

Detection and Quantification of Specific IgE Antibodies against Eight *Malassezia* Species in Sera of Patients with Atopic Dermatitis by Using an Enzyme-Linked Immunosorbent Assay

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Received May 8, 2006; in revised form, August 9, 2006. Accepted August 10, 2006

Abstract: The lipophilic yeast *Malassezia*, a member of the cutaneous microflora, is an exacerbating factor in atopic dermatitis (AD). Of the 11 currently recognized species, *M. globosa* and *M. restricta* are found to frequently colonize the skin of AD patients. In this study, we attempted to quantify specific IgE antibodies against eight *Malassezia* species, namely, *M. dermatitis*, *M. furfur*, *M. globosa*, *M. obtusa*, *M. pachydermatis*, *M. slooffiae*, *M. sympodialis*, and *M. restricta*, in sera from AD patients by using an enzyme-linked immunosorbent assay (ELISA). The specific IgE value against *M. restricta* was greater than those against other *Malassezia* species. Competitive ELISA inhibition tests revealed that *M. restricta* contained species-specific as well as shared antigens. Therefore, *M. restricta* could be considered as a candidate diagnostic antigen for detecting anti-*Malassezia* IgE in sera from AD patients.

Key words: Atopic dermatitis, ELISA, Inhibition test, *Malassezia* species, Specific IgE

Malassezia species are yeasts that require lipids for growth, and they frequently colonize lipid-rich areas, such as the face, neck, and shoulders, on human bodies (3, 4, 9, 21). Although the *Malassezia* species are a part of the cutaneous microflora, they are associated with various skin diseases, including pityriasis versicolor, folliculitis, seborrheic dermatitis, and atopic dermatitis (AD) (1, 14). During the last decade, many studies have investigated the relationship between AD and *Malassezia* species. AD patients have specific serum IgE antibodies (Ab) against *Malassezia* (7, 15). Treatment of AD patients with antifungal agents decreases *Malassezia* colonization and the severity of eczematous lesions, suggesting that these species play a major role in AD (12, 22). Currently, 11 species are recognized in the genus *Malassezia*: *M. dermatitis*, *M. furfur*, *M. globosa*, *M. japonica*, *M. obtusa*, *M. slooffiae*, *M. sympodialis*, *M. restricta*, *M. yamatoensis*, *M. nana*, and *M. pachydermatis* (5, 6, 16, 18, 19). The last two species are typically found in animals. Using a PCR-based nonculture method, Sugita et al. detected both *M.*

globosa and *M. restricta* in almost all AD patients, while the remaining *Malassezia* species were observed in less than 40% of AD patients (17); this suggests that both *M. globosa* and *M. restricta* play an important role in AD.

Currently, two different assay systems, namely, AlaSTAT Microplate Allergen specific IgE (Diagnostic Products Corporation, Los Angeles, Calif., U.S.A.) with antigens from *M. furfur* TIMM 2782 (=CBS 1878) (2, 7) and CAP RAST FEIA (Pharmacia Diagnostics AB, Uppsala, Sweden) with antigens from *M. sympodialis* ATCC 42132, are commercially available to determine the serum values of *Malassezia*-specific IgE antibodies (24). Although the serum IgE values measured by the two assay systems show a good correlation, discrepancies have sometimes been observed between these assay systems (8). This may be attributable to the differences in the antigens employed in the assay. Therefore, it is important to find an appropriate diagnostic antigen for the detection of serum IgE Ab to *Malassezia* in patients with AD. In this study, we attempted to quantify specific IgE Ab against eight

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Abbreviations: AD, atopic dermatitis; CBS, Centraal Bureau voor Schimmelcultures; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

Table 1. Profiles of the serum samples of three AD patients used in the competitive ELISA inhibition tests

Serum No.	Age	Sex	Severity of AD	Total IgE IU/ml	AlaSTAT class	<i>M.globosa</i> U/ml	<i>M.restricta</i> U/ml	<i>M.slooffiae</i> U/ml	<i>M.sympodialis</i> U/ml	<i>M.dermatitis</i> U/ml	<i>M.furfur</i> U/ml	<i>M.obtusa</i> U/ml	<i>M.pachydermatis</i> U/ml
#12	27	male	severe	36,551	6	67.6	93.3	45.9	75.3	65.2	27.8	19.5	56.8
#117	27	female	moderate	17,300	6	11.1	47.2	6.6	16.1	37.9	21.4	37.7	8.4
#126	15	male	moderate	3,120	6	12.9	66.7	43.2	13.1	48.7	38.6	34.7	10.0

Malassezia species, including *M. dermatitis*, *M. furfur*, *M. globosa*, *M. obtusa*, *M. pachydermatis*, *M. slooffiae*, *M. sympodialis*, and *M. restricta*, by using an enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Subjects. Sera from 20 AD patients, including 11 males and 9 females, aged 15 to 64 years (mean age, 33.4 ± 11.4 years) were used in this study. The initial inclusion of AD patients was on the basis of an AlaSTAT titer for *M. furfur*-specific IgE Ab >3 IU/ml. The study was approved by the ethics committee at Meiji Pharmaceutical University.

Preparation of *Malassezia* antigens. *Malassezia dermatitis* (CBS 9196), *M. furfur* (CBS 1878), *M. globosa* (CBS 7966), *M. obtusa* (CBS 7876), *M. pachydermatis* (CBS 1879), *M. slooffiae* (CBS 7956), *M. sympodialis* (CBS 7222), and *M. restricta* (CBS 7877) were grown on modified Leeming and Notman agar (10 g glucose, 10 g polypeptone, 8 g bile salts, 2 g yeast extract, 0.5 g glycerol monostearate, 10 ml glycerol, 5 ml Tween 60, 20 ml Olive oil) (Oxoid, Hampshire, U.K.) containing 1% Tween 40, 0.2% glycerol, and 50 µg chloramphenicol/ml (Sankyo, Tokyo) at 32 C for 5 days. The cultures were harvested and washed five times with phosphate-buffered saline (PBS). The cells were suspended in three volumes of PBS and subjected to mechanical disruption by using a French press cell (Aminco, St. Louis, Mo., U.S.A.) at 18,000 pounds/square inch. The cell suspension was centrifuged at 14,500 rpm for 1 hr, and the supernatant was used as a source of antigen.

Determination of specific IgE antibodies against *Malassezia* species. The IgE antibody value against each *Malassezia* species was determined using the AlaSTAT Microplate system (Diagnostic Products Corporation) with slight modifications (20, 23). Briefly, the antigens extracted from the eight *Malassezia* species were adjusted to a protein concentration of 250 µg/ml with PBS (pH 7.2). The wells of a microtiter plate (Corning, New York, N.Y., U.S.A.) were coated with 100 µl of each antigen for 18 to 20 hr at 4–8 C. Fifty microliters of serum sample was added to each well of the antigen-coated microtiter plate, followed by peroxidase-labeled anti-IgE antibodies. After adding TMB

(3,3',5,5'-tetramethylbenzidine dissolved in H₂O₂) substrate solution, the absorbance at 650 nm was measured at 10-sec intervals for 5 min. The IgE antibody value (units/ml) of the patients' samples was calculated using a standard curve based on a reference serum in accordance with the 2nd IRP 75/502 of the World Health Organization.

Competitive ELISA inhibition test. Competitive ELISA inhibition tests were performed to determine the specificity of IgE binding to each *Malassezia* antigen. Sera from three AD patients were used in this assay (Table 1). The serum samples were diluted 1/5–1/7 in PBS. Starting from 1 mg/ml, 10-fold serial dilutions of extracts from the eight *Malassezia* species were prepared in PBS and used as inhibiting fluid-phase antigens. A 25-µl aliquot of the patients' sera was mixed with an equal volume of the inhibiting fluid-phase antigens overnight at 4–8 C. The mixture was then added to each well of an antigen-coated microtiter plate. The same steps were followed as those for ELISA. After studying the control samples, in which an equal volume of PBS was preincubated instead of inhibitors, the inhibition of specific IgE binding was expressed as % inhibition.

Statistical analysis. We used Fischer's exact test to compare the IgE antibody values between the groups. A *P* value of <0.05 was considered statistically significant. The Spearman's rank order correlation test was used for correlation analysis.

Results

Specific IgE Antibody Values against Each of the Eight Malassezia Species

The specific IgE antibody value against each *Malassezia* species in the sera from 20 AD patients was 15.4 ± 22.9 U/ml (mean $\pm$ standard deviation) for *M. globosa*, 38.5 ± 33.2 U/ml for *M. restricta*, 20.5 ± 30.9 U/ml for *M. slooffiae*, 19.0 ± 27.1 U/ml for *M. sympodialis*, 29.9 ± 28.0 U/ml for *M. dermatitis*, 15.9 ± 18.9 U/ml for *M. furfur*, 15.8 ± 21.7 U/ml for *M. obtusa*, and 13.8 ± 24.8 U/ml for *M. pachydermatis* (Fig. 1). The specific IgE value against the *M. restricta* antigen in the patients' sera was significantly ($P < 0.05$) higher than those against the other 5 *Malassezia* species—*M. glo-*

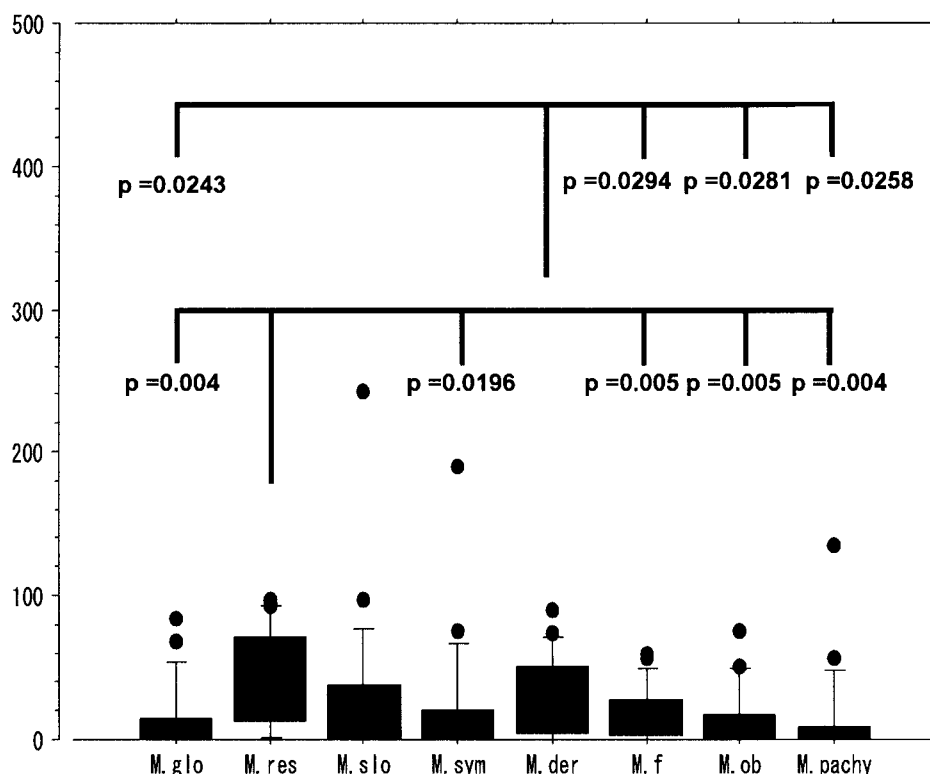


Fig. 1. The species-specific IgE values of eight *Malassezia* species in sera from 20 AD patients determined using an ELISA method. Abbreviations: M.glo, *M. globosa*; M.res, *M. restricta*; M.slo, *M. slooffiae*; M.sym, *M. sympodialis*; M.der, *M. dermatitis*; M.f, *M. furfur*; M.ob, *M. obtusa*; M.pachy, *M. pachydermatis*.

Table 2. The correlation coefficients between the specific IgE values against the eight *Malassezia* species and the total IgE values in sera from 12 AD patients

	Total IgE ^{a)}	<i>M.globosa</i>	<i>M.restricta</i>	<i>M.slooffiae</i>	<i>M.sympodialis</i>	<i>M.dermatitis</i>	<i>M.furfur</i>	<i>M.obtusa</i>
<i>M.globosa</i>	0.604							
<i>M.restricta</i>	0.644	0.623						
<i>M.slooffiae</i>	0.344	0.851	0.467					
<i>M.sympodialis</i>	0.593	0.941	0.534	0.951				
<i>M.dermatitis</i>	0.555	0.778	0.534	0.680	0.731			
<i>M.furfur</i>	0.306	0.705	0.754	0.692	0.662	0.663		
<i>M.obtusa</i>	0.277	0.760	0.564	0.831	0.779	0.764	0.884	
<i>M.pachydermatis</i>	0.468	0.942	0.591	0.937	0.987	0.707	0.719	0.791

^{a)} Determined by only 12 AD patients' sera.

bosa, *M. sympodialis*, *M. furfur*, *M. obtusa*, and *M. pachydermatis*. Table 2 presents the correlation coefficients between each of the specific IgE values against the eight *Malassezia* species and the total IgE values in sera from the 12 AD patients. The specific IgE value against the *M. restricta* antigen showed a higher correlation with the total IgE value ($r=0.644$) than that against all the other *Malassezia* species antigens. We also found that the specific IgE value against the *M. restricta* antigen showed a relatively poor correlation with that against all the other *Malassezia* species anti-

gens ($r=0.467$ – 0.623), except the *M. furfur* antigen ($r=0.754$).

Competitive ELISA Inhibition Test

To investigate the antigen specificity of each *Malassezia* species, competitive ELISA inhibition tests were performed. Figure 2 shows the inhibition of IgE binding to *M. globosa* and *M. restricta* antigens with different concentrations of fluid-phase antigens from the eight *Malassezia* species. As shown in Fig. 2a, the fluid-phase antigens from all *Malassezia* species

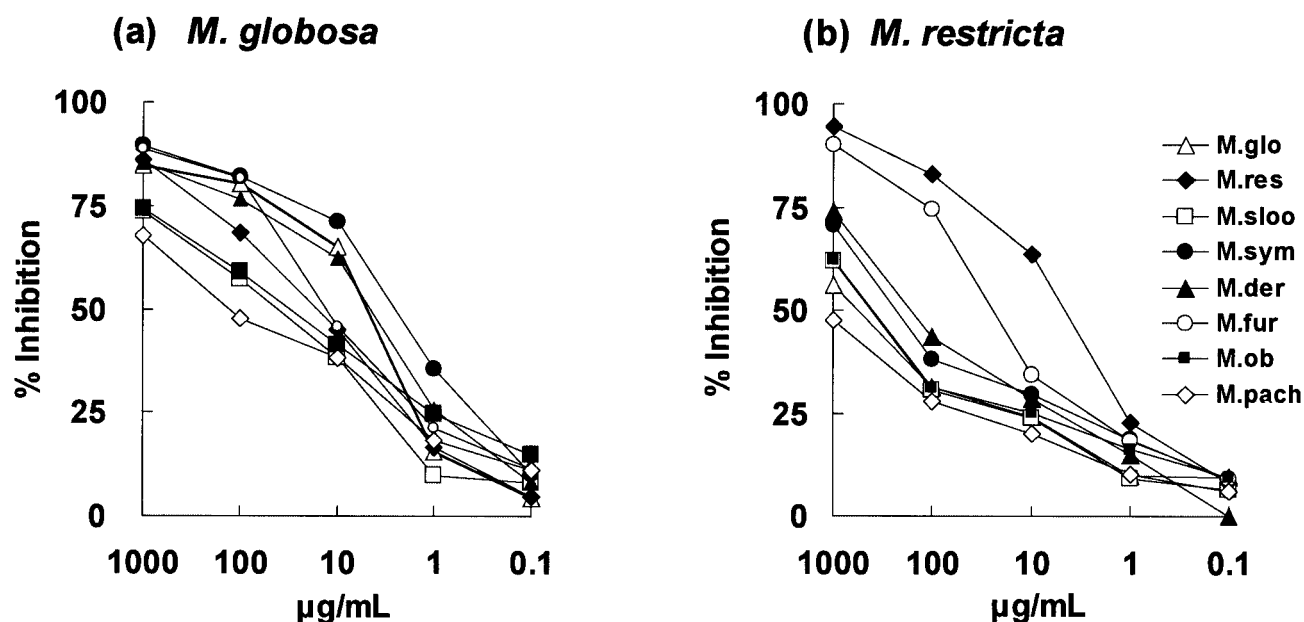


Fig. 2. Inhibition of IgE binding to solid-phase antigens from (a) *Malassezia globosa* and (b) *Malassezia restricta* by using fluid-phase antigens from eight *Malassezia* species.

Table 3. The 50% inhibition levels of IgE binding to solid-phase antigens from each *Malassezia* species

Solid-phase antigen	50% inhibition level (µg/ml)							
	Fluid-phase antigen (inhibitor antigen)							
	<i>M.globosa</i>	<i>M.restricta</i>	<i>M.slooffiae</i>	<i>M.sympodialis</i>	<i>M.dermatitis</i>	<i>M.furfur</i>	<i>M.obtusa</i>	<i>M.pachydermatis</i>
<i>M.globosa</i>	5.6±1.0	16.3±4.6	17.8±3.0	7.4±4.9	45.4±29.7	14.2±8.7	194.2±216.8	29.9±6.1
<i>M.restricta</i>	117.8±54.3	5.2±0.3	278.8±82.5	83.0±16.2	135.4±39.6	38.6±31.4	157.9±88.1	563.2±231.7
<i>M.slooffiae</i>	36.0±15.0	7.0±3.0	1.1±0.1	10.7±4.5	68.5±43.0	5.0±1.5	26.3±7.6	238.4±117.1
<i>M.sympodialis</i>	5.3±1.1	18.0±4.9	53.8±17.9	1.8±0.4	2.8±0.6	11.5±5.7	45.0±39.6	51.5±26.8
<i>M.dermatitis</i>	11.3±4.0	21.0±4.3	92.0±44.0	3.6±0.5	1.1±0.1	7.3±1.3	202.0±230.7	201.7±89.1
<i>M.furfur</i>	42.7±20.9	2.9±0.3	12.0±7.8	18.7±3.5	6.4±2.6	0.6±0.2	1.1±0.2	16.5±9.2
<i>M.obtusa</i>	9.1±1.5	11.3±3.3	63.8±79.6	3.5±2.6	99.0±68.3	3.1±0.6	2.2±2.0	332.8±178.5
<i>M.pachydermatis</i>	43.7±11.3	11.3±2.2	203.3±183.1	32.4±10.8	12.4±13.8	3.1±2.1	41.3±27.8	7.9±2.0

Data represent mean ± standard deviation obtained from the sera of three AD patients.

markedly inhibited IgE binding to solid-phase *M. globosa* antigen in a concentration-dependent manner (75–90% inhibition at the highest concentration). The binding of IgE to the *M. restricta* antigen was markedly inhibited only by the fluid-phase *M. furfur* antigen (Fig. 2b). Table 3 presents the 50% inhibition levels of IgE binding to the solid-phase antigens from each of the *Malassezia* species. The binding of IgE to the *M. restricta* antigen was hardly inhibited by the fluid-phase antigens from all the other *Malassezia* species (50% inhibition levels were 38.6–563.2 µg/ml), whereas the fluid-phase antigen from this species produced a marked inhibition (50% inhibition level was 5.2 µg/ml). On the other hand, the fluid-phase *M. restricta* antigen produced a remarkable inhibition of IgE binding to the solid-phase antigens from all the other *Malassezia*

species (50% inhibition levels were 2.9–21.0 µg/ml). These results suggest that *M. restricta* contains species-specific as well as common antigens that are shared by all the *Malassezia* species tested in this study.

Discussion

The *Malassezia* yeasts play a role in the pathogenesis of AD (12, 22). Hence, serum analysis for the presence of IgE antibodies against *Malassezia* can be a useful tool for the diagnosis of AD. Since 11 *Malassezia* species have been recognized to date, it is essential to consider the use of appropriate antigens for the detection of IgE antibodies to *Malassezia* in patients with AD. Sugita et al. (17) have reported that the skin of 90% of the patients showed the presence of *M. globosa* and *M.*

restricta, *M. sympodialis* and *M. furfur* were detected in 40% of the patients. On the other hand, *M. slooffiae* DNA was found in less than 7% of the patients. This suggests that both *M. globosa* and *M. restricta* play an important role in the pathogenesis of AD. Using sera from AD patients, Koyama et al. (10) determined the IgE reactivity against five *Malassezia* species by Western blot analysis. They reported that the extracts from *M. globosa*, *M. restricta*, *M. slooffiae*, *M. sympodialis*, and *M. furfur* reacted with serum IgE antibodies from 83%, 56%, 50%, 74%, and 65% of AD patients, respectively. Based on an immunoblotting assay, Zargari et al. (24) reported that *M. globosa* and *M. restricta* have more IgE-binding components than other *Malassezia* species. However, thus far, only a few quantitative studies involved serological analysis for each of the recognized *Malassezia* species in AD patients. In this study, we quantified the specific IgE values against eight *Malassezia* species in sera from 20 AD patients by using ELISA.

Quantitative ELISA demonstrated that the sera from AD patients contained a high titer of IgE antibodies against *M. restricta* (Fig. 1). We also found that the specific IgE value against *M. restricta* was closely correlated with the total IgE value. Since the total IgE is the sum total of multiple individual allergen-specific IgE levels, it is conceivable that a high titer of specific anti-*M. restricta* IgE may reflect the elevated total IgE value in AD patients. However, since increased levels of total IgE may be detected even in situations without an allergic background, the measurement of total IgE has a poor positive or negative predictive value for the serodiagnosis of AD.

Our inhibition study demonstrated that IgE binding to the *M. restricta* antigen was hardly inhibited by antigens from the other *Malassezia* species (Fig. 2b); this suggests that *M. restricta* contains a considerable amount of species-specific antigens. Only the fluid-phase *M. furfur* antigen inhibited IgE binding to the solid-phase antigens from *M. restricta*. This suggests that *M. furfur* and *M. restricta* share antigens to a considerable extent. This finding was supported by the result that in sera from AD patients, the specific IgE value against *M. restricta* showed a good correlation with that against *M. furfur* (Table 2). We also found that IgE binding to the *M. globosa* antigen was efficiently inhibited by antigens from all other *Malassezia* species, except *M. obtusa*. This indicates that *M. globosa* contains a considerable amount of antigens that are common to other species (Fig. 2a and Table 3). This finding was consistent with the result that the specific IgE values against *M. globosa* showed a good correlation with that against the other species. All these

findings are in agreement with the results of previous studies indicating that the *Malassezia* species contain both species-specific and common allergenic components that react with IgE antibodies (10, 24).

Based on Western blot analysis, Zargari et al. (24) reported a 54% inhibition of IgE binding to the *M. sympodialis* antigen by the *M. globosa* antigen and a 60% inhibition by the *M. restricta* antigen. These inhibitory values were lower than that for the other species. In contrast, our competitive ELISA inhibition tests demonstrated that IgE binding to the *M. sympodialis* antigen was efficiently inhibited by *M. globosa* and *M. restricta* antigens (50% inhibition levels were 5.3 and 18.0 µg/ml, respectively). This discrepancy may be due to the differences in the assay systems employed. Our ELISA inhibition test facilitates more quantitative comparisons of cross-reactivity.

Although Koyama et al. (11) previously extracted *Malassezia* antigens from intact organisms with 2-mercaptoethanol (2ME), we prepared antigens from an extract of mechanically homogenized cells. We followed this procedure because the protein concentration of the antigens in the 2ME extract was considerably lower than that of the antigens produced by mechanical homogenization (data not shown). Furthermore, we detected only trace levels of IgE antibodies against the 2-ME extracts from *M. globosa* and *M. restricta* (data not shown). The preparation of microbial antigens is often influenced by culture conditions such as the medium, incubation time, and temperature, even when the same strain is used (13). However, when we used three different preparations of *M. restricta* antigens for detecting IgE antibodies in sera from AD patients, no significant batch-to-batch variation in the preparations was observed (data not shown).

In conclusion, quantitative ELISA demonstrated that the specific IgE value against *M. restricta* was greater than those against other *Malassezia* species. Competitive ELISA inhibition tests revealed that *M. restricta* contained species-specific as well as shared antigens. These findings suggest that *M. restricta* could be considered as one of the candidates for the identification of IgE antibodies in AD patients suspected to have *Malassezia* sensitization.

This study was supported in part by research grants from the Japan Society for the Promotion of Science (#16590127) (TS), the Ministry of Education, Culture, Sports, Science and Technology, Japan for an Open Research Center Project (TS and YI), and the IFO Foundation (TS).

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