FUNCTIONAL ROLES OF TWO POLYPEPTIDE CHAINS THAT COMPOSE AN ANTIGEN-SPECIFIC SUPPRESSOR T CELL FACTOR

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Our previous studies (1) have demonstrated that a soluble factor (TsF)[1] obtained from primed suppressor T cells or suppressor T cell hybridomas specific for keyhole limpet hemocyanin (KLH) is composed of two discrete polypeptide chains having molecular weights of 45,000 (heavy chain) and 28,000 (light chain), respectively, and mediates the suppression of antihapten IgG plaque-forming cell (PFC) response in a carrier-specific and genetically restricted manner (2). The heavy chain possesses the two functional domains, such as the antigen-binding moiety and the putative constant region determinant (Ct) (3). The light chain carries the I-J antigenic determinant (1).

Furthermore, the two polypeptide chains have also been demonstrated to be essential for the functional expression of the TsF activity (4, 5). Neither the heavy or light chain can exert any biological activity by itself, whereas the mixture of these two molecules reconstitutes the active suppressor function. The requirement of the two chains in the functional expression of TsF has also been reported by other investigators using different antigen-specific TsF (6, 7).

We have previously reported (8) that the 11S and 13S mRNA coding for the heavy and light chains of KLH-TsF are fractionated and translated in *Xenopus laevis* oocytes, and also that the mixture of the translated heavy and light chains successfully reconstitutes the active form of TsF that mediates the KLH-specific and genetically restricted suppressor activity (8).

Based on the above findings, it is clear that the two-chain TsF is distinct from TsF composed of a single molecule that carries the antigen-binding moiety and the I-J antigenic determinant, as reported by Krupen et al. (9). However, the functional roles of the two polypeptide chains in the expression of the TsF activity have not been determined. The use of the mRNA translates of TsF permits us to investigate the biological properties of the heavy and light chains of TsF.

In this communication, we describe the functional roles of the heavy and light chains of TsF in suppressing the antibody response in an antigen-specific and

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[1] *Abbreviations used in this paper:* BPV, *Bordetella pertussis* vaccine; DNP, 2,4-dinitrophenyl; GT, $Glu^{50}Tyr^{50}$; KLH, keyhole limpet hemocyanin; OVA, ovalbumin; PFC, plaque-forming cell; PMSF, phenylmethylsulfonylfluoride; TsF, suppressor T cell factor.

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genetically restricted fashion. The heavy chain mediates the antigen specificity and the light chain works as an element to determine the restricting specificity of TsF.

Materials and Methods

Animals. C57BL/6 CrSlc (H-2^b), C3H/HeJ CrSlc (H-2^k), and BALB/c CrSlc (H-2^d) mice were purchased from Shizuoka Experimental Animal Laboratory Co. Ltd., Hamamatsu, Japan. B10.A(3R) and B10.A(5R) mice were raised in our animal facility.

Antigens. KLH was purchased from Calbiochem-Behring Corp., San Diego, CA. Ovalbumin (OVA), recrystallized five times, was obtained from Sigma Chemical Co., St. Louis, MO. Dinitrophenylated KLH (DNP-KLH) and OVA (DNP-OVA) were prepared by coupling with 2,4-dinitrobenzenesulfonic acid under alkaline conditions. *Bordetella pertussis* vaccine (BPV) was purchased from the Chiba Serum Institute, Chiba, Japan.

Preparation of TsF. KLH-TsF and OVA-TsF were prepared as described previously (10). Briefly, mice were intraperitoneally injected twice with 200 μg KLH or OVA at a 10-d interval. 10 d after the second immunization, single-cell suspensions of their thymocytes were prepared, adjusted to 1×10^8 cells/ml in phosphate-buffered saline containing 0.2 mM phenylmethyl sulfonylfluoride (PMSF), and disrupted by freezing and thawing 10 times. The freeze-thaw materials were centrifuged at 20,000 *g* for 1 h at 4°C.

Immunoadsorbents. Conventional anti-I-J^k and anti-I-J^b antiserum were raised in our laboratory as previously described (10). Immunoadsorbents were prepared by coupling the gamma globulin fractions of anti-I-J antisera, KLH, or OVA to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals Piscataway, NJ) at 5–10 mg protein/ml packed gel as previously described (10). 1 ml of protein-conjugated Sepharose beads was packed into a 1-ml plastic syringe. For the preparation of the KLH- or OVA-binding heavy chain, the KLH- or OVA-primed C57BL/6 thymocyte extracts were applied to the first column composed of anti-I-J^b. The effluent was further incubated with the second KLH or OVA column. The absorbed materials were eluted with 0.175 M glycine HCl buffer, pH 3.2. For preparation of the I-J-positive light chain, KLH-primed thymocyte extracts equivalent to 1.5×10^7 cells were reacted with the KLH column. The effluent was successively applied to the second anti-I-J^b or anti-I-J^k column. The acid eluate was neutralized and dialyzed against phosphate-buffered saline, pH 7.2. In some experiments, the effluent from the antigen column was used as the source of the light chain of TsF. The effluent or eluate was divided and added to six cultures per group (extract equivalent to $\sim 2.0 \times 10^6$ cells/culture).

Preparation of mRNA and Its In Vitro Translation. The preparation of the mRNA used was described by Chirgwin et al. (11). The production and characterization of C57BL/6-derived KLH-TsF-producing hybridomas were described previously (2, 8). The cultured hybridoma cell pellets (10^9 cells) were resuspended in 10 ml of a guanidium thiocyanate solution (4.2 M guanidium thiocyanate, pH 7.0, containing 25 mM sodium citrate, 0.5% *N*-laurylsarcosin, and 0.1% antiform A). The cell suspensions were homogenized with a potter homogenizer. The homogenized materials were overlayed on a CsCl solution (5.7 M $CsCl_2$, 0.1 M EDTA, pH 7.0) (ratio of materials/$CsCl_2$ solution, 2:1, vol/vol) in a polyallomer tube (1.3 × 13 cm, 13 PA tube; Hitachi Koki Co., Ltd., Tokyo) and centrifuged at 230,000 *g* for 15 h at 15°C by a swing rotor. The pellets were rinsed with cold 80% ethanol, dried, and resuspended in distilled water. The RNA solution (1 mg/ml), which had been adjusted at a final concentration of 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM EDTA and 0.5 M KCl, was applied to the column (1.5 × 5 cm) composed of 5 ml of oligo(dT)-cellulose type 7 (P-L Biochemicals, Inc., Milwaukee, WI). Poly (A)-positive RNA (mRNA) were eluted with Tris-HCl buffer containing 0.1 mM EDTA, pH 7.4, under monitoring at 260 nm with a UV monitor (model UA-500; ISCO, Lincoln, NE), pelleted with ethanol precipitation, and dissolved in distilled water (5 mg/ml). mRNA was further fractionated by 5–22% linear sucrose density gradient centrifugation at 230,000 *g* for 16 h with a swing rotor. 30–50 nl of the fractionated mRNA of size 11S and 13S was injected into fully grown oocytes from healthy, adult female *Xenopus*

laevis using a fine micropipette under an inverted microscope. 10 oocytes injected with the same fraction of mRNA were incubated at 20°C for 36 h in 100 μl of a sterile Barth's medium (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM Hepes, pH 7.4, containing 10 mg/ml penicillin and 10 mg/ml streptomycin sulfate). The translation products were prepared by centrifugation of oocyte homogenates at 14,000 *g* for 20 min. The supernate was collected, added to 0.2 mM PMSF, and stored at −80°C until use. The 11S and 13S mRNA translation products were used as the I-J^b-positive light chains and KLH-binding heavy chains, respectively. Usually, either the 11S or 13S mRNA product was used at a final concentration of 1.25 μl/ml.

Assay for Suppressor Activity. The assay system to detect suppressor activity was described previously (2). Briefly, 4×10^6/ml spleen cells of mice primed with 100 μg of DNP-KLH or DNP-OVA and 1×10^9 BPV were cultured for 5 d in a well (200 μl) of a Mishell-Dutton culture plate with RPMI 1640 enriched with 10% fetal calf serum, in the presence of 0.1 μg/ml of DNP-KLH or DNP-OVA. The mixture of the isolated heavy and light chains was added to the culture at the start of cultivation. Anti-DNP IgG PFC were enumerated using DNP-coupled sheep erythrocytes as described (10).

Results

Element Mediating the Antigen Specificity of TsF. The heavy chain per se carries the specific affinity for the native antigen. It is therefore likely that the heavy chain determines the antigen specificity of TsF. To test this possibility, the heavy chains of TsF with KLH or OVA specificity were isolated from the conventional KLH- or OVA-primed thymocyte extracts by two successive immunoadsorbent columns of KLH or OVA and anti-I-J^b. The isolated heavy chain with KLH- or OVA-binding activity was mixed with the 11S mRNA translation products of the I-J-positive light chain (Table I). The mixture of the isolated heavy and light chains from KLH- or OVA-primed thymocyte extracts suppressed the anti-DNP

TABLE I

Reconstitution of Antigen-specific TsF Activity with 11S mRNA Translation Products and Isolated OVA- or KLH-binding Chains

Materials obtained from:		Anti-DNP IgG PFC response to:	
Light chain*	Heavy chain‡	DNP-KLH	DNP-OVA
—	—	2,310 ± 410§	950 ± 130
TsF	KLH-TsF	550 ± 260	830 ± 150
TsF	OVA-TsF	2,510 ± 460	160 ± 100
—	KLH-TsF	2,640 ± 400	800 ± 140
—	OVA-TsF	2,290 ± 410	860 ± 240
11S mRNA translates	13S mRNA translates	620 ± 460	860 ± 130
11S mRNA translates	—	2,410 ± 440	830 ± 70
11S mRNA translates	KLH-TsF	900 ± 290	1,070 ± 160
11S mRNA translates	OVA-TsF	2,350 ± 510	180 ± 90

* I-J-positive light chains were purified by two successive immunoabsorbents that had been applied with C57BL/6-derived KLH-primed thymocyte extracts equivalent to 1.5×10^7 cells (see Materials and Methods). Eluate was divided and added to six cultures per group (TsF equivalent to 2.0×10^6 cells per culture). 1.25 μl/culture of the 11S mRNA translation product was used.

‡ KLH- or OVA-binding heavy chains were purified by two successive immunoabsorbents that had been applied with C57BL/6-derived KLH- or OVA-primed thymocyte extracts equivalent to 1.5×10^7 cells. Eluate was divided and added to six cultures per group. 1.25 μl/ml of the 13S mRNA translate was used.

§ Arithmetic means of PFC numbers of six cultures ± SD.

IgG PFC response in an antigen-specific manner. Similarly, the translated light chain and the KLH-binding heavy chain suppressed only the responses against DNP-KLH but not those to DNP-OVA, whereas the mixture in combination with the OVA-binding heavy chain mediated the TsF function in the responses to DNP-OVA but not to DNP-KLH. However, the 11S mRNA product of the I-J-positive light chain or the isolated heavy chain by itself had no functional activity. Thus the antigen specificity of TsF entirely depends on the function of the antigen-binding heavy chain.

Element Mediating the Genetic Restriction Specificity of TsF. Similar experiments to determine the genetic restriction specificity of TsF were carried out. In these experiments, the light chains with different I-J antigenic determinants were purified by two successive immunoadsorbent columns using the anti-I-J^k or anti-I-J^b and antigen column. The isolated light chain, which did not show functional activity by itself, was mixed with the translated KLH-binding heavy chain. The activity of the mixture was investigated in the responses of C3H (H-2^k) and C57BL/6 (H-2^b) mice primed with DNP-KLH. The results shown in Table II demonstrate that the I-J^k-positive light chain from C3H and the 13S mRNA translation product of the KLH-binding chain suppressed the responses of syngeneic C3H but not allogeneic C57BL/6 mice. When the same translated heavy chain was mixed with the I-J^b-positive isolated light chain from C57BL/6, C57BL/6 but not C3H response was preferentially suppressed.

The same results were obtained when the effluent of primed thymocyte extracts from the antigen column was used as the source of the light chain. Since the thymocyte extracts contain free heavy and light chains as well as combined forms of these two chains, the free light chain can be obtained in the effluent

TABLE II

Reconstitution of Genetically Restricted TsF Activity with 13S mRNA Translation Products and Isolated I-J-positive Light Chains

Materials obtained from:		Anti-DNP IgG PFC response in:	
Light chain*	Heavy chain‡	C57BL/6	C3H
—	—	4,800 ± 660§	1,410 ± 390
C57BL/6 TsF	C57BL/6 TsF	1,230 ± 500	1,320 ± 330
C3H TsF	C3H TsF	3,930 ± 710	280 ± 100
C57BL/6 TsF	—	3,830 ± 510	1,420 ± 340
C3H TsF	—	3,840 ± 320	1,360 ± 440
11S mRNA translates	13S mRNA translates	1,130 ± 460	1,230 ± 300
—	13S mRNA translates	4,070 ± 540	1,200 ± 380
C57BL/6 TsF	13S mRNA translates	1,770 ± 600	1,280 ± 290
C3H TsF	13S mRNA translates	3,970 ± 670	270 ± 180

* I-J-positive light chains were purified by two successive immunoabsorbent columns that had been applied with thymocyte extracts equivalent to 1.5×10^7 cells (see Materials and Methods). Eluate was divided and added to six cultures per group (TsF equivalent to 2.0×10^6 cells per culture). The 11S mRNA translation product was the same as used in Table I.

‡ KLH-binding heavy chains were purified by two successive immunoabsorbent columns that had been applied with KLH-primed C57BL/6 or C3H thymocyte extracts equivalent to 1.5×10^7 cells (see Materials and Methods). Eluate was divided and added to six cultures per group. The 13S mRNA translation product was the same as in Table I.

§ Arithmetic means of PFC numbers of six cultures ± SD.

from the antigen column. The effluent of either BALB/c or C57BL/6 thymocyte extract from the KLH column was mixed with the translated heavy chain and tested for suppressor activity. As demonstrated in Table III, the BALB/c (H-2^d) but not C57BL/6 (H-2^b) antibody response was predominantly suppressed when the effluent of BALB/c TsF was mixed with the 13S mRNA translation product of the heavy chain. Similarly, the C57BL/6 light chain with the translated heavy chain could not suppress the BALB/c responses.

It is thus strongly suggested that the light chain determines the genetic specificity of TsF and also that there is no genetic preferential combination between heavy and light chains in the functional expression of the TsF activity.

The Restricting Specificity of the B10.A(3R) or B10.A(5R) Light Chain. For further analysis of the genetic specificity of the restricting element of TsF, the free light chains of the B10.A(3R) and B10.A(5R) TsF were obtained in the effluent from the antigen column. They were then mixed with the 13S mRNA translation product of the heavy chain. As shown in Table IV, the light chain from B10.A(3R) TsF and the translated heavy chain suppressed the responses of C57BL/6 (H-2^b) but not those of C3H (H-2^k) mice. Similarly, only C3H responses were exclusively suppressed when the B10.A(5R) but not B10.A(3R) light chain was used. It is apparent that the B10.A(3R) and B10.A(5R) TsF provide the light chains with I-J^b and I-J^k antigenic determinants, respectively.

Discussion

The functional roles of the two polypeptide (heavy and light) chains that compose the TsF which mediates the antigen specific and genetically restricted suppressor function were investigated by the use of the isolated heavy and light

TABLE III

Reconstitution of TsF Activity with 13S mRNA Translation Products and Effluent of C57BL/6 or BALB/c Thymocyte Extracts from Antigen Column

Materials obtained from:		Anti-DNP IgG PFC response in:	
Light chain*	Heavy chain‡	C57BL/6	BALB/c
—	—	2,470 ± 360§	2,440 ± 400
C57BL/6 TsF	C57BL/6 TsF	340 ± 100	2,880 ± 420
BALB/c TsF	BALB/c TsF	2,280 ± 270	250 ± 200
C57BL/6 TsF	—	2,390 ± 360	2,350 ± 360
BALB/c TsF	—	2,410 ± 260	2,490 ± 400
11S mRNA translates	13S mRNA translates	320 ± 300	ND‖
—	13S mRNA translates	2,210 ± 180	2,550 ± 710
C57BL/6 TsF	13S mRNA translates	220 ± 60	1,830 ± 350
BALB/c TsF	13S mRNA translates	2,290 ± 360	200 ± 110

* BALB/c- or C57BL/6-derived KLH-primed thymocyte extracts equivalent to 1.5×10^7 cells were applied to the KLH column. Effluent from the column was used as light chains, divided, and added to six cultures per group. The 11S mRNA translate was the same as used in Table I.

‡ BALB/c- or C57BL/6-derived KLH-binding heavy chain was purified by two successive immunoabsorbent columns that had been applied with BALB/c- or C57BL/6 KLH-primed thymocyte extracts equivalent to 1.5×10^7 cells. Eluate was divided and added to six cultures per group. The 13S mRNA translate was the same as used in Table I.

§ Arithmetic means of PFC numbers of six cultures ± SD.

‖ Not done.

TABLE IV

Genetic Specificity of Restricting Element of TsF Obtained from B10.A(3R) and B10.A(5R) Mice

Materials obtained from:		Anti-DNP IgG PFC response in:	
Light chain*	Heavy chain‡	C57BL/6	C3H
—	—	1,910 ± 360§	510 ± 200
B10.A(3R) TsF	B10.A(3R) TsF	230 ± 210	600 ± 230
B10.A(5R) TsF	B10.A(5R) TsF	2,100 ± 390	30 ± 40
B10.A(3R) TsF	—	2,020 ± 370	480 ± 230
B10.A(5R) TsF	—	1,870 ± 600	570 ± 240
11S mRNA translates	13S mRNA translates	650 ± 460	450 ± 90
—	13S mRNA translates	1,910 ± 710	400 ± 180
B10.A(3R) TsF	13S mRNA translates	690 ± 220	430 ± 40
B10.A(5R) TsF	13S mRNA translates	1,870 ± 470	30 ± 60

* B10.A(3R)- or B10.A(5R)-derived KLH-primed thymocyte extracts equivalent to 1.5×10^7 cells were applied to the KLH column. Effluent from the column was used as light chains, divided, and added to the six cultures per group. The 11S mRNA translate was the same as used in Table I.

‡ B10.A(3R)- or B10.A(5R)-derived KLH-binding heavy chain was purified by two successive immunoabsorbents that had been applied with B10.A(3R) or B10.A(5R) KLH-primed thymocyte extracts equivalent to 1.5×10^7 cells. Eluate was divided and added to six cultures per group. The 13S mRNA translation product was the same as used in Table I.

§ Arithmetic means of PFC numbers of six cultures ± SD.

chains from conventional TsF or of in vitro translated mRNA products of heavy and light chains of TsF.

The mixture of translated heavy or light chain together with the isolated free heavy or light chain from the conventional TsF successfully reconstituted the active form of TsF that mediates the antigen-specific and genetically restricted suppressor function. Therefore, shuffled experiments using the heavy chains with different antigen-binding specificities and the light chains having distinct I-J antigenic determinants were performed to determine the biological roles of the two chains of TsF.

To determine the functional role of the heavy chain, the 11S mRNA translated light chain was mixed with the OVA- or KLH-binding heavy chain isolated from the conventional TsF. Since the heavy chain by itself possesses the specific ability to bind native antigen, it is likely that the heavy chain determines the antigen specificity. In fact, the combination of the OVA-binding heavy chain with the translated light chain only suppressed the response to DNP-OVA, but not to DNP-KLH (Table I). Similarly, the mixture of the same light chain and the KLH-binding heavy chain preferentially suppressed the anti-DNP-KLH response. It is clear that the heavy chain mediates the antigen specificity of TsF.

On the other hand, the light chain with I-J antigenic determinant seems to be the element that determines the genetic restriction specificity of TsF (Tables II, III, and IV), because the suppression of antibody response was observed only when the histocompatibility between the light chain and the responding cell was identical. In the experiments investigating the genetic specificity of the restricting element, the light chains were prepared in two different ways. First, the I-J-positive molecules were isolated by the anti-I-J column and were tested for their haplotype-specific suppressor activity (Table II). Second, since the suppressor T cell extracts contained the free light chain, it was possible to obtain the light

chain in the effluent from the antigen column. In this preparation, the light chain was able to be separated with no relation to the anti-I-J. Therefore, the restriction specificity of the light chain could be investigated without bias. As shown in Tables III and IV, it is likely that the genetic specificity of the restricting element is mediated by the histocompatible I-J-positive molecule, since the genetic difference between B10.A(3R) and B10.A(5R) is putatively I-J.

The molecular genetic studies on the I-J gene reported by Steinmetz et al. (12), Kobori et al. (13), and Kronenberg et al. (14) have suggested that there is no genetic differences between the I-A and the I-E subregions of B10.A(3R) and B10.A(5R), in which the I-J subregion has been mapped (15). However, the results in Table IV demonstrate that B10.A(3R) produces the light chains with I-J^b haplotype, and B10.A(5R) provides the I-J^k positive light chains. It is thus clear that the light chains with I-J antigenic determinants work as elements mediating the genetic specificity of TsF. The cloning of the genes coding for the I-J-positive light chain will solve the discrepancies between the functional results and the molecular genetic data.

Lei et al. (6) have also investigated the possibility of interchanging the idiotype-bearing and I-J-positive chains between two different poly($Glu^{50}Tyr^{50}$)-specific TsF (GT-TSF) with I-J^b or I-J^k determinants. The results, however, indicated that only the homologous I-J and idiotype-bearing chains can reconstitute the active suppressor molecule. This is not true in our case. In our present study, no genetic preference was observed in the functional association between the heavy and light chains (Tables II, III, and IV). This discrepancy may be due to the difference between the methods of preparation of the two chains of TsF. In the experiment of Lei et al. (6), GT-TsF was treated with a reducing agent and the two chains were separated by using the appropriate anti-I-J columns. In contrast, we prepared the free heavy and light chains isolated from the extracted TsF without reduction. Therefore, the treatment of TsF with a reducing agent may interfere with the heterologous association of the two chains. In any event, the ability to exchange I-J-bearing chains from different haplotypes shown in our study would suggest that the association of the I-J-bearing and antigen-binding chains does not seem to be placed by an I-J anti-I-J-type interaction, as proposed by others (7, 16, 17).

The Igh-linked restriction on the expression of the TsF activity reported by Yamauchi et al. (7) was not observed in our present experiments. The light chain from B10.A(5R) (Igh^b, I-J^k) but not B10.A(3R) (Igh^b, I-J^b) TsF suppressed the responses of C3H (Igh^j, I-J^k) (Table IV). Moreover, our recent studies[2] using the inducible suppressor hybridoma have supported the results in Table IV. The C3H.SW (Igh^j, I-J^b) or CWB (Igh^b, I-J^b) but not C3H (Igh^j, I-J^k) TsF can activate the C57BL/6 (Igh^b, I-J^b)-derived inducible acceptor-suppressor hybridoma. It is thus apparent that the Igh restriction is not necessary for the expression of TsF function in the KLH system. The antigen-specific and Igh-restricted TsF reported by Yamauchi et al. (7) is obtained from Lyt-1^+2^- inducer suppressor T cells, whereas KLH-TsF is derived from Lyt-2^+ suppressor T cells (18). Therefore, different T cell subsets make distinct TsF that may act in the different

[2] Sumida, T., I. Takei, and M. Taniguchi. Activation of acceptor-suppressor hybridoma with antigen-specific suppressor T cell factor of two chain type. Manuscript submitted for publication.

stages of the immunoregulation. In any case, it is important to know how the I-J-positive light chain gives the genetic restriction in the regulatory cell interactions. Our present studies are focused on answering these questions.

Summary

The functional roles of the two polypeptide chains that compose the T cell suppressor factor (TsF) that mediates the antigen-specific and genetically restricted suppressor function were studied by using the heavy or light chains isolated from the conventional TsF or the 11S and 13S mRNA translation products of TsF. Either the heavy or the light chain of mRNA translation products reconstitutes the active TsF that suppresses the antibody response in an antigen-specific and genetically restricted manner when it is combined with the isolated heavy or light chain from the conventional TsF. As a consequence, the antigen-binding heavy chain mediates the antigen specificity of TsF. On the other hand, the I-J-positive light chain works as an element to determine the genetic restriction specificity. Thus, the identity of the histocompatibility between the I-J haplotypes on the light chain and the responding cell is essential for the functional expression of TsF. No genetic preference, however, was observed, in the association of the heavy and light chains of TsF.

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