dismutase
rhTrx: Recombinant human thioredoxin
SI: Stimulation index
sTCC: Skin-derived T-cell clone
TCC: T-cell clone
TCL: T-cell line

wheat and maize thioredoxins has been demonstrated in patients with baker's asthma.[14]

Most phenotypic studies on allergen-specific T cells derived from patients with AD have focused on the T_H1/T_H2 paradigm.[1-4] Positive atopy patch test (APT) reactions to *M sympodialis* (previously designated *M furfur)* in patients with AD correlate with a T_H2-like peripheral blood mononuclear cell response.[15] More recently, other T_H cell subsets, such as T_H17 and T_H22 cells, have been shown to play a role in skin pathogenesis and remodeling.[16,17]

In the present study we show, for the first time, cross-reactivity of T-cell recognition between Mala s 13 and hTrx of T cells at a clonal level. Our data indicate that autoreactive T cells are characterized by a cytokine secretion profile encompassing not only the T_H1 and T_H2 phenotypes but also the T_H17 and T_H22 phenotypes, which might have relevance for the pathomechanism of AD.

METHODS

Allergens and allergen extracts

M sympodialis extract was prepared from American Type Culture Collection strain 42132 (ATCC, Manassas, Va), as previously described.[18] rMala s 6, rMala s 11, rMala s 13, and recombinant human thioredoxin (rhTrx) were produced as $[His]_6$-tagged fusion proteins in *Escherichia coli* and purified by means of Ni^{2+} affinity chromatography, as previously described.[13,19,20] *Aspergillus* species lyophilisate was kindly provided by Alk-Abelló (Arzneimittel GmBH, Wedel, Germany). Endotoxin levels were assessed with the QCL-1000 endotoxin kit (Cambrex Bio Science, Walkersville, Md). The endotoxin levels for the antigens (Mala s 13 and hTrx) and allergen extract *(M sympodialis)* used in all the *in vitro* assays were less than 1 EU/μg.

Study patients and control subjects

Three adult patients with chronic relapsing AD for more than 5 years and fulfilling the criteria of Hanifin and Rajka[21] who were sensitized to *Malassezia* species (class ≥3; Immuno-CAP m227; Phadia, Uppsala, Sweden) were included (Table I).[22] These patients had specific IgE antibodies to rMala s 13 and rhTrx determined by means of ELISA and inhibition assays, as described previously,[12] and had positive APT reactions to *M sympodialis* extract and rMala s 13 (Table I). Three patients with AD and 3 adults with psoriasis who were all nonsensitized to *Malassezia* species and 5 adult nonatopic healthy subjects were enrolled as control subjects. This study was approved by the ethics committee of the Hannover Medical School (MHH).

Lymphocyte proliferation assays

PBMCs from all subjects enrolled in the study were isolated from heparinized blood samples (60 mL) by means of density centrifugation on Ficoll Hypaque (Fresenius Kabi Norge AS, Oslo, Norway; density = 1.077 g/mL). The PBMCs (2×10^5/200 μL) were then cultured for 6 days in the presence or absence of 2.5 μg/mL of either rMala s 13 or rhTrx in Iscove medium (Biochrom KG, Berlin, Germany) supplemented with 4% human heat-inactivated AB serum, 2 mM/L glutamine, 50 mg/mL of gentamicin/L, 100 μg/mL penicillin and streptomycin, and nonessential amino acids (designated as culture medium) in 96-well microtiter plates. During the last 16 hours of culture in a humidified atmosphere containing 5% CO_2 at 37°C, tritiated thymidine (2 μCi per well; Amersham Buchler, Braunschweig, Germany) was added, and the incorporated radioactivity was measured by means of scintillation counting. Both rMala s 13 and rhTrx were titrated at concentrations of 0.1, 1, 2.5, and 10 μg/mL in triplicates. PHA (10 μg/mL; Sigma GmbH, Deisenhofen, Germany) was used as a positive control. Results were expressed as the stimulation index (SI), with an SI of greater than 2 taken as a positive score.

APTs

APTs were performed on the uninvolved back skin of all 3 *Malassezia* species–sensitized patients with AD by using 12-mm Finn chambers. Extracts from *M sympodialis* (30 μL at a concentration of 1 mg/mL and in parallel of 5 mg/mL) and rMala s 6, 11, and 13 (from each antigen, 30 μL at a concentration of 100 μg/mL, 1 mg/mL, and 4 mg/mL) were applied in addition to *Aspergillus* species extract (20 μL at a concentration of 5 mg/mL) and the vehicle PBS (30 μL) as a negative control. After 48 hours, the patch test results were evaluated and scored according to the revised European Task Force on Atopic Dermatitis key for APT result reading.[22] A punch biopsy specimen of 5 mm was obtained after achievement of local anesthesia with mepivacaine (Scandicaine; AstraZeneca GmbH, Wedel, Germany) 1% from each positive patch test lesion. Two thirds of the biopsy specimens were used for generating skin-derived TCCs (sTCCs).

Preparation of single cells from skin biopsy specimens of positive APT lesions

Skin biopsy specimens from positive APT lesions were incubated in Hanks solution (for 5 hours at 37°C) containing collagenase (0.1%), DNAse (20 μg/mL), dispase (1.2 U/mL; Boehringer, Mannheim, Germany), hyaluronidase (0.1%, Sigma), and 10% FCS, as previously described.[23] Biopsy cell suspensions were washed and filtered through nylon gauze. The percentage of T cells was assessed by means of flow cytometry with an antibody to CD3 (Becton Dickinson GmbH, Heidelberg, Germany).

Generation of T-cell lines and T-cell clones

T-cell lines (TCLs) generated in the presence of rMala s 13 were obtained by using a technique previously described.[23] Briefly, PBMCs (1.5×10^6/mL) from the 3 patients with AD and the control subjects were stimulated with 2.5 μg/mL rMala s 13 in 24-well flat-bottom culture plates (3524; Costar, Cambridge, Mass). After 5 days, 10 U/mL human rIL-2 (Boehringer) was added. At day 7, T-cell blasts were enriched by means of density centrifugation and used in antigen restimulation tests to assess their specificity to rMala s 13. In addition, TCLs were also generated from the biopsy specimens of the positive APT reactions to rMala s 13 and *M sympodialis* extract by using the same procedure. Cell suspensions obtained from biopsy specimens of positive APT reactions were used as a source of T cells.

For generation of T-cell clones (TCCs), Mala s 13–specific TCLs were seeded (0.3 cells per well) on 96-well round-bottom plates (Nunclone) in the presence of 2×10^5 irradiated allogeneic PBMCs and 10 U/mL PHA. TCCs were expanded at weekly intervals with fresh allogeneic irradiated feeder cells and rIL-2. Cells of expanded TCLs (5×10^4) were restimulated with rMala s 13 (2.5 μg/mL) or rhTrx (2.5 μg/mL) in the presence of 1×10^5 autologous irradiated (50 Gy) PBMCs. For generation of TCCs from the skin biopsy specimens, the protocol mentioned above was followed, starting with stimulating the entire single-cell suspension obtained from the positive APT biopsy specimen with 2.5 μg/mL rMala s 13.

Antigen restimulation test

An 8-day interval between the last round of expansion was allowed before antigen specificity of the TCLs and TCCs were tested in a restimulation assay. After repeated washings, 5×10^3 T cells were stimulated with 2.5 μg/mL of either rMala s 13 or rhTrx in the presence of 7.5×10^4 irradiated autologous PBMCs. All cultures were performed in triplicates. On day 5, cultures were

TABLE I. Characteristics of *Malassezia*–sensitized patients with AD and results of the APTs

Patient no.	Age (y)	Specific IgE (KU/L) *Malassezia* species (m227)	Specific IgE rMala s 13 (OD/nm)	Specific IgE rhTrx (OD/nm)	APT* rMala s 13
1	66	38.4	2.8	0.3	+
2	21	47.1	3.2	0.3	+
3	39	57.7	2.4	1.8	++++

*Scoring of APT reactions according to the revised European Task Force on Atopic Dermatitis key for APT reading[22]: +, erythema and infiltration; ++, erythema and a few papules; +++, erythema and many or spreading papules; ++++, erythema and vesicles.

pulsed with 2 μCi of tritiated thymidine per milliliter (Amersham) for 4 hours. The β decay was counted in a liquid scintillation counter, and results of triplicate cultures were expressed as the mean. The SI was defined by the ratio of mean counts per minute of stimulated to unstimulated cultures. A TCC was scored positive for antigen reactivity when the SI was greater than 2. In TCL restimulation assays rMala s 11 (2.5 μg/mL), recombinant human manganese superoxide dismutase (MnSOD; 2.5 μg/mL), and human serum albumin (5 μg/mL, Sigma) were used as negative controls. In both TCL and TCC restimulation assays, PHA (10 μg/mL) was used as a positive control.

Blocking experiments

Inhibition of antigen-induced T-cell activation was investigated by incubating randomly selected blood-derived TCCs (bTCCs) and sTCCs generated from patients with AD with rhTrx in the presence or absence of 5 μg of mAb specific for HLA-DR (clone G46-6).[12] An isotype-matched mAb (Zymed Laboratories, San Francisco, Calif) was used as a control. Proliferation was measured by means of tritiated thymidine uptake, as described above.

Phenotyping of TCCs and cytokine assays

Established TCCs were stained with allophycocyanin-labeled anti-CD3, anti-CD4, anti-CD8, or anti–cutaneous lymphocyte antigen (CLA; BD Biosciences, San Jose, Calif). Flow cytometry was performed with a FACSCAN (Becton Dickinson, Heidelberg, Germany). Mala s 13–specific TCCs were washed and incubated with irradiated autologous PBMCs in the presence of rhTrx (2.5 μg/mL) or Mala s 13 (2.5 μg/mL) for 5 days. Cytokine levels in supernatants were measured in duplicates by means of ELISA (Ready-Set-Go human ELISA; Biocarta, Hamburg, Germany) for IFN-γ, IL-5, IL-13, IL-10, IL-17, and IL-22, according to the manufacturer's instructions. TCCs were assigned T_H phenotype as follows: T_H1, IFN-γ/IL-5 (or IL-13) greater than 10; T_H2, IL-5 (or IL-13)/IFN-γ greater than 5, as previously described[11]; T_H17, amount of IL-17 equaled or exceeded 500 pg/mL; and T_H22, amount of IL-22 equaled or exceeded 500 pg/mL.

Stimulation of human primary keratinocytes with inflammatory cytokines and detection of release of thioredoxin

Primary cultures of normal human keratinocytes were prepared from foreskin of children undergoing surgery, as described previously.[24] In brief, the single-cell suspension of keratinocytes was cultured in serum-free keratinocyte growth medium (Keratinocyte Growth Medium 2 Kit; PromoCell GmbH, Heidelberg, Germany). All cell cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37°C and were used at passages 2 to 5. Hydrocortisone-free medium was used for all experiments. The purity of keratinocytes was verified by the expression of the epithelial marker cytokeratin (anti-human cytokeratin antibody, clone MNF-116; DakoCytomation, Hamburg, Germany). All cells (>95%) were found to be uniformly positive for cytokeratin. Triplicates of keratinocyte cultures (n = 8) were stimulated with the following cytokines: IFN-γ (50 ng/mL), IL-4 (50 ng/mL), TNF-α (50 ng/mL), and IFN-γ (50 ng/mL) plus TNF-α (50 ng/mL) for 24 and 48 hours. After these 2 time points, the supernatants were collected and ultracentrifuged at 100,000 for 1 hour. The clear supernatants were immediately snap-frozen in liquid nitrogen. The release of thioredoxin into the supernatants was assayed by using a commercially available ELISA kit for hTrx-1 (YoungInfrontier, Seoul, South Korea). The lower detection limit was 0.39 ng/mL. The viability of the keratinocytes at the 48-hour time point was assayed with the ToxiLight Kit (Lonza, Nottingham, United Kingdom).

TABLE II. SIs for lymphocyte proliferation tests for patients with AD sensitized to *Malassezia* species

Patient no.	Mala s 13	hTrx
AD1	14.1	12.5
AD2	21.2	16.8
AD3	30.1	23.1

Statistical analysis

Statistical analyses were performed with the Student paired *t* test or the Mann-Whitney *U* test when data points did not show Gaussian distribution. *P* values of less than .05 were considered significant. The program GraphPad Prism version 3.02 (GraphPad Software, Inc, San Diego, Calif) was used for statistical analysis.

RESULTS

Lymphocyte proliferation assays

PBMCs from all patients with AD, as well as those from patients with psoriasis and healthy control subjects, were stimulated with different concentrations of rMala s 13 and rhTrx to ascertain the optimum concentration of Mala s 13 and hTrx to be used in the T-cell cloning experiments and subsequent restimulation tests. In PBMCs from the 3 sensitized patients with AD, both Mala s 13 and hTrx induced a pronounced lymphocyte proliferation at a concentration of 2.5 μg/mL (Table II). Therefore we used both Mala s 13 and hTrx at this concentration in all subsequent experiments. PBMCs from the control subjects did not proliferate in the presence of rMala s 13 or rhTrx (SI < 2, data not shown).

APTs

All 3 patients with AD sensitized to *Malassezia* species (as characterized in Table I) had a positive APT reaction to rMala s 13 after 48 hours, and patient AD3 also had a highly positive reaction to the *M sympodialis* extract.

None of the patients with AD had positive APT reactions to any of the other recombinant *M sympodialis* allergens used, their homologous human proteins, or the negative PBS control.

Mala s 13–specific TCLs generated from the peripheral blood of patients with AD are cross-reactive with hTrx

Nine TCLs each were generated from peripheral blood of the 3 patients with AD with IgE sensitization to *Malassezia* species, 3

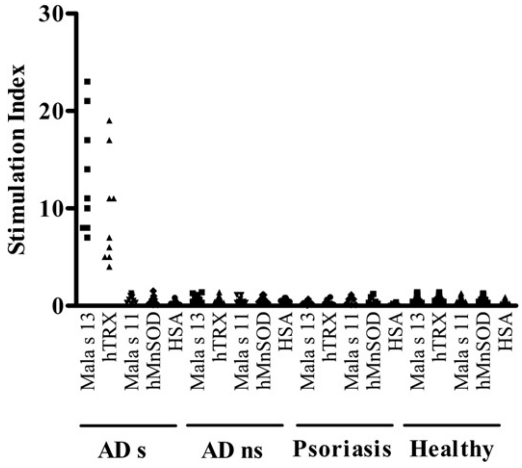

FIG 1. TCLs from patients with AD generated in the presence of Mala s 13 proliferate in the presence of Mala s 13 and hTrx. TCLs generated in the presence of Mala s 13 from the peripheral blood of patients with AD with *(AD s)* and without *(AD ns)* IgE sensitization to *Malassezia* species, patients with psoriasis, and healthy control subjects were stimulated with rMala s 13, rhTrx, rMala s 11, recombinant human MnSOD, and human serum albumin *(HSA)* for 5 days. Proliferation was assessed by means of incorporation of tritiated thymidine, and results are expressed as the SI. The counts per minute value for the medium control in this experiment was 672.5 ± 96.5 (mean ± SEM), which is indicative of efficient thymidine incorporation.

patients with AD without sensitization to *Malassezia* species, and 3 patients with psoriasis in the presence of rMala s 13 and 12 TCLs from the peripheral blood of 5 healthy control subjects.

TCLs generated from the patients with AD without IgE sensitization to *Malassezia* species, from patients with psoriasis, and from healthy control subjects did not show proliferative responses to rMala s 13 or rhTrx (SI < 2, Fig 1). Moreover, none of the TCLs from the patients with psoriasis or healthy subjects reacted to either rMala s 11 or recombinant human MnSOD (Fig 1).

All TCLs generated from patients with AD sensitized to *Malassezia* species showed a proliferative response to both rMala s 13 and rhTrx. Mala s 13–reactive TCLs did not respond to the other recombinant *M sympodialis* allergens, such as rMala s 11, its human homologue recombinant human MnSOD, or human serum albumin (Fig 1).

Mala s 13–specific TCCs generated from patients with AD are cross-reactive with hTrx

In total, 65 TCCs were generated from the peripheral blood of the 3 sensitized patients with AD. Antigen restimulation tests revealed that 44 of 65 bTCCs were specific for Mala s 13 (n = 15 TCCs from patient AD1, n = 12 TCCs from patient AD2, and n = 17 TCCs from patient AD3; see Table E1 in this article's Online Repository at www.jacionline.org). Forty-three of 44 TCCs were cross-reactive with hTrx (Fig 2, *A*). Eighty-five TCCs were generated from the 3 rMala s 13–positive APT reactions (n = 25 TCCs from patient AD1, n = 19 TCCs from patient AD2, and n = 41 TCCs from patient AD3; see Table E1). Seventy-one of 85 sTCCs were reactive to rMala s 13 and cross-reactive with rhTrx (Fig 2, *B*). Twenty-four sTCCs could be generated from the *M sympodialis* extract–positive APT reaction from patient AD3. Interestingly, 14 of 24 TCCs generated from skin out of this lesion were specific both for Mala s 13 and cross-reactive with hTrx (Fig 2, *C*).

Mala s 13–specific TCCs generated from blood and skin of patients with AD are mainly $CD4^+$ and express CLA

Forty-three of 44 bTCCs and 69 of 71 sTCCs were $CD4^+$, whereas 1 of the bTCCs and 2 of the sTCCs were $CD8^+$. All Mala s 13–reactive bTCCs and sTCCs clearly stained positive for the skin-homing receptor CLA: mean fluorescence intensities for bTCCs and sTCCs were 65.3 ± 21.4 and 68.9 ± 21.8, respectively. This was in a similar range as the mean fluorescence intensities for CD4 for the same TCCs, which were 81.7 ± 23.9 and 84.7 ± 13.1 for bTCCs and sTCCs, respectively. These values represent the means ± SEMs (subtracted for the isotype control values).

MHC class II restriction of the CD4 T-cell response

An mAb directed to HLA-DR was used in inhibition experiments to investigate whether the Mala s 13 specificity and hTrx cross-reactivity of the TCCs were dependent on antigen presentation by MHC class II molecules. Addition of a blocking anti–HLA-DR led to a marked reduction in cell proliferation (73% to 94% decrease, $P < .0001$ calculated compared with the isotype control). In contrast, an isotype-matched control mAb had no significant effect (see Fig E1 in this article's Online Repository at www.jacionline.org). Blocking with an antibody against HLA-DR/DP/DQ also abrogated the cell proliferation (data not shown).

Mala s 13–specific TCCs cross-reactive with hTrx belong to T_H1, T_H2, T_H17, and T_H22 cell phenotypes

Forty-four bTCCs and 85 sTCCs shown to be Mala s 13 reactive were stimulated with rhTrx for 5 days, and the supernatants were analyzed for various cytokines by means of ELISA. Forty-five percent of the bTCCs and 35% of the sTCCs were of the T_H1 phenotype, followed by TCCs of the T_H2 phenotype (Fig 3). We could also identify bTCCs and sTCCs with T_H17 and T_H22 phenotypes. Notably, we could generate TCCs (13%) of the T_H2/T_H17 phenotype only from the skin and not from the blood of the patients with AD. TNF-α was coproduced only by T_H22 TCCs and not by T_H1, T_H2, or T_H17 TCCs (Fig 3). Some of the T_H17 TCCs belonged to the T_H1/T_H17 and T_H2/T_H17 subsets. Only 1 sTCC secreted significant amounts of IL-10 (data not shown). We further analyzed the cytokine profile of Mala s 13–specific TCCs, which had been stimulated with Mala s 13. We found that the cytokine secretion pattern was similar to that of the same TCCs stimulated with the autoantigen hTrx (see Table E2 in this article's Online Repository at www.jacionline.org).

Mala s 13– and hTrx-specific TCCs cannot be generated from healthy control subjects

Thirteen bTCCs that had initially grown in the presence of rMala s 13 and 13 bTCCs that had grown in the presence of rhTrx from 2 healthy subjects were assessed for their specificity to the respective antigens. TCLs and TCCs were generated from the blood of healthy control subjects by using the same protocol that was used for the generation of TCLs and TCCs from patients with AD. Only 2 bTCCs generated in the presence of Mala s 13 (B7 and B16) were reactive at baseline level to rMala s 13, as indicated by their SI. None of the bTCCs generated in the presence of hTrx reacted with hTrx (see Table E3 in this article's Online Repository at www.jacionline.org).

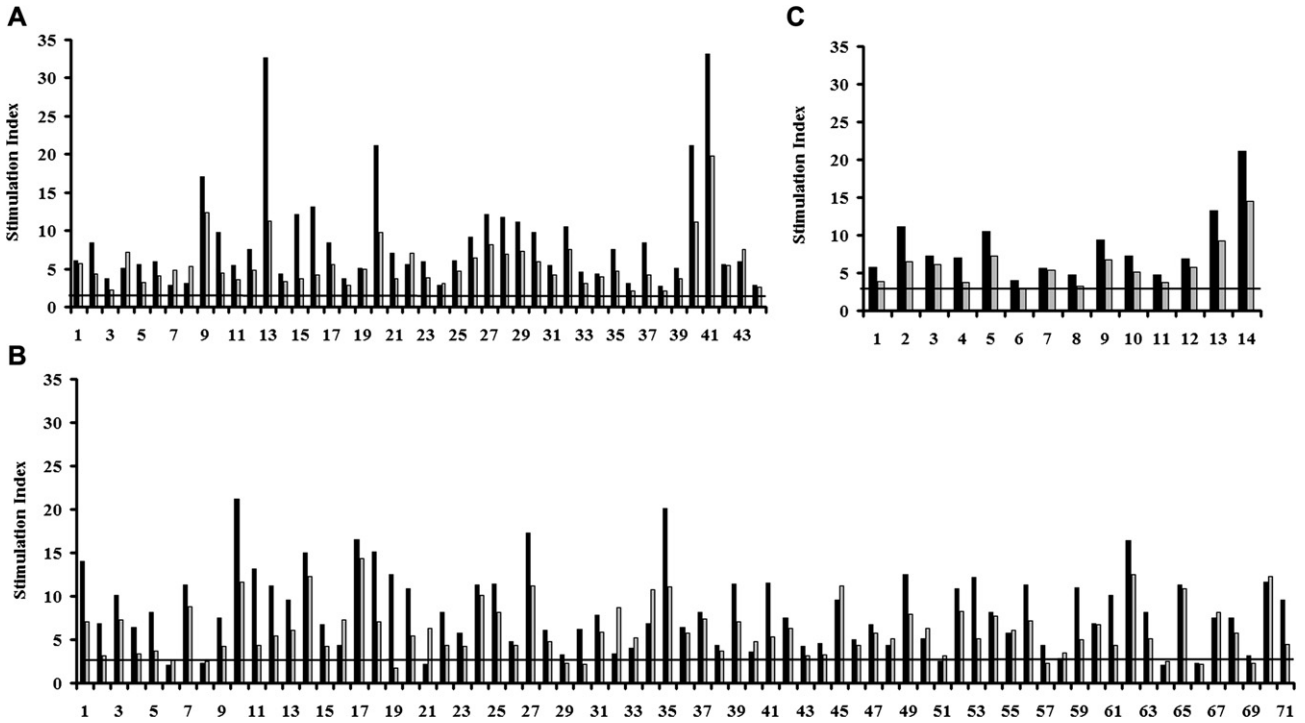

FIG 2. High degree of cross-reactivity of Mala s 13 with hTrx in TCCs. Forty-four TCCs generated from blood and 71 TCCs generated from positive APT lesions were specific for Mala s 13. The SIs for the antigen restimulation tests with TCCs performed with rMala s 13 *(black bars)* or with rhTrx *(gray bars)* are indicated. The *horizontal line* indicates the cutoff SI value of 2. **A**, Mala s 13–specific bTCCs generated from the blood of patients with AD sensitized to *M sympodialis*. **B**, Mala s 13–specific sTCCs generated from biopsy specimens of positive APT reactions to rMala s 13. **C**, Mala s 13–specific sTCCs generated from a biopsy specimen of a positive APT reaction to *M sympodialis* extract.

IFN-γ stimulates the release of thioredoxin from human primary keratinocytes, which is augmented by TNF-α

Human primary keratinocytes were stimulated with various inflammatory cytokines to investigate whether thioredoxin is released from human keratinocytes under inflammatory conditions so that it becomes available to immune cells in the skin compartment. Keratinocytes when stimulated with IFN-γ (50 ng/mL) for 48 hours released thioredoxin into supernatants, which was readily detected by using a commercially available thioredoxin ELISA. This effect was further augmented by TNF-α (50 ng/mL). Stimulation with IL-4 (50 ng/mL) did not lead to the release of thioredoxin at any of the time points tested (Fig 4). The keratinocytes were still viable at the 48-hour time point, which was determined by using the ToxiLight Kit, which assays the release of adenylate kinase. The amount of adenylate kinase released from keratinocytes stimulated with all the cytokines mentioned above at the 48-hour time point was similar to that seen in nonstimulated cells (data not shown).

DISCUSSION

Autoreactivity to human proteins has been postulated to be involved in the pathogenesis of AD in a subgroup of patients.[4,7,8] Autoreactivity at the IgE level between fungal allergens and their homologous human proteins has been well characterized in the last decade.[4,8,9] Often autoreactivity is a consequence of cross-reactivity as a result of molecular mimicry.[9] At the T-cell level, cross-reactivity with possible clinical relevance for AD among food allergens, such as Bet v 1 and Api g 1, has been identified.[11]

Previously, Schmid-Grendelmeier et al[25] have shown that the fungal MnSOD (Mala s 11 and Asp f 6) and the homologue human MnSOD are able to induce specific T-cell proliferation in patients with AD with specific IgE sensitization to human MnSOD. Interestingly, in these patients it was also possible to induce eczematous lesions through APTs by applying human MnSOD on the unaffected skin for 48 hours. In another study it was demonstrated that hTrx and its fungal counterpart from *M sympodialis* thioredoxin (Mala s 13) were able to elicit positive APT reactions in sensitized subjects after 48 hours.[13]

IgE cross-reactivity between human and fungal allergens has been well characterized on a molecular basis in sensitized patients with AD.[12,13,19] Here we report cross-reactivity at the T-cell level between the fungal thioredoxin Mala s 13 from the skin-colonizing yeast *M sympodialis* and its human homologue hTrx. We found that Mala s 13–specific T lymphocytes are fully cross-reactive with hTrx, irrespective of whether the TCCs were derived from blood or skin.

We excluded nonspecific induction of proliferation by hTrx by demonstrating that rhTrx failed to induce proliferation in PBMCs from nonsensitized patients with AD, healthy control subjects, and patients with psoriasis. Because Mala s 13– and hTrx-specific TCLs could not be generated from the peripheral blood of the control subjects, such autoreactivity appears to be specific for AD and, in particular, for those who are IgE sensitized to

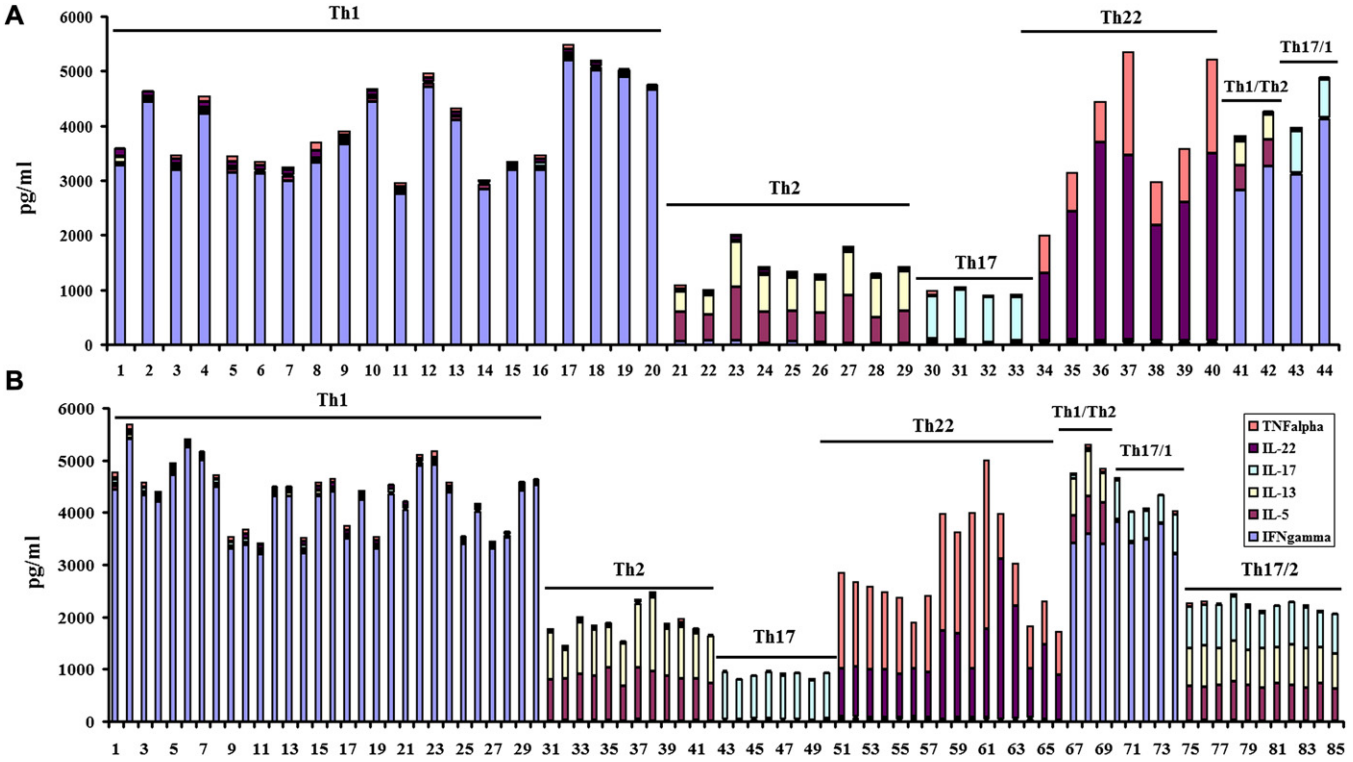

FIG 3. Cytokine secretion profile of blood- and skin-derived Mala s 13–specific TCCs stimulated with hTrx. Forty-four bTCCs **(A)** and 85 sTCCs **(B)** shown to be hTrx reactive were stimulated with rhTrx for 5 days, and the supernatants were analyzed for various cytokines by means of ELISA.

Malassezia species. Moreover, cross-reactivity in patients with AD was largely HLA-DR restricted, as demonstrated by inhibition experiments with MHC class II–specific antibodies, which also precludes induction of nonspecific proliferation by thioredoxin.

M sympodialis has been reported as the most frequent skin-colonizing yeast in both patients with AD and healthy subjects.[9,26] Approximately 50% of adults with AD show immediate-type skin reactions, have specific serum IgE, or both against *M sympodialis*.[27] Sensitization to the yeast occurs almost exclusively in patients with AD.[28] The main cause for this specific sensitization might be the disrupted skin barrier function in patients with AD, facilitating allergen uptake. The AD skin has been shown to have a slightly alkaline pH, which facilitates allergen release from *M sympodialis*.[29] Therefore it is postulated that the preferential release of these allergens in AD skin and the disrupted skin barrier seen in patients with AD lead to specific sensitization,[6,7] corroborating our above finding that we could generate Mala s 13–specific TCCs only from patients with AD and not from healthy persons or patients with psoriasis.

In the present study all the TCCs expressed the skin-homing receptor CLA, and all but 3 Mala s 13–specific TCCs were $CD4^+$. It cannot be excluded that a selection of the $CD4^+$ phenotype was due to cell-culture conditions. However, in a recently published study the majority of TCCs specific for α-nascent polypeptide-associated complex, another autoallergen with possible relevance for AD, were $CD8^+$.[30] Thus it appears that it is not the culture conditions but rather the antigen that selects the phenotype.

Previous studies have shown that the skin-homing receptor CLA is present on the majority of skin-infiltrating T lymphocytes

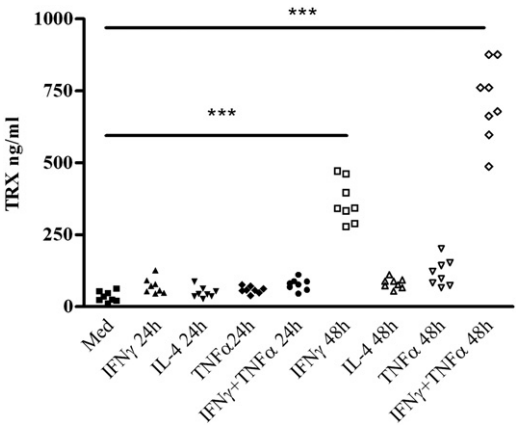

FIG 4. Human keratinocytes release thioredoxin *(TRX)*. Human primary keratinocytes (n = 8) were stimulated with various cytokines, as described in the Methods section, and the supernatants were assayed for the release of hTrx by means of ELISA. ***$P < .001$.

in patients with AD and other inflammatory skin diseases, but its expression is not restricted to defined functional and phenotypic T-cell subsets.[31] Expression of CLA on Mala s 13–specific T lymphocytes supports the view that they might have a pathogenic role because CLA can direct the cells toward skin where the T cell–derived cytokines might potentiate an inflammatory response.

The majority of the TCCs generated in our study were of the T_H1 phenotype, followed by TCCs of the T_H2 phenotype. This

finding is different from cytokine profiles that have been described for TCCs that had been generated from APT lesions reactive to inhalant allergens, in which the majority of the allergen-specific TCCs generated from APT lesions were of the T_H2 phenotype.[4]

Recently, the knowledge of T_H cell–derived cytokines has expanded to include new members, such as IL-17 and IL-22.[16,17] T cells secreting IL-22 can be induced by staphylococcal exotoxins.[32] In this study 16% of Mala s 13/rTrx–specific bTCCs and 19% of the sTCCs secreted IL-22. These TCCs also cosecreted TNF-α. When these TCCs cosecrete different cytokines, this could be due to the presence of subclones or due to different states of activation of individual cells. This observation will be further investigated with freshly generated TCCs from blood and skin in the future.

The proportion of IL-17–secreting Mala s 13/hTrx–specific TCCs was higher in sTCCs (32%) versus bTCCs (18%). In addition, Mala s 13–specific TCCs of the T_H17 subsets, such as T_H17/T_H1 and T_H17/T_H2, could be identified. This confirms data from Eyerich et al,[16] who have recently reported the T_H17 TCC. Of note, the T_H17/T_H2 TCCs could only be generated from the skin, whereas T_H17/T_H1 TCCs could be generated from both blood and skin in our study.

The role of IFN-γ in the pathogenesis of skin disorders has been well characterized.[4] IL-17 and IL-22 both seem to have specific effects on skin pathogenesis and remodeling, with some protective effects as well.[16,17,33] AD lesions harbor autoreactive T cells. Therefore inflammatory cytokines, such as IFN-γ, IL-17, and IL-22 secreted by autoreactive Mala s 13–specific T cells, might contribute to the inflammatory processes in the skin.

hTrx is a redox-sensing cytoplasmic enzyme,[34] and it still remains to be shown how and when hTrx is released from cutaneous cells to become available for interaction with Mala s 13–specific T cells. It has been previously shown that hTrx can be detected in human sera, and it is released from a variety of primary cells, such as fibroblasts, B cells, and T cells. To investigate whether hTrx could be released from human keratinocytes, we treated primary human keratinocytes with inflammatory cytokines, such as IFN-γ, TNF-α, and IL-4. After 48 hours, we could detect substantial amounts of hTrx in the culture supernatants of IFN-γ–treated keratinocytes, which was enhanced in a synergistic way by TNF-α, whereas IL-4 had no effect. Therefore in an inflammatory milieu characteristic of chronic AD lesions, hTrx can be released, which then becomes available for cross-reactivity with Mala s 13–specific T cells.

In summary, our data provide evidence for T cell–mediated cross-reactivity between a human protein, thioredoxin, and the fungal protein Mala s 13. We demonstrate this for both peripheral blood–derived and APT-induced, skin lesion–derived T cells. Although it has already been shown that allergen-specific T cells can secrete the newly described cytokines IL-17 and IL-22,[16,33] we show for the first time that cross-reacting T cells also have the propensity to secrete IL-17 and IL-22. Therefore thioredoxin-autoreactive skin-homing T cells might contribute to the pathogenesis of AD by perpetuating skin inflammation and chronification of eczema in patients with AD sensitized to *Malassezia* species.

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Key messages

- **Mala s 13–specific TCCs with T_H1, T_H2, T_H17, and T_H22 phenotypes generated from patients with AD are fully cross-reactive with hTrx.**
- **In patients with AD sensitized to *Malassezia* species, T cell–mediated cross-reactivity to Mala s 13 and the homologous hTrx might be relevant for cutaneous inflammation.**

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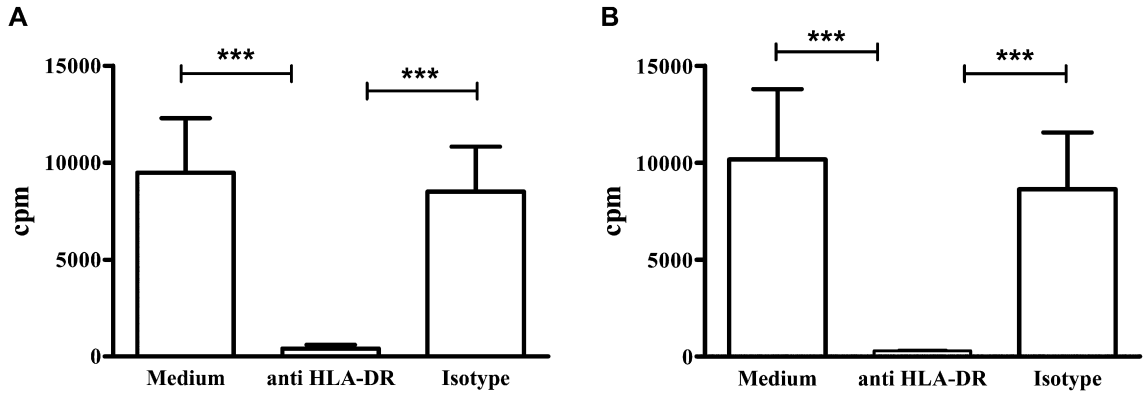

FIG E1. Suppression of proliferation of Mala s 13/hTrx–specific TCCs in the presence of a blocking antibody against HLA-DR. Ten randomly selected Mala s 13–specific bTCCs **(A)** and 10 randomly selected Mala s 13–specific sTCCs **(B)** generated from positive APT reactions to rMala s 13 were restimulated with rhTrx in the presence or absence of a blocking antibody against HLA-DR. An isotype-matched control antibody was used as a negative control. ***$P < .001$.

TABLE E1. Numbers and cytokine patterns of TCCs generated from blood and skin from the 3 patients with AD (AD1-AD3) sensitized to *Malassezia* species

	T_H1	T_H2	T_H1/T_H2	T_H17	T_H17/T_H1	T_H17/T_H2	T_H22
Blood TCCs							
AD1 (n = 15)	5	4	0	1	1	0	4
AD2 (n = 12)	6	2	1	1	1	0	1
AD3 (n = 17)	9	3	1	2	0	0	2
Skin TCCs							
AD1 (n = 25)	9	5	1	2	1	3	4
AD2 (n = 19)	7	2	0	3	1	2	4
AD3 (n = 41)	14	5	2	3	3	6	8

TABLE E2. Cytokine profile (in picograms per milliliter) of TCCs generated from blood and positive APT lesions to Mala s 13 of patient 3 with AD (AD3) with the allergen Mala s 13 in the presence of irradiated autologous PBMCs

	Phenotype	IFN-γ	IL-5	IL-13	IL-17	IL-22	TNF-α
Blood TCCs							
12	T_H1	**5173**	63	31	8	17	55
13	T_H1	**4327**	41	9	31	11	71
14	T_H1	**3562**	36	14	4	39	29
16	T_H1	**6327**	28	36	24	7	62
17	T_H1	**3678**	48	26	35	41	113
28	T_H2	141	**860**	**587**	77	83	48
29	T_H2	97	**640**	**733**	32	46	108
32	T_H17	91	17	21	**917**	49	72
33	T_H17	79	38	7	**764**	83	22
39	T_H22	57	19	27	9	**3537**	**2567**
40	T_H22	82	41	52	25	**2493**	**1352**
Skin TCCs							
17	T_H1	**5187**	43	7	13	23	91
20	T_H1	**4835**	27	24	25	17	63
25	T_H1	**6433**	21	11	43	42	122
26	T_H1	**3452**	19	39	22	51	74
29	T_H1	**4954**	37	41	19	24	57
40	T_H2	98	**659**	**704**	41	44	61
41	T_H2	121	**848**	**553**	71	36	73
42	T_H2	44	**621**	**662**	26	61	101
49	T_H17	88	27	35	**1281**	73	109
50	T_H17	132	44	11	**883**	110	68
83	T_H17/T_H2	84	**653**	**446**	**1103**	65	78
84	T_H17/T_H2	57	**519**	**487**	**837**	42	59
85	T_H17/T_H2	97	**612**	**523**	**772**	11	83
65	T_H22	44	26	31	12	**4891**	**2792**
66	T_H22	61	14	16	51	**3327**	**2321**

The values in boldface indicate the amount of cytokines produced which is above the cutoff values described in the Methods section.

TABLE E3. Lack of proliferation of blood-derived TCCs from healthy subjects to rMala s 13/rhTrx

TCC	SI* (rMala s 13)	SI* (rhTrx)
B1	0.1	0.9
B2	0.1	0.6
B3	0.7	0.4
B4	0.9	0.3
B7	2.3	0.6
B8	0.7	0.7
B13	1.1	1.3
B15	0.9	1.0
B16	2.8	0.9
B18	0.7	0.6
B19	0.4	0.4
B20	1.1	0.3
B22	0.8	0.6

*SI values obtained from restimulation tests for rMala s 13– and rhTrx-specific TCCs generated from 2 healthy subjects. Two TCCs (B7 and B16) proliferated in the presence of rMala s 13 and were not cross-reactive to hTrx.