

## Letter to the Editor

**Differential cytokine induction by the human skin-associated autoallergen thioredoxin in sensitized patients with atopic dermatitis and healthy control subjects**

To the Editor:

The term autoallergy describes IgE reactivity to autoantigens, which can be found in a subgroup of patients with atopic dermatitis (AD).<sup>1</sup> Thioredoxin belongs to the group of autoallergens that are also described as panallergens because these evolutionary highly conserved proteins are present in many allergenic sources. Human thioredoxin (hTrx) is a homologue of the *Malassezia sympodialis* allergen Mala s 13, and both proteins have been shown to cross-react at the IgE level *in vitro*, as well as to induce immediate and late-phase skin reactions *in vivo*.<sup>2</sup> Because as many as 50% of adults with AD are sensitized to *M. sympodialis* and this sensitization has been associated with more severe phenotypes of AD,<sup>3</sup> autosensitization to hTrx might have a significant effect on exacerbation of the disease.

We have recently shown cross-reactivity between Mala s 13 and hTrx at the T-cell level.<sup>4</sup> Proliferation to Mala s 13 or hTrx could only be induced in T-cell lines generated in the presence of Mala s 13 from PBMCs of patients with AD who were sensitized to *Malassezia* species but not from nonsensitized patients with AD, patients with psoriasis, or healthy control subjects. The cross-reactive T-cell lines were mainly CD4<sup>+</sup>, and all were positive for the skin-homing receptor cutaneous lymphocyte antigen. They could be assigned to the T-cell subsets T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17, and T<sub>H</sub>22. Furthermore, release of hTrx from primary keratinocytes was induced by stimulation with IFN- $\gamma$ , which might exacerbate atopic skin inflammation in sensitized patients through activation of hTrx-specific T cells.<sup>4</sup>

The present study aimed to compare cytokine induction by hTrx and Mala s 13 in circulating mononuclear cells from patients with AD who were sensitized or not to *Malassezia* species and from healthy donors. Patients' characteristics are listed in Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). The T<sub>H</sub>2 cytokine IL-13 was detected at significantly higher levels in supernatants of hTrx-stimulated PMBCs from patients with AD who were sensitized to *Malassezia* species compared with those from healthy donors, which was not the case for patients with AD who were not sensitized to *Malassezia* species (Fig 1, A). Levels of the T<sub>H</sub>1 cytokine IFN- $\gamma$  were significantly increased in supernatants from control PBMCs stimulated with Mala s 13, and a similar trend was observed for hTrx stimulation compared with levels seen in patients with AD who were not sensitized to *Malassezia* species (Fig 1, A). A number of other innate or T-cell cytokines (eg, IL-12p40 and IL-17) were induced efficiently both by hTrx and Mala s 13 in PBMCs from both patients with AD and healthy donors (Hradetzky et al, manuscript in preparation). Compared with  $\alpha$ -chain of the nascent polypeptide-associated complex ( $\alpha$ -NAC), another autoallergen whose cytokine effects we have studied before,<sup>E1,E2</sup> hTrx induced secretion of IFN- $\gamma$  and IL-10 more efficiently (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

The most prominent difference regarding hTrx-induced cytokine secretion between patients with AD and control donors was observed for IL-10: PBMCs from patients with AD who were

sensitized to *Malassezia* species secreted significantly lower levels of this anti-inflammatory cytokine in response to hTrx than those from the healthy donors, whereas there was no significant difference for non-*Malassezia* species-sensitized patients (Fig 1, B). The same was also true when patients were grouped according to IgE sensitization to Mala s 13 or hTrx (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Compared with the autoantigen hTrx, the exogenous allergen Mala s 13 induced lower levels of IL-10 in PBMCs from patients with AD who were sensitized to *Malassezia* species, as well as from healthy donors. Because Mala s 13 and hTrx share about 40% sequence identity,<sup>2</sup> the recognition of non-hTrx-cross-reactive epitopes on Mala s 13 might promote inflammation instead of tolerance induction. IL-10 was mainly secreted by monocytes, as shown in Fig 1, C.

Because it is known that monocytes from donors with the extrinsic form of AD express higher levels of the high-affinity IgE receptor than those from nonatopic donors,<sup>5</sup> we further assessed whether specific IgE to Mala s 13 and hTrx was directly involved in IL-10 and IL-13 regulation in monocytes. Therefore we treated PBMCs from patients with AD who were sensitized to Mala s 13 with lactic acid to remove surface-bound IgE.<sup>E3,E4</sup> By using this treatment, surface levels of IgE on monocytes were reduced (Fig 1, D), and IL-10 secretion was significantly increased after Mala s 13 stimulation but not after hTrx stimulation (Fig 1, E). In contrast, IL-13 secretion could be reduced after lactic acid treatment followed by hTrx stimulation but not by Mala s 13 stimulation (Fig 1, E). An explanation for these discrepancies might be the small fraction of Mala s 13- and hTrx-specific IgE in relation to the patient's total serum IgE levels. In fact, the increase in IL-10 levels after IgE stripping and stimulation with Mala s 13 correlated with the *Malassezia* species-specific IgE/total IgE ratio (Spearman  $r = 0.536$ ,  $P = .048$ ). In turn, this can be explained by the correlation of monocyte surface levels of Fc $\epsilon$ RI with total serum IgE levels.<sup>6</sup>

Our findings are in contrast to those of previous reports showing an increased IL-10 secretion of Fc $\epsilon$ RI-activated monocytes.<sup>7,8</sup> This might be due to differences in the experimental setup, such as the use of monoclonal IgE and anti-IgE for receptor cross-linking compared with our assay by using 1 distinct allergen, as well as due to different cell-culture conditions and the time point of analysis. Using anti-IgE stimulation, we could also upregulate IL-10 in PMBCs from patients with AD who were sensitized to *Malassezia* species (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Therefore this discrepancy between massive IgE receptor cross-linking with anti-IgE and the potentially weaker cross-linking resulting from application of native allergens should be considered in future studies.

The major findings of this study are (1) an IgE-dependent upregulation of the T<sub>H</sub>2 cytokine IL-13 by hTrx and (2) an impaired upregulation of IL-10 by hTrx in patients with AD who were sensitized to Mala s 13 and hTrx. IL-10 promotes the development of regulatory dendritic cells and T cells and therefore tolerance, and it is involved in a number of processes involved in the downregulation of a local inflammatory response.<sup>9</sup> The relative lack of antigen-specific IL-10 secretion in PBMCs from patients with AD who were sensitized to *Malassezia* species might contribute to the perpetuation of the local cutaneous

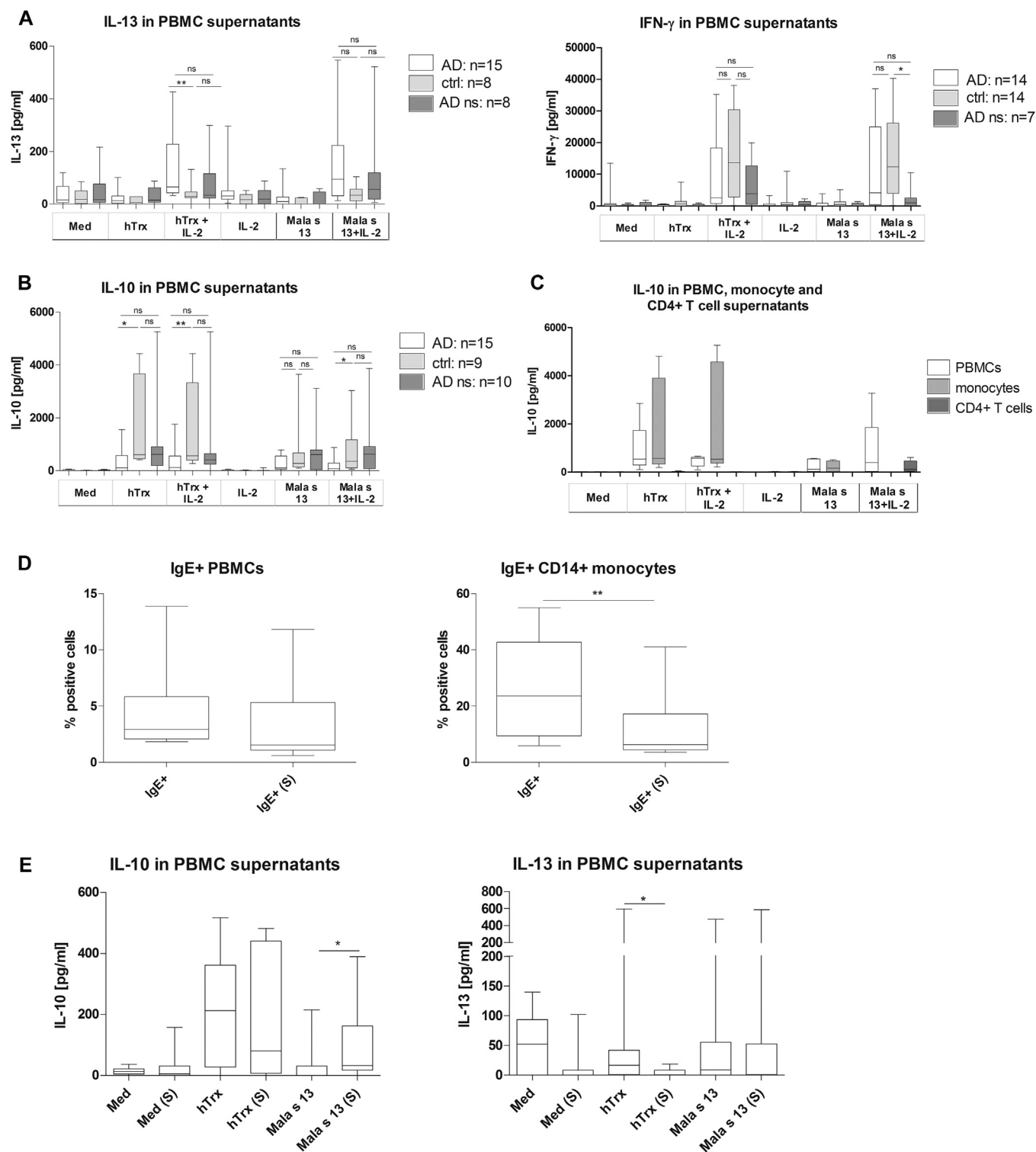


**FIG 1.** Variable cytokine induction by hTrx and Mala s 13 in PBMCs from patients with AD who were sensitized or not to *Malassezia* species and healthy donors. **A** and **B**, PBMCs from patients with AD who were sensitized to *Malassezia* species (AD), healthy donors (ctrl), and patients with AD who were not sensitized to *Malassezia* species (AD ns) were stimulated with 2.5  $\mu$ g/mL hTrx or Mala s 13 with or without IL-2 for 72 hours. Supernatants were analyzed for levels of IL-13 and IFN- $\gamma$  (Fig 1, A) or IL-10 (Fig 1, B) by means of ELISA. **C**, PBMCs, isolated monocytes, or isolated CD4 $^{+}$  T cells from 5 healthy donors were stimulated with hTrx and Mala s 13 with or without IL-2 for 3 days. Supernatants were analyzed for levels of IL-10 by means of ELISA. **D** and **E**, PBMCs from 10 patients with AD who were sensitized to Mala s 13 were treated with PBS or stripping buffer for 10 minutes. Cells were analyzed for surface IgE through flow cytometry (Fig 1, D) or stimulated afterward as indicated for 3 days, and supernatants were analyzed for levels of IL-10 and IL-13 by using ELISA (Fig 1, E). Medians and minimums/maximums are shown. \* $P < .05$  and \*\* $P < .01$ . ns, Not significant; S, stripping pretreatment.

inflammation in patients with AD and to further development of T<sub>H</sub>2- and IgE-mediated immune responses. Moreover, specific sensitization against hTrx can directly be involved in disease exacerbation in patients with AD because hTrx can be released from dermal or epidermal cells and might then promote inflammatory responses by activating specific T cells and cells expressing receptors for IgE.

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## METHODS

### Allergens and allergen extracts

Recombinant Mala s 13 and hTrx were produced as [His]<sub>6</sub>-tagged fusion proteins in *Escherichia coli* and purified by means of Ni<sup>2+</sup> affinity chromatography, as previously described.<sup>E5</sup> Recombinant  $\alpha$ -NAC was expressed in *E. coli* as a soluble protein and purified by means of Ni<sup>2+</sup> affinity chromatography, as described previously.<sup>E6</sup> The LPS concentration of hTrx was 10 ng/mg protein, corresponding to 25 pg/mL in the working concentration, and the LPS content of Mala s 13 was 1.5 ng/mg protein, corresponding to 3.75 pg/mL in the working concentration. The highest LPS concentration of 25 pg/mL was used as a control in all experiments.

### Blood donors

Blood was obtained from patients with AD who provided informed consent and were recruited from the outpatient clinic of the Department of Dermatology and Allergy, Hannover Medical School, Germany. Only patients without any immunosuppressive treatment were admitted. Sensitization to *Malassezia* species mix (m227) was analyzed by using Phadia with ImmunoCAP (Phadia AB, Uppsala, Sweden). Values of greater than 3.5 kU/L were regarded as positive for *Malassezia* species-specific IgE. Patients were tested for sensitization to Mala s 13 or hTrx by using ELISA. OD values at least 2.5 times greater than those of the negative control sera were regarded as positive.

Buffy coats from healthy voluntary blood donors provided by the Institute of Transfusion Medicine, Hannover Medical School, were used as control samples. The study was approved by the local ethics committee of Hannover Medical School and conducted according to the Declaration of Helsinki principles.

### Preparation of cells

PBMCs were isolated by means of density gradient centrifugation on Ficoll (Pan-Biotech, Aidenbach, Germany). Cells were cultured in Iscove medium (Biochrom KG, Berlin, Germany) supplemented with 4% human heat-inactivated AB serum, 2 mmol/L glutamine, 50 mg/mL gentamicin, 100 mg/mL penicillin and streptomycin, and nonessential amino acids. For some experiments, monocytes and CD4<sup>+</sup> T cells were isolated from PBMCs by means of magnetic cell sorting (Monocyte Isolation Kit II, CD4<sup>+</sup> T Cell Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany).

### Cell stimulation

The following stimulants were used: hTrx (2.5  $\mu$ g/mL), Mala s 13 (2.5  $\mu$ g/mL),  $\alpha$ -NAC (2.5  $\mu$ g/mL), IL-2 (50 U/mL; Roche Applied Science, Mannheim, Germany), and LPS (25 pg/mL; Sigma-Aldrich, Munich, Germany). Cells cultured at a density of  $1 \times 10^6$ /mL for all experiments were stimulated for 3 days for generation of supernatants. IgE stripping was

performed with a buffer of 0.13 mol/L NaCl, 0.05 mol/L KCl, and 0.01 mol/L lactate for 10 minutes at room temperature. Fc $\epsilon$ RI cross-linking was performed with human myeloma IgE (1  $\mu$ g/mL; Calbiochem, San Diego, Calif) for 1 hour at 37°C, followed by application of polyclonal rabbit anti-human IgE (20  $\mu$ g/mL; Dako, Glostrup, Denmark) for 1 hour at 37°C.

### Flow cytometric analysis

IgE stripping was assessed by staining PBMCs with a fluorescein isothiocyanate-labeled anti-CD14 antibody (Beckman Coulter, Krefeld, Germany) and a biotin-coupled anti-human IgE antibody (BD Biosciences, Heidelberg, Germany) detected with Streptavidin-allophycocyanin (BD Biosciences). Ten thousand cells were analyzed with the FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest software (Becton Dickinson).

### ELISA

Culture supernatants were taken 3 days after stimulation, centrifuged, and stored at -20°C. ELISAs were performed according to the manufacturer's instructions and measured with a FluoStar Reader (BMG Labtech GmbH, Ortenberg, Germany). ELISA kits from eBioscience (IL-10, IL-13, and IFN- $\gamma$ ; eBioscience, San Diego, Calif) were used.

### Statistical analyses

Statistical analysis was performed with the Wilcoxon signed-rank test or Mann-Whitney test (GraphPad Prism; GraphPad Software, San Diego, Calif).

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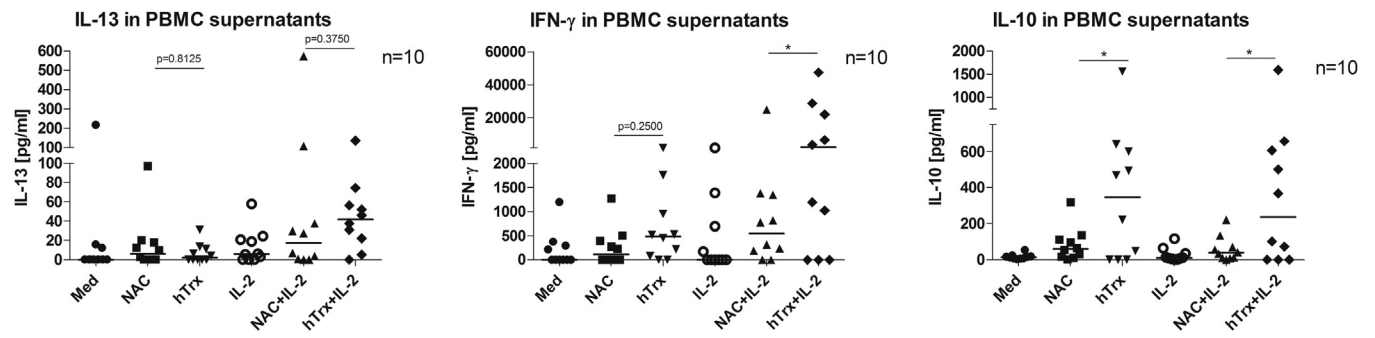


**FIG E1.** hTrx is more efficient in IFN- $\gamma$  and IL-10 induction in PBMCs from patients with AD than the autoallergen  $\alpha$ -NAC. PBMCs from patients with AD who were sensitized or not sensitized to the respective allergens were stimulated with 2.5  $\mu$ g/mL hTrx or 2.5  $\mu$ g/mL  $\alpha$ -NAC with or without IL-2 for 72 hours. Supernatants were analyzed for cytokine levels by means of ELISA. Medians are shown. \* $P < .05$ .

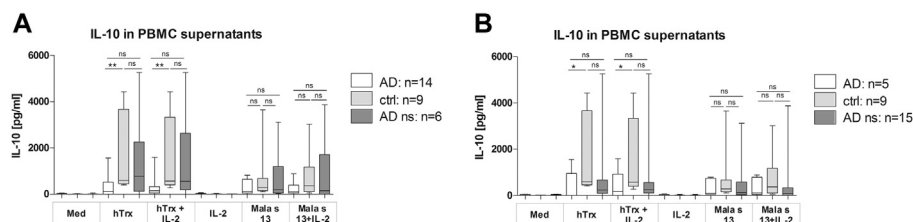


**FIG E2.** IgE sensitization to Mala s 13 or hTrx similarly affects IL-10 secretion in PBMCs from the respective sensitized patients with AD compared with control subjects. **A**, PBMCs from patients with AD who were sensitized to Mala s 13 (*AD*), healthy donors (*ctrl*), and patients with AD who were not sensitized to the respective antigen (*AD ns*) were stimulated with 2.5  $\mu$ g/mL hTrx or 2.5  $\mu$ g/mL Mala s 13 with or without IL-2 for 72 hours. Supernatants were analyzed for levels of IL-10 by means of ELISA. **B**, PBMCs from patients with AD who were sensitized to hTrx (*AD*) were compared with PBMCs from healthy donors (*ctrl*) and patients with AD who were not sensitized to the respective antigen (*AD ns*) under similar conditions, as described for Fig E2, A. Medians and minimums/maximums are shown. \* $P < .05$  and \*\* $P < .01$ . *ns*, Not significant.

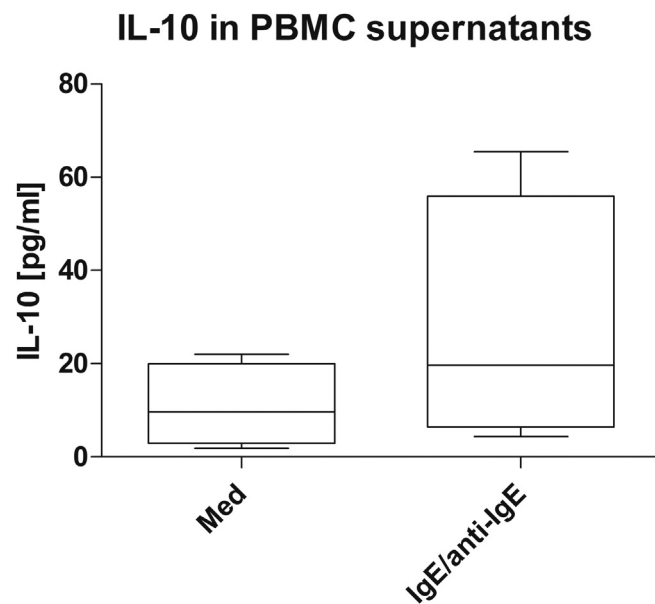


**FIG E3.** IL-10 is upregulated in PBMCs after monoclonal FcεR cross-linking. PBMCs from 4 patients with AD were stimulated with human myeloma IgE for 1 hour, washed, stimulated with rabbit anti-human IgE antibody for 1 hour, washed, and incubated for 24 hours. Supernatants were taken and analyzed for levels of IL-10 by means of ELISA.

**TABLE E1.** Patients' characteristics

|                                                             | Patients with AD and positive<br><i>Malassezia</i> species-specific IgE results | Patients with AD and negative<br><i>Malassezia</i> species-specific IgE results |
|-------------------------------------------------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| No. (male/female)                                           | 24 (15/9)                                                                       | 9 (3/6)                                                                         |
| Median age, y (range)                                       | 38 (20-75)                                                                      | 35 (22-73)                                                                      |
| Median total IgE, kU/L (range)                              | 2,678 (119-16,740)                                                              | 228 (4-37,870)                                                                  |
| Median <i>Malassezia</i> species-specific IgE, kU/L (range) | 27.25 (3.86-100)                                                                | 0.33 (0-3.36)                                                                   |
| Mala s 13-specific IgE                                      | 78%                                                                             | 44%                                                                             |
| hTrx-specific IgE                                           | 30%                                                                             | 33%                                                                             |
| Median SCORAD score (range)                                 | 34.95 (8.6-89.4)                                                                | 34.25 (11-57.5)                                                                 |