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Yersinia enterocolitica Provides the Link between Thyroid-Stimulating Antibodies and Their Germline Counterparts in Graves’ Disease

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Graves’ disease results from thyroid-stimulating Abs (TSAbs) activating the thyrotropin receptor (TSHR). How TSAbs arise from early precursor B cells has not been established. Genetic and environmental factors may contribute to pathogenesis, including the bacterium Yersinia enterocolitica. We developed two pathogenic monoclonal TSAbs from a single experimental mouse undergoing Graves’ disease, which shared the same H and L chain germline gene rearrangements and then diversified by numerous somatic hypermutations. To address the Ag specificity of the shared germline precursor of the monoclonal TSAbs, we prepared rFab germline, which showed negligible binding to TSHR, indicating importance of somatic hypermutation in acquiring TSAb activity. Using rFab chimeras, we demonstrate the dominant role of the H chain V region in TSHR recognition. The role of microbial Ags was tested with Y. enterocolitica proteins. The monoclonal TSAbs recognize 37-kDa envelope proteins, also recognized by rFab germline. MALDI-TOF identified the proteins as outer membrane porin (Omp) A and OmpC. Using recombinant OmpA, OmpC, and related OmpF, we demonstrate cross-reactivity of monoclonal TSAbs with the heterogeneous porins. Importantly, rFab germline binds recombinant OmpA, OmpC, and OmpF confirming reactivity with Y. enterocolitica. A human monoclonal TSAb, M22 with similar properties to murine TSAbs, also binds recombinant porins, showing cross-reactivity of a spontaneously arising pathogenic Ab with Y. enterocolitica. The data provide a mechanistic framework for molecular mimicry in Graves’ disease, where early precursor B cells are expanded by Y. enterocolitica porins to undergo somatic hypermutation to acquire a cross-reactive pathogenic response to TSHR. The Journal of Immunology, 2013, 190: 000–000.

Graves’ disease is an organ-specific Ab-mediated autoimmune disease, where thyroid-stimulating Abs (TSAbs) to the thyroid-stimulating hormone receptor (TSHR) mimic thyroid-stimulating hormone (TSH) to activate the receptor, leading to hyperthyroidism. Other Abs to the TSHR include thyroid-stimulating blocking Abs (TSBAb) and neutral Abs (1). The Ab response to TSHR is therefore diverse in terms of epitope recognition and function. Earlier studies on TSAbs have indicated a restricted clonal response to TSHR with TSAbs confined to IgG1 isotype and γ-L chain usage (2–4). Recent genetic studies in inbred mice strains have implicated the IGH V region gene locus in TSAb development (5, 6). Monoclonal TSAbs with powerful agonist activity have been characterized from mice undergoing experimental Graves’ disease following immunization by genetic delivery of plasmid or adenovirus encoding TSHR or the extracellular region of the receptor, known as TSHR A-subunit (7–9). The monoclonal TSAbs derived from experimental models of Graves’ disease have been shown to be pathogenic in vivo, confirming their role in disease pathogenesis (8–10). Importantly, human monoclonal TSAbs from patients with Graves’ disease have also been derived, providing detail into their molecular and biochemical properties, and pathogenicity in vivo (11–13). A major advance has been the delineation of the atomic structures of immune complex of human monoclonal TSAb, M22 and human monoclonal TSBAb, and K1-70 with human TSHR A-subunit (14–16). The structures reveal the epitopes for M22 and K1-70 to be dependent on discontinuous determinants on leucine-rich repeat (LRR) regions of TSHR A-subunit (14, 15). Importantly, TSHR residues R38, K58, R80, H105, and K129 were critical for recognition of the receptor by M22 (14). However, despite these insights into the molecular basis of TSHR recognition and stimulation by these pathogenic Abs, there is limited information available on their germline V region genes, and no knowledge on the putative Ag recognized by the germline Igs and its relationship to TSHR in Graves’ disease.

The etiology of Graves’ disease is not well understood but is known to be complex, involving a combination of genetic and...
environmental factors (17). Danish twin studies have estimated 25% of the predisposition to Graves’ disease to be contributed by environmental factors (18). Infection with the bacterium Yersinia enterocolitica has been implicated in the pathogenesis of Graves’ disease since the 1970s (19–21). Y. enterocolitica was shown to contain high-affinity binding sites for the hormone TSH as well as TSHR Abs from patients with Graves’ disease (22–24). Immunization of mice with envelope proteins from Y. enterocolitica led to production of anti-TSHR Abs and supports the concept of molecular mimicry between Y. enterocolitica Ags and TSHR (25). Bioinformatics searches have reported on regions of sequence homology between Y. enterocolitica outer membrane porin (OmpM) and TSHR (26). Recently, a murine neutral mAb to TSHR (mAb A9) (27), whose linear epitope has been mapped to the TSHR A-subunit (28), was shown to bind Y. enterocolitica OmpF (29).

Thus, overall, a large number of studies indicate Y. enterocolitica to be potentially important in Graves’ disease. With the development of monoclonal TSAbs with pathogenic properties in vivo, their relationship with Y. enterocolitica and Graves’ disease remains to be elucidated. We have previously described two monoclonal TSAbs, KSAb1 (IgG2a/k) and KSAb2 (IgG2a/k), with strong agonist activity in the low nanogram range, developed from a single animal undergoing experimental Graves’ disease (9, 10). Detailed genetic analysis revealed both TSAbs to have undergone numerous somatic hypermutations (SHM) and to be clonally related, because they use the same germine Ig V region gene rearrangements (30). This gave an opportunity to study the Ag specificity of their germine Ig genes, which by extensive SHM give rise to TSAb activity with consequent pathogenesis. In the current study, we have expressed the germine gene arrangements of KSAb1 and KSAb2 as rFab (termed rFab germine) to assess whether it displays autoreactive potential by binding to TSHR. In other studies, the contribution of H and L chain to TSAb activity was explored by chimeric constructs expressing germine and mature H and L chains. Given the important role of microbial Ags in promoting autoimmune disease (31, 32), we investigated the reactivity of the TSAbs and the rFab germine with Y. enterocolitica proteins. Our study reveals new insights into the development of TSAbs from their germine Ig genes and demonstrates a compelling pathogenic role for the Y. enterocolitica porin family in Graves’ disease.

Evaluation of binding of rFabs to TSHR

TSHR reactivity of the rFabs was tested in four different assay systems, including Western blotting followed by detection with sensitive ECL. The first assay used an in vitro radioligand binding inhibition assay that measures the inhibition of binding of 125I-TSH to detergent-solubilized TSHR in a commercial kit (34) (Thermo Fisher Scientific). The second assay was flow cytometry, and 0.5 × 10⁶ GFP-2 cells (35) were incubated with rFab at 10 and 1 μg/ml for 1 h on ice. Isotype-matched control mAbs were used for the anti-TSHR Abs 4C1 (IgG1) and KSAb1 (IgG2a); for purified rFab germine and rFab KSAb1, the control rFab 96/3 was used. Cells were washed twice with PBS containing 2% BSA and 0.05% sodium azide and incubated with PE-conjugated goat anti-mouse IgG (Abcam) for 30 min on ice in the dark. Cells were washed twice and resuspended. Cytometric analysis was performed on the same day on a FACSCanto II Flow Cytometer (BD Biosciences). The third assay was a functional bioassay based on stimulation of TSHR in Chinese hamster ovary cells stably transfected with human TSHR (JP09 cells) for quantitative measurement of intracellular cAMP performed in duplicate samples as described previously (9). Supernatants from JP09 cells stimulated with each rFab preparation were measured by cAMP measurement using commercial RIA kits (Enzo Life Sciences) (9). The fourth assay for detection of binding of the rFab preparations to TSHR relied on Western blotting and sensitive ECL detection system.

Y. enterocolitica: serotypes and preparation of proteins

Y. enterocolitica colonies were grown on Congo red magnesium oxalate plates, and plasmid-positive colonies were selected for growth in Yersinia-Luria-Bertani broth overnight at 30°C. Whole-cell lysate samples were prepared by centrifugation of 1 ml of overnight cultures at 13,000 rpm for 1 min, resuspension in 1× Laemmli buffer, and sonication in an ice-water slurry for 30 bursts of 1 s each. Envelope proteins were isolated from Y. enterocolitica as previously described (36) and stored aliquoted at −20°C.

Affinity enrichment of Y. enterocolitica with affinity matrix-coupled Ab

KSAb1 IgG was covalently coupled to cyanogen bromide-activated Sepharose 4B (10 mg IgG/ml gel slurry) (Sigma-Aldrich), according to the manufacturer’s instructions. Pelleted cells of overnight cultures of Y. enterocolitica were resuspended in lysis buffer containing Triton X-100 (1% v/v) and hen egg lysozyme (1 mg/ml) and incubated at 37°C for 30 min, followed by sonication as above. After brief centrifugation to pellet the cellular debris, the supernatant containing solubilized Y. enterocolitica protein extract was removed, and protease inhibitors were added and incubated with KSAb1–Sepharose 4B affinity matrix. After overnight incubation with gentle agitation in the cold room, the resin was washed extensively, prior to elution of the bound proteins with 0.1 M glycine-HCl (pH 2.5) for 5 min, followed by neutralization with 1 M Tris HCl (pH 8). After dialysis in PBS, protein concentration was measured, and the sample was run on SDS-PAGE gels for protein identification, as described below.

Protein identification by mass spectrometry

The 37-kDa band was excised from an SDS-PAGE gel stained with Coomassie blue and sent for MALDI-TOF analysis to the Protein
and Nucleic Acid Chemistry Laboratory (University of Leicester) using an Applied Biosystems 477 Protein Sequencer (Life Technologies).

**Recombinant Omp protein expression**

The OmpA synthetic gene (GenScript) cloned in pUC vector was subcloned into pET22b vector (Merck Millipore). The OmpC and OmpF genes were also available cloned into pET22b vector. The plasmids were individually transformed into BL21 (DE3) competent cells (Merck Millipore). The recombinant proteins were expressed by isopropyl β-D-thiogalactoside (IPTG) induction.

**SDS-PAGE and Western blotting**

Purified rFab and *Y. enterocolitica* protein preparations were analyzed by 12% gels under reducing conditions and electro-transferred to HyBond membrane (GE Healthcare). Purified human TSHR protein (Novus Biologicals) was used to evaluate binding of rFab germline by sensitive ECL detection. Membranes were blocked overnight with PBS-5% milk powder/0.05% Tween 20 at room temperature and probed with purified rFab preparations (1 μg/ml) or mouse anti-TSHR mAbs KSAb1, KSAb2, or mAb A9 (specific for amino acid residues 214–222 of TSHR) (27, 28) (1 μg/ml) as primary Abs, followed by detection with HRP-goat anti-mouse whole IgG conjugate (NA931; GE Healthcare) (diluted 1:1000 in PBS-5% milk powder/0.05% Tween 20). The blots probed with the TSAb M22 (RSR, Cardiff, U.K.) were developed with HRP-sheep anti-human IgG whole IgG conjugate (NA933; GE Healthcare) (diluted 1:10,000 in PBS-5% milk powder/0.05% Tween 20). The HRP blots were developed with Amersham ECL Prime Western blotting Detection Reagent (GE Healthcare) and exposed to Amersham Hyperfilm ECL (GE Healthcare). Molecular weight ladders include Protein Ladder 10–250 kDa (New England Biolabs U.K., Hitchin, U.K.) and Full-Range Rainbow Molecular Weight Markers (GE Healthcare).

**Molecular modeling**

Homology models for OmpA, OmpC, and OmpF were created using the University of California San Francisco Chimera package (37) and Modeler version 9.8 (38). Cartoon representations for Supplemental Fig. 3 were created using PyMOL Molecular Graphics System, version 1.3r2. Electrostatic potential surfaces were created using CCP4MG (39) and color-coded, according to electrostatic potential (calculated by the Poisson–Boltzmann solver within CCP4MG). The probe radius used was 1 Å.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 3 Software (GraphPad). The p values were calculated using nonparametric Mann–Whitney U test. The p value ranges are indicated in the figures as follows: *p* > 0.05; **p** > 0.01. Errors bars were calculated as SEM.

**Results**

**Expression of KSAb1 and KSAb2 germline V region genes as rFab**

To determine the Ag binding specificity of the Fab encoded by the shared germline genes of KSAb1 and KSAb2, the germline IGH and IGK nucleotide sequences were synthesized as a gene cassette for in-frame cloning (Supplemental Fig. 1) into the bacterial expression vector pAK19 for rFab expression (30). The rFabs were purified by Ni-affinity chromatography and analyzed by SDS-PAGE with Coomassie Blue staining (Fig. 1A, lane i) and Western blotting developed by ECL with a HRP-conjugated anti-penta His tag Ab (Fig. 1A, lane ii).

**Evaluation of reactivity of rFab germline to TSHR**

Four assay systems of 1) radioligand binding inhibition assay, 2) flow cytometry, 3) TSHR stimulation bioassay, and 4) Western blotting in combination with sensitive ECL were used to evaluate Ab binding to TSHR (34). Purified rFab KSAb1 or anti-TSHR mAbs were used as a positive control and rFab 96/3, specific for a pancreatic islet cell Ag IA-2, served as a negative control (33) for the bacterial produced Abs.

**Radioligand binding inhibition assay.** rFab KSAb1 displaced the binding of 125I-TSH to the receptor in the assay, confirming the integrity of the rFab preparations (Fig. 1B). Evaluation of rFab germline showed only marginal inhibition of 125I-TSH binding, similar to that obtained with the negative control rFab 96/3 (Fig. 1B).

**Flow cytometry.** GPI9-5 cells (Chinese hamster ovary cells expressing high levels of lipid anchored TSHR A-subunit) (35) were used in flow cytometry to determine binding of rFab germline to TSHR (Fig. 1C). The anti-TSHR mAb 4C1, used as a positive control showed binding to >90% cells. KSAb1 IgG (isotype control, IgGk) and purified rFab KSAb1 displayed binding to TSHR, but rFab germline showed no binding to TSHR, which was comparable to the negative control, rFab 96/3 (Fig. 1C).

**TSHR stimulation bioassay:** Agonist activity was assessed by TSHR stimulation bioassay, resulting in accumulation of intracellular cAMP in JP09 cells (9). KSAb1 IgG induced maximal cAMP response, equivalent to that obtained with a suboptimal dose of bTSH and forskolin. rFab KSAb1 induced significant cAMP stimulation; however, the rFab germline did not stimulate cAMP production. Thus, rFab germline failed to show binding to TSHR in different assay systems of competition assay, flow cytometry, and bioassay (Fig. 1D).

**Western blotting and ECL.** We used a commercial source of purified TSHR protein, produced in a wheat-germ extract cell-free system, with the receptor of predicted molecular mass of 84 kDa. In Western blotting and ECL, the TSHR protein was recognized by the anti-TSHR mAb A9, comigrating at 84 kDa, but rFab germline showed no binding to the TSHR. Isotype-matched mAb and rFab 96/3 used as controls showed no binding to TSHR protein (Fig. 1E).

**Evaluation of TSHR binding specificity and TSAb activity of chimeric rFabs**

To evaluate the individual role of mature H and L chains of KSAb1 in determining binding to TSHR, a variety of chimeras of mature and germline IGH and IGK genes were constructed and expressed as rFab.

**Radioligand binding inhibition assay.** The chimera constructed with mature IGH and IGK, termed rFab H-mature, showed >35% inhibition of 125I-TSH binding to TSHR in the radioreceptor assay; this inhibition was reproducible at high concentrations (100 μg/ml). The converse chimera, encoded by germline IGH and mature IGK genes, termed rFab L-mature, failed to show inhibition of 125I-TSH binding at any of the concentrations tested. We also constructed a chimera of KSAb1 H chain in combination with mature L chain derived from a non-thyroid Ag-specific rFab; this chimera was termed rFab L irrelevant. Interestingly, this chimeric rFab preparation failed to show any binding to TSHR in the radioreceptor assay (Fig. 2A).

**TSHR stimulation bioassay.** The chimeric rFabs were also evaluated in a TSHR stimulation assay. rFab H mature induced a weak but statistically significant positive cAMP stimulation response in the assay. rFab L mature and rFab L irrelevant do not display any measurable binding of the TSHR. Although the L chain does not have a dominant role in TSHR binding, it is critical for Ag binding as replacement with an irrelevant L chain leads to loss of all binding. The data show that the H chain of KSAb1 IgG contains the key determinants for recognition of TSHR (Fig. 2B).

**Determination of binding of TSAb and rFabs to Y. enterocolitica proteins**

We examined the reactivity of rFab germline with *Y. enterocolitica* preparations. Two serotypes of *Y. enterocolitica*, O:3 and O:8, reported to be associated with Graves’ disease (22, 24), were selected for preparation of whole-cell lysates and envelope proteins. Whole-cell lysate and envelope protein preparations of *Y. enter-
Y. enterocolitica differ subtly between the two serotypes in SDS-PAGE (Fig. 3A). Western blotting on whole-cell lysate and envelope protein preparations with KSAb1 IgG and KSAb2 IgG showed binding to a Y. enterocolitica protein of ∼37 kDa in both preparations of both serotypes (Fig. 3D, 3F). Isotype-matched controls IgG2b and IgG2a did not show any reactivity to the whole-cell lysate and envelope proteins (Fig. 3E, 3G, respectively). rFab germline also showed binding to an approximate 37-kDa Y. enterocolitica protein in both serotypes (Fig. 3B). The negative control, rFab 96/3, did not show any binding to the Y. enterocolitica proteins (Fig. 3C).

Affinity enrichment of Y. enterocolitica proteins bound by KSAb1 IgG and identification by MALDI-TOF

To characterize the 37-kDa band in Y. enterocolitica lysate (serotype O:8) recognized by the rFabs, the protein was first enriched by Ab affinity purification, prior to MALDI-TOF analysis. The bacterial lysate was incubated with KSAb1 IgG conjugated to...
separose 4B matrix, and bound proteins were eluted in acidic conditions and immediately neutralized with 1 M Tris base. As control, BSA coupled to Sepharose 4B was used. A single band of ~37 kDa was eluted when analyzed by SDS-PAGE and Western blotting performed to confirm binding by KSAb1 (Fig. 3H). The 37-kDa protein was present in the Y. enterocolitica lysate (pre-incubation with KSAb1 IgG) and in the eluate lane. The protein band was only present in the lysate fraction and absent in the eluate fraction from BSA–Sepharose 4B affinity matrix, demonstrating that the 37-kDa protein bound specifically to KSAb1 IgG. A narrow gel plug in the 37-kDa range was excised and analyzed by MALDI-TOF, which identified the two most abundant proteins as OmpA and OmpC (Supplemental Fig. 2A).

Expression of Y. enterocolitica porins as recombinant proteins and reactivity with TSAb and rFab germline

The identified porins of Y. enterocolitica, OmpA and OmpC, were cloned and expressed as recombinant proteins with a His-tag. Because expression of OmpC is reciprocal to expression of the related OmpF in Y. enterocolitica (40), we also cloned OmpF for expression as recombinant protein. The nucleotide sequence of OmpA of Y. enterocolitica was obtained from PubMed (accession number 008800) and synthesized as a synthetic gene for in-frame cloning and expression in the vector pET22b. OmpC and OmpF of Y. enterocolitica were available cloned in pET32b. Expression of the three recombinant porins was analyzed by SDS-PAGE and showed strong IPTG-inducible expression (Fig. 4A), but OmpF showed leaky expression because it was expressed in the absence of IPTG induction (Fig. 4A). Binding of KSAb1 IgG to recombinant OmpA, OmpC, and OmpF in total bacterial cell extracts was tested by Western blotting and revealed binding to all the three porins, which was similar to the binding obtained with KSAb2 IgG (Fig. 4B). Isotype control IgG2b and IgG2a mAbs did not show any reactivity (Fig. 4B). Evaluation of rFab germline showed strong binding to OmpC and OmpF, with weaker binding to OmpA; control rFab 96/3 did not bind to any of the porins (Fig. 4B). We next evaluated the binding of the human TSAb, M22, to the recombinant porins, which showed binding to OmpF and OmpC (Fig. 4B).

Structural correlates of Y. enterocolitica Omp F, Omp C and Omp A proteins with TSHR A-subunit for binding of TSAb

Because OmpF and OmpC belong to the same family of porins, there are large regions of amino acid sequence homology, but the two porins show no homology with OmpA (Supplemental Fig. 2B).

In addition, there are no regions of significant similarity between the amino acid sequences of the TSHR A-subunit and the Y. enterocolitica porins that may provide insight into the basis of this cross-reactivity (Supplemental Fig. 2C). We therefore looked for structural correlates by creating homology models of OmpC, OmpF, and OmpA from Y. enterocolitica and comparing their properties with the known crystal structure of TSHR-A subunit bound to M22 Fab (14) (Supplemental Fig. 3A, 3B). The β sheet topology of TSHR A-subunit bears some similarity to the porin β barrel, but because the majority of the porin barrel is embedded in the membrane, we did not investigate this further but focused our attention on the extracellular loops of the porins (41, 42). Given that all five N-terminal TSHR residues that are critical for M22 binding are positively charged (R38, K58, R80, H105, and K129) (14), we examined the electrostatic potential surface of the extracellular loops of the porins for clues to cross-reactivity. Both OmpC and OmpF are overall negatively charged in this region but do possess solvent accessible arginine residues, which within the trimer may create a molecular surface of appropriately similar dimensions to the concave Ab-binding surface of TSHR A-subunit, which are possible candidates for mAb binding (Supplemental Fig. 3C, 3D). We speculate that these features may lead to weak reactivity with rFab germline that is then expanded by numerous SHM in the CDR regions to acquire TSHR binding. The known flexibility of the porin extracellular loops may further facilitate engagement with rFab. In evaluating this for OmpA, which is a monomer (43), two protruding positively charged residues can be identified that may contribute to TSAb cross-reactivity; the monomeric nature of OmpA may explain the weaker reactivity we report here (Supplemental Fig. 3E, 3F).

Discussion

The murine TSAbs, KSAb1 and KSAb2, share a number of characteristics with the pathogenic anti-TSHR Abs present in Graves’ disease patients, including high-affinity binding for TSHR, strong agonistic activity to the receptor and in vivo pathogenicity (9, 10). KSAb1 and KSAb2 are thus equivalent to the TSAbS present in Graves’ disease patients (11, 12). Importantly, both KSAb1 and KSAb2 are derived from the same original precursor B cell as they use identical germline gene rearrangements, thus giving an opportunity to evaluate the Ag specificity of their germline precursor (30). This led to an important finding, described to our knowledge for the first time in Graves’ disease, that the germline revertant of the two TSAbs, when expressed as rFab, does not bind TSHR. A variety of assay systems (34) with differing sensitivities of detection of TSHR were used to evaluate binding of rFab germline to TSHR. One of the assays used to
enumerate the Ag specificity of rFab germline was Western blotting in conjunction with ECL to ensure that the detection sensitivities were commensurate when TSHR and recombinant Omp proteins were tested for binding. It could be argued that Western blotting would not be as sensitive a detection technique as the radioligand binding, flow cytometry, or TSHR stimulation bioassay (cAMP induction) because it destroys the conformation of the protein, and some Abs and their targets are conformationally sensitive. However, it was included in this study as the rFab germline was evaluated for binding Omp proteins by Western blotting.

The lack of binding of rFab germline to TSHR was unlikely to be due to the integrity of the rFab preparations, because rFabs prepared from KSAb1 and KSAb2 continue to interact with TSHR as effectively as their IgG counterparts (30). We therefore conclude that the germline B cell Ab was not reactive with the target

![Figure 3](http://www.jimmunol.org/download.png)
autoantigen, TSHR, and that hypermutation of the germline Ab was necessary to confer TSHR autoreactivity. Somatic hypermutation serves to increase affinity for an Ag after exposure. However, hypermutation may also introduce cross-reactivity to other target Ags. Indeed, it has been shown in humans that there is an increase in autoreactive cells in IgG memory B cells compared with naïve B cells (44). Therefore it is possible to hypothesize that during the process of generating memory cells to an original pathogen challenge, the memory B cells acquire cross-reactivity with TSHR so that when the mice were later challenged with TSHR, they used the memory cells from the previous challenge. This is not a new concept, because there have been reports of other autoantibody-mediated autoimmune conditions, where the germline revertants of pathogenic Abs in systemic lupus erythematosus (45–48) and more recently in Pemphigus vulgaris (49) fail to recognize the target Ag of the somatically hypermutated, mature Ab. However, we provide evidence for this hypothesis by identification of the pathogen recognized by the germline form of the Ab to TSHR in experimental Graves’ disease.

In our studies using rFab chimeras constructed of combinations of mature and germline H and L chains, we show the important role of the H chain in binding TSHR and TSAb activity. The important role of the H chain for TSAb was already known from earlier genetic analysis studies from recombinant inbred mice strains (5, 6). Nevertheless, the L chain also appears to be important for TSAb activity, because substitution with L chain from a mAb of non-thyroid Ag specificity, leads to rFab chimera preparation that does not bind TSHR. This finding of restricted L chain usage for TSAb activity after SHM events suggests that agonistic activity to the TSHR is mediated by a particular conformation of Ab that can perhaps be attained by a few combinations of IGH and IGK rearrangements. These observations are in line with tentative findings on the common usage of certain gene rearrangements in monoclonal TSAb from experimental Graves’ disease (7, 8, 30) and the human monoclonal TSAb from patients with autoimmune hyperthyroidism (11, 12). Although limited information is available, there appears to be notable similarity in the gene usage by mouse and human monoclonal TSAb (Supplemental Table I). Among the murine monoclonal TSAb generated in independent laboratories using different genetic delivery immunization procedures (7–9, 30), there is considerable restriction in the both H and L chain gene usage. For example, usage of H chain genes, IGHVIS1, IGHDQ52, and IGHJ2, and L chain genes, IGKV6-13 and IGKJ2 or IGKJ5, is common among the mouse TSAb.

Similarly, despite fewer human monoclonal TSAb and TSBAbs reported, a number of common Ig gene families appear to be used repeatedly (Supplemental Table I).

A second important finding in this study was the identification of the pore forming proteins of *Y. enterocolitica*, OmpA, OmpC, and OmpF that were recognized by the monoclonal TSAb in both the serotypes O:3 and O:8 of *Y. enterocolitica*. These porins are located in the outer membrane of the bacterium and hence accessible to the immune system. Interestingly, OmpC and OmpF belong to the classical family of porins, with similar structures and function, but they differ substantially from the nonclassical family of porins such as OmpA (50). The expression of OmpC and OmpF on the bacterial cell surface is regulated reciprocally, dependent on the growth temperature conditions (40). In this study, the culture of *Y. enterocolitica* was grown at 30°C, when OmpA is known to be overexpressed preferentially to OmpF (40), which also explains the MALDI-TOF identification of OmpC as one of the components of the excised 37-kDa band. Both the murine monoclonal TSAb and the human monoclonal TSAb, M22, evaluated in this study show reactivity with OmpC and OmpF, indicating display of important cross-reactive determinants with the TSHR A-subunit. However, the nature of the cross-reacting determinant on *Y. enterocolitica* OmpC and OmpF with TSHR A-subunit is not readily apparent. Although OmpC and OmpF show high-sequence homology, they do not show any extended regions of amino acid sequence with significant homology with TSHR A-subunit, which may readily explain the nature of the cross-reactive determinants. Structural studies have shown that OmpC and OmpF adopt β barrel structures, with the extracellular regions adopting loop conformations (41, 42); it is possible that some of these conformations may mimic the TSAb epitopes in the LRR region of TSHR A-subunit. However, the pore function of OmpC and OmpF is dependent on adopting a trimeric structure (50), which may multiply the accessibility of the cross-reactive determinants to TSHR. Recent studies have elucidated the atomic structure of the immune complex of TSHR A-subunit associated with M22 Fab, which have identified critical residues in the LRR region of TSHR.
A-subunit important in contact with residues in the CDRs of M22 for the high-affinity binding (14). The important contact amino acid residues in the receptor for M22 have been identified as R38, K58, R80, H105, and K129 (14). To date, there are no reports on the structure of TSHR A-subunit in complex with murine monoclonal TSAs, although mutational analysis of TSHR A-subunit has been used to study their conformational analysis (8, 51). Remarkably, identical residues K58, R80, and H105 in the LRR region of TSHR A-subunit, common to the M22 epitopes on the receptor, were identified as important determinants for the murine TSAbs, IRI-SAb2 binding and activation of the receptor (8). Thus, although knowledge on the epitopes on TSHR A-subunit of murine TSAbs is limited to a small number of TSAbs (8, 16), the fact that KSAb1 and KSAb2 are comparable in their stimulating properties to the murine IRI-SAb2 and human M22 mAbs (52) makes it likely that amino acids R38, K58, R80, H105, and K129 important for M22 interaction are also important for binding and stimulation function KSAb1 and KSAb2 evaluated in this study. The restricted number of the amino acid determinants on the receptor essential for TSAbs activity is supported by the finding of restricted germline gene usage of mouse and human TSAbs (Supplemental Table I), which perhaps can only be mediated by a particular conformation of Ab that can be attained by a few combinations of H and L chains. However, in addition to the reactivity of KSAb1 and KSAb2 (and rFab germline) to Y. enterocolitica OmpF and OmpC, they also react with OmpA, which suggests that there may be subtle differences in their interaction with TSHR A-subunit, compared with that of M22, because the latter does not recognize OmpA.

It has been difficult to define the molecular basis of the cross-reactivity of TSAbs with Y. enterocolitica Omps. In the absence of sequence similarities between the TSHR A-subunit and the Y. enterocolitica Omps, we examined instead structural correlates between the TSHR A-subunit and homology models of OmpC, OmpF, and OmpA from R38, K58, R80, H105, and K129 residues in the LRR region of TSHR A-subunit essential for activation of the receptor (Supplemental Fig. 3A, 3B). The extracellular loops of both OmpC and OmpF harbor arginine residues that are electrostatically complimentary to the rFab CDR loops and thus may be analogous to the arginine residues of TSHR A-subunit that are critical for its engagement with M22 Fab (Supplemental Fig. 3C, 3D). Given the similar molecular dimensions between the Omps’ flexible extracellular loops and the TSHR epitope, we speculate that these features may lead to weak but biologically significant reactivity with rFab germline that is then expanded by numerous SHM in the CDRs to acquire TSHR binding. What is remarkable is that after the numerous SHM events, which leads to recognition of TSHR (30), the ability to react with the Y. enterocolitica porins is maintained, resulting in the TSAbs acquiring cross-reactive properties. The monomeric nature of OmpA may explain the weaker reactivity we report in this study (Supplemental Fig. 3E, 3F). A characterization of the role of the extracellular loops awaits further testing, for example by mutagenesis.

In another recent study using a neutral mAb to TSHR generated in our laboratory, mAb A9 (27), cross-reactivity with Y. enterocolitica OmpF was reported (29). Reactivity of Graves’ disease patient serum with Y. enterocolitica OmpF also was demonstrated. Homology models showed the molecular basis of the cross-reactivity between TSHR A-subunit and Y. enterocolitica OmpF restricted to a short stretch of eight amino acids containing five identical residues. mAb A9 reacts with recombinant preparations of Y. enterocolitica OmpF in our laboratory (C.E. Hargreaves and J.P. Banga, unpublished observations), but because this mAb recognizes a nonpathogenic epitope on TSHR A-subunit that is different from the TSAbs determinants, it was not explored further.

In conclusion, the remarkable finding that the germine precursor of TSAbs does not react with the TSHR, but instead binds the porins of Y. enterocolitica suggests a mechanism for activation of the early B cells, resulting by SHM to gain cross-reactivity with TSHR with TSAbs activity and extend our knowledge on the pathogenesis of Graves’ disease. An understanding of how this occurs in individuals with the appropriate genetic susceptibility and constitutional factors will provide a greater insight into the molecular basis of this common condition.

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Disclosures

J.P.B. and D.D.-W. receive revenue from a patent on TSAbs mAbs for diagnosis of thyroid autoimmune disease. The other authors have no financial conflicts of interest.

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