Type 1 diabetes is a chronic autoimmune disease in which genetic predispositions affect the immune system, leading to a loss of T cell tolerance to β cells and consequent T cell–mediated destruction of insulin-producing islet cells. Genetic studies have suggested that PRSS16 is linked to a diabetes susceptibility locus of the extended HLA class I region in humans. PRSS16 encodes what we believe to be a novel protease, thymus-specific serine protease (TSSP), which shows predominant expression in thymic epithelial cells and is suspected to have a restricted role in the class II presentation pathway. Consistently, Tssp is necessary for the intrathymic selection of few class II–restricted T cell receptor specificities in B6 mice. To directly assess the role of Tssp in autoimmune diabetes, we generated Tssp-deficient (Tssp°) NOD mice. While remaining immunocompetent, Tssp° NOD mice were protected from diabetes and severe insulitis. Diabetes resistance of Tssp° NOD mice was a property of the CD4 T cell compartment that is acquired during thymic selection and correlated with an impaired selection of CD4 T cells specific for islet antigens. Hence, in the NOD mouse, Tssp is a critical regulator of diabetes development through the selection of the autoreactive CD4 T cell repertoire.
Thymus-specific serine protease controls autoreactive CD4 T cell development and autoimmune diabetes in mice

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Type 1 diabetes is a chronic autoimmune disease in which genetic predispositions affect the immune system, leading to a loss of T cell tolerance to β cells and consequent T cell–mediated destruction of insulin-producing islet cells. Genetic studies have suggested that PRSS16 is linked to a diabetes susceptibility locus of the extended HLA class I region in humans. PRSS16 encodes what we believe to be a novel protease, thymus-specific serine protease (TSSP), which shows predominant expression in thymic epithelial cells and is suspected to have a restricted role in the class II presentation pathway. Consistently, Tssp is necessary for the intrathymic selection of few class II–restricted T cell receptor specificities in B6 mice. To directly assess the role of Tssp in autoimmune diabetes, we generated Tssp-deficient (Tssp−/−) NOD mice. While remaining immunocompetent, Tssp−/− NOD mice were protected from diabetes and severe insulitis. Diabetes resistance of Tssp−/− NOD mice was a property of the CD4 T cell compartment that is acquired during thymic selection and correlated with an impaired selection of CD4 T cells specific for islet antigens. Hence, in the NOD mouse, Tssp is a critical regulator of diabetes development through the selection of the autoreactive CD4 T cell repertoire.

Introduction

Type 1 diabetes (T1D) is a complex autoimmune disease characterized by a T cell–mediated destruction of pancreatic β cells. In NOD mice, which spontaneously develop diabetes with many characteristics of human T1D, it is now clear that both CD4 and CD8 T cells contribute equally to disease development, since absence of either subset prevents insulitis and diabetes (reviewed in ref. 1). However, in both mice and humans, the most important genetic determinants in diabetes susceptibility lie in the MHC locus and in particular the MHC class II locus. The human and mouse class II susceptibility molecules HLA-DQβ1/DQα1 and I-Aβ2 contain a substitution of the Asp57 amino acid of the β chain that impacts the peptide repertoire presented by these class II molecules and alters the stability of the peptide–MHC complexes (2–4). It is noteworthy that the peculiar class II I-Aβ2 molecule is associated with an increased frequency of autoreactive CD4 T cells (5).

PRSS16 was initially described as a gene of the extended HLA region linked to a diabetes susceptibility locus in humans (6, 7). PRSS16 encodes a serine protease, thymus-specific serine protease (TSSP), that belongs to the family of lysosomal ProXaa serine exopeptidases that includes lysosomal prolyl carboxypeptidase and dipeptidyl peptidase II/VII. TSSP is predominantly expressed by thymic epithelial cells (TECs) of the cortex (cTECs) (8, 9). Expression in the endosomal compartment of cTECs and homology with endosomal proteases led to the hypothesis that TSSP might contribute to the generation of the peptide repertoire presented by MHC class II molecules in the thymus and consequently to T cell repertoire selection (8, 9). Tssp-deficient (Tssp−/−) B6 mice showed normal CD4 T cell development in the thymus and normal numbers of peripheral CD4 T cells expressing polyclonal TCRs, indicating that Tssp, in contrast to cathepsin L, has no quantitative impact on T cell repertoire selection (10, 11). However, the thymic development of CD4 T cells expressing I-Aβ1–restricted TCR transgenes (OT-II and Marilyn) is impaired in Tssp−/− B6 mice, while the development of TCR transgenic CD8 T cells (OT-I) proceeded normally (11). Thymic expression of Tssp is therefore necessary for the selection of some MHC class II–restricted TCRs. These different results argue for a restricted role of Tssp in the MHC class II presentation pathway in the thymus.

The adverse selection of self-reactive T cells specific for islet antigens (Asgs) during T cell development in the thymus is considered as a major, though not the sole, contributor to diabetes development (12–15). Indeed, the peripheral T cell repertoire of NOD mice is highly autoreactive and includes specificities for several islet Asgs (16). Diabetogenic T cells target different islet Asgs, including insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated protein 2 (IA-2), phogrin (IA-2β), and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and T cells specific for insulin, GAD65, and IGRP are present in peripheral lymphoid organs of naive NOD mice, indicating defective thymic tolerance to these autoantigens (17–21). Given the implication of Tssp in the intrathymic selection of some T cell specificities in B6 mice, we examined the possibility that Tssp may control the selection of autoreactive T cells essential for diabetes initiation/development and consequently autoimmune diabetes in the NOD mouse.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2011;121(5):1810–1821. doi:10.1172/JCI43314.
Results

Tssp° NOD mice are resistant to insulitis and diabetes. To evaluate the role of Tssp in the development of autoimmune diabetes, we generated Tssp° NOD mice by backcrossing Prss16°−/− B6 mice onto the NOD background for up to 13 generations (see Supplemental Methods and Supplemental Figure 1 for genetic characterization; supplemental material available online with this article; doi:10.1172/JCI43314DS1). Heterozygous Prss16°−/− mice were intercrossed at the sixth (N6F2), tenth (N10F2), and thirteenth (N33F2) backcross generation to generate Tssp° NOD mice and WT control littermates. While WT control female mice at the 6-backcross generation developed diabetes as early as 14 weeks of age, with a penetrance close to 60% by 30 weeks of age, Tssp° NOD littermates were completely protected from disease (Figure 1A). Similarly, Tssp° NOD mice at the 10- and 13-backcross generation were fully resistant to diabetes development, further supporting the conclusion that protection is, in these mice, due to the absence of Tssp and not to background gene effects (data not shown and Figure 1B).

Regarding insulitis, we found that while nondiabetic WT control mice showed an age-dependent progressive insulitis characterized by peri-, marked, or full insulitis, Tssp° mice showed only limited infiltration even at 40 weeks of age (Figure 1C).

Tssp deficiency therefore reduced the severity of insulitis and prevented spontaneous autoimmune diabetes in NOD mice. The remarkable phenotype of Tssp° mice suggests that Tssp deficiency may affect a critical and early event in diabetes initiation/development.

Tssp° NOD mice are immunocompetent. In agreement with previous reports (10, 11), Tssp° NOD mice showed normal T cell development and no global defect in positive or negative T cell selection in the thymus (Supplemental Figure 2). Furthermore, the Vβ-segment usage by mature peripheral CD4 and CD8 was similar in Tssp° and WT control mice (Supplemental Figure 3). Altogether, these results indicate that there are no global defects in positive and negative selection of polyclonal T cells in Tssp° NOD mice. Moreover, there was no alteration of the frequency of γδ T cells and NK and NKT cells in Tssp° NOD mice (data not shown).

In the periphery, the T cell populations of lymphoid organs were also normal in Tssp° NOD mice (Supplemental Figure 4). There was no difference with control mice in terms of CD5 expression level or in the frequency of T cells with effector/memory phenotype, as evidenced by the comparable levels of expression of CD44, CD69, and CD25 (Supplemental Figure 4, B–D). Concerning functional aspects, we found that the in vitro proliferative response to polyclonal activators and the in vivo response to peptide immunization of Tssp° CD8 T cells were comparable to those of WT controls (Figure 2, A and B). Furthermore, the cytotoxic function of alloreactive CD8 T cells was not altered in Tssp° NOD mice (Figure 2C). Similarly, the in vitro proliferation induced by anti-CD3 stimulation and the in vitro differentiation of Tssp° CD4 T cells into Th1 and Th2 effector cells proceeded normally (Figure 3, A and B). Likewise, the Ig response to T-dependent Ags was also normal in Tssp° NOD mice, further indicating that activation and differentiation of CD4 T cells into...
we found very high levels of Tssp° NOD mice are not intrinsically resistant to T1D development. To decipher the mechanism of diabetes resistance of Tssp° NOD mice, we first determined whether Tssp deficiency might interfere with the access of lymphocytes to pancreatic islets or with the susceptibility of the mutant pancreas to immune attack. We therefore adoptively transferred splenocytes from prediabetic, WT control NOD female mice into Tssp° NOD/SCID mice. Under these conditions, we also evaluated the ability of the Tssp° environment, including DCs, to support the activation of diabetogenic T cells. Diabetes onset and penetrance were comparable, regardless of whether or not the NOD/SCID hosts expressed Tssp (Figure 4A). Tssp° NOD mice are therefore not intrinsically resistant to autoimmune diabetes, suggesting that the diabetes resistance of Tssp° NOD mice is an attribute of their immune system. To test this hypothesis, we performed the reverse experiment and transferred into Tssp-sufficient NOD/SCID female mice splenocytes from 6-week-old Tssp° or WT control NOD female donors. All NOD/SCID mice injected with WT splenocytes developed diabetes within 8 to 15 weeks after transfer (Figure 4B). In contrast, diabetes incidence remained very low in NOD/SCID mice reconstituted with Tssp° splenocytes (Figure 4B). Indeed, diabetes onset was markedly delayed relative to that of controls, starting at 17 to 20 weeks after transfer, and disease penetrance remained limited, with less than 27% of the mice developing disease at 35 weeks after adoptive transfer. Hence, the diabetes resistance of Tssp° NOD mice reflects a property of their immune system.

Diabetes resistance of Tssp° NOD mice is a property of the CD4 T cell subset. Published in situ hybridization studies showed that Tssp is predominantly expressed by cTECs but not to significant levels in the hematopoietic compartment (8, 9, 22). In agreement, we found very high levels of Prss16 mRNA in cTECs but not in medullary TECs. Though at lower levels, Prss16 was also expressed by thymic DCs and mainly by mature CD4+ thymocytes (CD4–single-positive [CD4-SP] thymocytes) (Supplemental Figure 5). In the periphery, naive and activated B cells also expressed Prss16, but, importantly, peripheral DCs did not express detectable levels of Prss16 mRNA (Supplemental Figure 5).

Since Tssp is related to lysosomal serine proteases, it may have a role in the antigen-processing pathway and thus affect the processing/presentation of pancreatic β cell determinants by peripheral APCs and consequently diabetogenic T cell activation. A defect in the processing/presentation of islet Ags by peripheral DCs is unlikely to contribute significantly to the full diabetes resistance of Tssp° NOD mice, since peripheral DCs did not express Tssp and since Tssp° DCs in Tssp° NOD/SCID mice efficiently activated diabetogenic T cells (Figure 4A and Supplemental Figure 5). Presentation of islet Ags by B cells may also contribute to T1D, though this lymphocyte subset may have additional implications in the pathogenesis of diabetes (23, 24). Since NOD B cells express Tssp, we evaluated the role of this subset in the reduced diabetes caused by mutant splenocytes, since peripheral DCs did not express Tssp° NOD splenocytes to induce diabetes upon transfer into NOD/SCID mice. For this experiment, donor splenocytes were depleted of the B cell subset, and these T cell populations were complemented with purified B cells of either genotype. Regardless of the origin of the B cell population (WT or Tssp°), the reconstituted NOD/SCID mice had a comparable diabetes incidence (Figure 4C). These results show that processing/presentation of islet Ags by B cells is not substantially affected in Tssp° NOD mice and suggest a role for T cells in the diabetes resistance of Tssp° NOD mice. Indeed, NOD/SCID mice reconstituted with Tssp° T cells together with WT B cells showed a reduced disease progression that was comparable to that observed after transfer of total Tssp° splenocytes (Figure 4C versus Figure 4B). To further examine, within the T cell subset, the relative role of CD4 or CD8 T cells in the reduced diabetes caused by mutant splenocytes in NOD/SCID mice, we depleted WT splenocytes of either CD4 or CD8 T cells and replaced them with the corresponding T cell subset isolated from either WT or Tssp° mice prior to adoptive transfer. The inoculum is therefore composed of WT B cells together with CD4 and CD8 T cells of either genotype. Control NOD/SCID mice infused with CD4 or CD8 T cell–depleted WT splenocytes did not develop diabetes, indicating that, under the condition used, both CD4 and CD8 subsets were required for disease progression (Figure 4D). Regardless of the origin of the CD8 T cell population, the reconstituted NOD/SCID mice had a comparable diabetes incidence (Figure 4D). In contrast, when CD4 T cells originated from Tssp° NOD mice, diabetes incidence and progression were reduced, with only 20% of the mice developing disease, starting at 17 weeks after transfer, as observed upon transfer of total Tssp° splenocytes (Figure 4D). Collectively, these results clearly indicate that CD4 T cells are mainly responsible for...
for the lowered capacity of Tssp⁺ splenocytes to cause diabetes in NOD/SCID hosts, further suggesting that diabetes resistance of Tssp⁺ NOD mice is a property of the CD4 T cell subset.

**Tssp⁺ NOD mice do not show exacerbated Treg function.** Among CD4 T cells, CD25⁺Foxp3⁺ Tregs are important negative regulators of autoreactive T cells, including diabetogenic T cells (25–28). We therefore determined whether increased Treg activity could at least in part explain the diabetes resistance of Tssp⁺ NOD mice. We found that the frequency and number of CD25⁺Foxp3⁺CD4⁺ Tregs was comparable in all lymphoid organs of WT and Tssp⁺ NOD mice (Supplemental Figure 6). We further examined the regulatory potential of Tssp⁺ lymphocytes by mixing Tssp⁺ and WT splenocytes in the NOD/SCID transfer system, reasoning that if Tssp⁺ splenocytes comprise an exacerbated T suppressor activity, diabetes induced by WT splenocytes should be substantially delayed in its onset and/or reduced in its severity by the copresence of Tssp⁺ splenocytes. We found that both the onset and the maximal penetrance of diabetes were the same in mice receiving a WT or a mixed inoculum (Figure 5A). Importantly, the lack of difference in the disease profiles of these 2 experimental groups was not due to an altered persistence/survival of Tssp⁺ Tregs, since the representation of WT and Tssp⁺ Tregs remained comparable 8 weeks after adoptive transfer (Figure 5B). To more directly assess the regulatory activity of Tssp⁺ CD25⁺CD4⁺ Tregs, we depleted the spleen population of prediabetic WT NOD mice of CD25⁺ T cells and complemented this population with the same number of CD25⁺CD4⁺ Tregs isolated from age-matched WT or Tssp⁺ NOD mice. We found that the addition of WT or Tssp⁺ CD25⁺CD4⁺ Tregs was equally able to significantly delay diabetes onset in this experimental system (Figure 5C). Collectively, these results show that Tssp⁺ splenocytes and Tregs do not express an exacerbated T suppressor activity.

Finally, when diabetes was induced by administration of cyclophosphamide (CY), Tssp⁺ NOD male mice were more resistant than WT control male mice (Figure 5D). Since CY treatment alters the immunosuppressive activity and promotes the apoptosis of CD25⁺CD4⁺ T cells (29), the reduced susceptibility of Tssp⁺ NOD mice to CY-induced diabetes also argues against an enforced T suppressor cell activity in NOD mice lacking Tssp. Hence, full resistance to diabetes in Tssp⁺ NOD mice is unlikely to be due to exacerbated immunosuppression mediated by CD4 Tregs.

**Tssp⁺ NOD mice respond to some but not all islet Ags.** The above results suggested that the diabetes resistance of Tssp⁺ NOD mice is a property of conventional CD4 T cells. Since thymic expression of Tssp is necessary for the development of some class II–restricted TCRs (11, 30), we considered the possibility that Tssp may likewise control the intrathymic selection of some islet-specific CD4 T cells, and, thus, its absence may introduce some holes in the autoreactive T cell repertoire. We therefore analyzed the response of Tssp⁺ and
WT control NOD mice to the immunodominant peptide of known islet autoantigens suspected to play a role in diabetes development (16). Mice were immunized with the corresponding Ag in CFA, and their recall response was analyzed in vitro. Quite unexpectedly in light of the foremost role of insulin in diabetes development (31, 32), we found that the frequency of IL-2–producing CD4 T cells specific for Ins1.29–31 and Ins49–66 was comparable in Tssp° and WT NOD mice (Figure 6). Likewise WT and Tssp° NOD mice responded similarly to the GAD206–220 peptide and the known IGRP immunodominant epitope (Figure 6). However, while all WT NOD mice responded to the IA-2β755–777 epitope of phogrin, a transmembrane protein found in the secretory granules of pancreatic islet cells, only 1 out of 5 Tssp° NOD mice analyzed responded to that epitope and at a lower level than that of control WT mice (Figure 6). Tssp° NOD mice are therefore tolerant to some islet Ags.

*Tssp is necessary for the intrathymic differentiation of some islet-specific CD4 T cells.* To further examine whether tolerance to IA-2β755–777 results from central or peripheral mechanisms, we generated retrogenic mice in which BM precursors from NOD/SCID mice were transduced with a bicistronic retrovirus encoding rearranged TCRα and TCRβ chains of a given, islet-specific CD4 T cell clone, along with EGFP, prior to injection into Tssp° or WT control NOD female donors. Hence, expression of the retrovirally encoded TCR is necessary for differentiation into DP and CD4-SP thymocytes.

We first examined the development of a GAD206-220–specific TCR (PA19; ref. 34). In agreement with the functional data, the intrathymic differentiation of thymocytes expressing this TCR was comparable, regardless of whether or not the host expressed Tssp. Indeed, the percentage of DP and CD4-SP thymocytes and the level of TCR expression by these 2 subpopulations were comparable for WT and Tssp° NOD/SCID hosts (Figure 7A and Supplemental Figure 7A). In sharp contrast, the differentiation of CD4 T cells expressing the IA18 TCR specific for the IA-2β755–777 epitope was impaired in Tssp° NOD/SCID hosts as compared with that of WT NOD/SCID hosts (Figure 7B and Supplemental Figure 7B). Injection of IA18-transduced NOD/SCID BM cells into Tssp-sufficient hosts (IA18 → WT) led to the development of DP and CD4-SP thymocytes expressing high levels of IA18 TCR (Figure 7B and Supplemental Figure 7B). Despite some interindividual variations, the thymocyte staining profile was dramatically altered when the same transduced BM cells were injected into Tssp° hosts (IA18 → KO; Figure 7B and Supplemental Figure 7B). Hence, DP immature thymocytes expressed low TCR levels, and the percentage of CD4-SP thymocytes was severely reduced in some mice. Furthermore, in all mice these CD4-SP cells expressed lower levels of TCR.

Collectively, these results show that the development of some islet-reactive CD4 T cells requires Tssp expression in the thymus. While IA-2β may be an autoantigen important for diabetes development, this Ag is unlikely an essential autoantigen, since NOD mice with...
targeted disruption of IA-2β have normal diabetes incidence (35–37). We therefore tested whether lack of Tssp expression in the thymus may likewise impair the differentiation of the diabetogenic NY4.1 TCR (38, 39). For these experiments, we took advantage of existing NY4.1 T cells between CD4+CD45.1+ (KO) or CD4+CD45.2+ (WT) splenic T cells (n = 3) is shown. (C) Diabetes incidence in NOD/SCID female mice after transfer of CD25− cell–depleted splenocytes isolated from 10-week-old NOD female donors either alone (none) or together with purified CD4+CD25+ Tregs was injected. Addition of CD4+CD25+ Tregs significantly delayed diabetes onset (P < 0.002). (D) Diabetes incidence of WT and Tssp−/− male NOD mice after 1 (15 days) or 2 (30 days) injections of CY. CY was injected on day 0 and 15. One representative experiment out of at least 2 performed is shown.

Figure 5
Diabetes resistance of Tssp−/− NOD mice is not due to exacerbated T cell–dependent immunosuppression. (A) Diabetes incidence in NOD/SCID female mice after transfer of 15 x 10^6 splenocytes isolated from young Tssp−/− or WT NOD female donors or a 1:1 ratio of both cell types (15 x 10^6 total cells). For statistical analysis, the different groups were compared with the WT control group, and significant P values are shown. (B) CD45.1 Tssp−/− and CD45.2 congenic NOD splenocytes were mixed at a 1:1 ratio prior to adoptive transfer into female NOD/SCID mice. Eight weeks later, spleen cells were stained for CD4, CD45.1, CD45.2, and Foxp3. One representative FACS profile with the mean percentage (± SD) of Foxp3− T cells between CD4+CD45.1+ (KO) or CD4+CD45.2+ (WT) splenic T cells (n = 3) is shown. (C) Diabetes incidence in NOD/SCID female mice after transfer of CD25− cell–depleted splenocytes isolated from 10-week-old NOD female donors either alone (none) or together with purified CD4+CD25+ Tregs was injected. Addition of CD4+CD25+ Tregs significantly delayed diabetes onset (P < 0.002). (D) Diabetes incidence of WT and Tssp−/− male NOD mice after 1 (15 days) or 2 (30 days) injections of CY. CY was injected on day 0 and 15. One representative experiment out of at least 2 performed is shown.
an NY4.1 ligand that can induce negative selection of thymocytes expressing this TCR, further suggesting that Tssp, in WT mice, impairs the presentation of this ligand.

Diabetes resistance of Tssp° NOD mice is acquired during T cell differentiation in the thymus. The above results showed that the intrathymic differentiation of some but not all islet-specific CD4 T cells was impaired in Tssp° NOD mice, suggesting that, likewise, the diabetes resistance of Tssp° NOD mice may result from altered intrathymic selection of polyclonal CD4 T cells that are actively involved in diabetes development. To address this issue, we generated BM chimeras. We first determined whether the absence of Tssp in the thymic epithelium was sufficient to alter the course of spontaneous diabetes in NOD mice by reconstituting lethally irradiated Tssp° or WT control NOD mice with BM cells from WT NOD donors. Knowing that the peripheral lymphoid and pancreatic environment was not contributing to the diabetes resistance of Tssp° NOD mice (Figure 4A), this group of BM chimeras permits the evaluation of the contribution of Tssp° thymic epithelium to the diabetes resistance of Tssp° mice. Though reconstitution of the peripheral CD4 and CD8 compartments was comparable for the different BM chimeras, diabetes incidence was significantly reduced in Tssp° hosts reconstituted with WT BM cells as compared with that of WT hosts (Figure 9). Since both groups of chimeras were reconstituted with WT BM cells, the results indicate that diabetes resistance is not an intrinsic property of Tssp° T cells but instead can be acquired by WT T cells upon maturation in a Tssp°, epithelial thymic environment.

The thymic epithelium shapes the T cell repertoire through both positive and negative selection of developing T cells, while BM-derived cells only induce negative selection (43–45). To assess the role of negative selection of diabetogenic T cells in the diabetes resistance of Tssp° NOD mice, we generated the reverse chimeras, in which Tssp° BM cells were injected into irradiated WT hosts. We found that only 1 out of 5 such chimeras became diabetic within the 25 weeks of observation, indicating that negative selection significantly contributes to diabetes resistance of Tssp° mice (Figure 9B). Collectively, these results show that diabetes resistance is acquired by T cells during their development in the thymus by a mechanism that involves negative selection and possibly also by the lack of positive selection of diabetogenic T cells.

Discussion
In this study we analyzed the role of Tssp in the development of autoimmune diabetes. We showed that Tssp° NOD mice are protected from severe insulitis and from diabetes, suggesting that protection affects a critical event in disease initiation. We further showed that the diabetes resistance of Tssp° NOD mice is a property of the CD4 T cell compartment that does not involve dominant tolerance. Instead, diabetes resistance was acquired during T cell differentiation in the thymus and correlated with impaired thymic differentiation of CD4 T cells specific for some islet Ags. Hence, diabetes resistance of Tssp° mice likely results from defective selection of CD4 T cells specific for autoantigen(s) essential for diabetes initiation/development.
In support of our hypothesis of a defective selection of some islet-reactive CD4 T cells, we found that Tssp⁺ NOD mice are tolerant to IA-2β755–777. We further showed that the development of IA-2β755–777–specific CD4 T cells and CD4 T cells expressing the diabetogenic NY4.1 TCR requires thymic expression of Tssp. Finally, mixed BM chimeras showed that diabetes resistance is imposed during thymic selection. Hence, by different approaches, we showed that in Tssp⁺ NOD mice the islet-reactive CD4 T cell repertoire is purged of some specificities that are essential for diabetes development. Diabetes resistance of Tssp⁺ mice may therefore result from either impaired selection of CD4 T cells that are specific for one or a few essential diabetogenic Ags or from an overall reduction of the frequency of islet reactive CD4 T cells targeting different islet Ags. We have analyzed the response of Tssp⁺ NOD mice to several islet Ags that are targeted by diabetogenic CD4 T cell clones and found normal responses to the immunodominant GADβ206–220 epitopes and the 3 known IGRP epitopes and normal frequency of insulin-specific CD4 T cells in both naive and primed Tssp⁺ NOD mice. Instead, we found only some specific holes in the autoreactive CD4 T cell repertoire of Tssp⁺ mice, suggesting that diabetes resistance more likely results from impaired selection of few essential diabetogenic CD4 T cell(s). Spontaneous CD4 T cell responses to IA-2β were detected in the NOD mouse strain, and IA-2β755–777–specific T cell clones can induce diabetes upon transfer into NOD/SCID mice, suggesting that IA-2β may be an autoantigen important for diabetes development (35, 36). IA-2β is, however, unlikely an essential autoantigen, since NOD mice with targeted disruption of IA-2β do not show significant protection from diabetes (37). Furthermore, NOD/SCID retrogenic mice expressing the IA-2β755–777–specific (IA18) TCR show some level of insulitis but do not develop diabetes (34). In contrast, the yet unknown Ag recognized by the NY4.1 TCR may be an essential Ag, since transgenic mice expressing this TCR show high diabetes incidence (38, 39). Identification of the NY4.1 ligand will be required to answer this question. Furthermore, Tssp⁺ NOD mice will likely permit the identification and characterization of novel islet Ags that are important in the pathogenesis of diabetes.

While all Tssp⁺ NOD mice remained free of diabetes, we found that 20%–30% of NOD/SCID mouse recipients of Tssp⁺ NOD splenocytes or CD4 T cells developed diabetes, though with a delayed onset. It is remarkable to note that a similar low disease incidence is observed in CY-treated Tssp⁺ NOD mice. Hence, Tssp⁺ NOD mice still harbor islet-reactive T cells, including diabetogenic T cells that remain silent under normal circumstances but may induce disease when subjected to homeostatic proliferation. Appearance of low disease incidence upon transfer of Tssp⁺ splenocytes into NOD/SCID BM cells is not surprising, since Tssp⁺ NOD mice have some islet-reactive T cells and since the homeostatic proliferation induced by T cell transfer into lymphopoenic host is known to favor the expansion and activation of self-reactive T cells and thus contributes to autoimmune diseases (46, 47). The low penetrance and delayed onset of disease suggest, however, that some CD4 T cells essential for diabetes initiation are underrepresented in the inoculum. Alternatively, the homeostatic proliferation could reveal the diabetogenic potential of otherwise nonpathogenic CD4 T cells that could induce disease in some mice. Whatever the interpretation, the results are consistent with the conclusion that diabetes resistance in Tssp⁺ NOD mice results from crippling of the autoreactive CD4 T cell repertoire that affects a limited but essential number of autoreactive T cell specificities.

A critical issue concerns the mechanisms by which Tssp deficiency may impair thymic development of some autoreactive CD4 T cells and prevent diabetes development. Reconstitution of lethally
irradiated Tssp° NOD hosts with WT splenocytes indicated that Tssp deficiency confined to nonhematopoietic cells could lead to disease protection. This result is consistent with a perturbed positive selection of diabetogenic T cell specificities by TECs lacking Tssp, although one cannot exclude that, under such conditions, negative selection, driven either directly by the thymic epithelium itself or indirectly by endogenous DCs presenting modified determinants captured from Tssp° epithelial cells, may also take place (43–45, 48). The thymic profile of NY4.1 → KO chimera is highly suggestive of reinforced negative selection of developing NY4.1 thymocytes by Tssp° TECs. We also show that Tssp° hematopoietic cells can induce the deletion of thymocytes expressing the NY4.1 TCR and contribute to disease resistance in BM chimeras, suggesting that Tssp° DCs present agonist ligand that can delete some autoreactive CD4 T cells. Tssp function is therefore not confined to cTECs but extends to other thymic APCs, likely DCs. In addition to a role in generating positively selecting ligand presented by cTECs (11), Tssp can therefore also impair the presentation of some self peptides by DCs and possibly also by cTECs and thus prevent tolerance induction to some self Ags. Since peripheral DCs do not express Tssp, even in WT NOD mice, these autoreactive T cells can be activated by peripheral DCs presenting the corresponding islet Ag and induce diabetes.

We show in this study that Tssp is necessary for the thymic development of some islet-specific CD4 T cells. In a parallel study in the NOD mouse, likewise, we found that Tssp is required for the development of CD4 T cells specific for 1 foreign protein Ag (hen egg lysozyme [HEL]) out of 6 tested (30). In this case too, impaired development of the corresponding functional CD4 TCR repertoire could be induced by Tssp° DCs, likely through presentation of an HEL-mimotope of sufficient affinity to delete HEL-reactive CD4 T cells. Tssp therefore contributes to the diversification of the functional CD4 T cell repertoire. These different observations raise the intriguing possibility that the primary function of Tssp in the immune system is to somehow limit the presentation of self antigens by thymic DCs and consequently limit thymic tolerance to increase the diversity of the functional CD4 T cell repertoire. While providing selective advantage to

Figure 8
Impaired thymic development of CD4 T cells expressing the diabetogenic NY.4.1 TCR in Tssp° NOD mice. A 1:1 mix of BM cells from NY4.1 TCR transgenic NOD mice and WT NOD/SCID BM cells (NY4.1 + WT) or Tssp° NOD/SCID BM cells (NY4.1 + KO) were i.v. injected into lethally irradiated NOD-Ca° (WT) or Tssp° NOD-Ca° mice (KO), as indicated (BM mix → host). (A) The CD4 versus CD8 profile of thymocytes and Vβ11 expression by DP and CD4-SP cells is shown for a representative chimera of each group and control NY4.1 transgenic mice (NY4.1 Tg). The percentage of DP and CD4-SP cells is shown in the dot plots as well as the frequency of Vβ11+ cells among DP and CD4-SP cells, respectively. (B) Absolute cell numbers of total thymocytes and Vβ11+DP and Vβ11+CD4-SP cells are expressed as mean values ± SEM of 5 individual chimeras for the 3 groups of chimeras. Significant P values are shown (*P = 0.02; **P = 0.01; ***P < 0.007; ****P < 0.0003). One representative experiment out of a total of 3 performed is shown.
clear infectious agents, such function may, as observed in the NOD mouse, lead to autoimmunity. Further characterization of Tssp function will help clarify this interesting possibility.

In conclusion our study provides compelling evidence that the serine protease Tssp is critical for thymic selection of CD4 T cells that are essential for diabetes development. The alteration in the intrathymic selection of the CD4 T cell repertoire observed in the NOD (this study) and B6 mouse (11) are consistent with a role for Tssp in the MHC class II presentation pathway. Compared with the known proteases of the class II pathway, such as the cathepsin family and asparagine endopeptidase, Tssp presents features that we believe to be unique. Indeed, this is the first example to our knowledge of a protease with a restricted impact on T cell repertoire selection and a major role in the development of autoreactive T cells. A critical issue is whether Tssp has a similar function in humans as that reported here for the NOD mouse. Mouse and human TSSP present 79% protein sequence homology and a similar tissue distribution. The structural similarities between the diabetes susceptible mouse and human MHC class II molecules and the similarities of the known MHC class II-restricted autoantigens in mice and humans suggest that the autoreactive T cell repertoire will also be conserved. It is therefore possible that the function of TSSP is conserved between the 2 species and that TSSP may also control diabetogenic CD4 T cell development in humans too.

Methods

Mice and assessment of diabetes and insulitis. NOD/LtJ and NOD/SCID (NOD.CB17-Prkdc<sup>scid</sup>tm1Mal/J) mice were purchased from the Charles Rivers Laboratories. NOD-Ca<sup>+</sup> mice and NY4.1 TCR transgenic mice (38) were from The Jackson Laboratory. NOD-Cd45.2 congenic mice were provided by Paola Romagnoli (Centre de Physiopathologie de Toulouse Purpan). Tssp<sup>+</sup> B6 mice (Prens16<sup>mic Colony</sup> mouse) (11) were backcrossed to NOD/LtJ for up to 13 generations, and Tssp<sup>+</sup> NOD, NOD/SCID, NOD-Ca<sup>+</sup>, and WT control mice were generated as described in the Supplemental Methods section. Ten days later, CD4 or CD8 T cells were isolated from draining LN cell suspensions from primed mice through 2 rounds of negative selection by using either anti-CD8<sup>+</sup> (H58.5.58) or anti-CD4 (H129-19-19-6) mAbs together with anti-FcγRIIb (2.4G2), anti-CD11b (M1/70), and anti-B220 (RA3-6B2) mAbs and anti-iat IgG-coated magnetic beads (Dynabeads, Invitrogen). For polyclonal activation, 2×10<sup>5</sup> to 4×10<sup>5</sup> purified CD4 or CD8 T cells were stimulated with titrated doses of anti-CD3 and 1 μg/ml of anti-CD28 mAb along with 10<sup>6</sup> irradiated (20 Gy) NOD splenocytes. For Ag responses, 4×10<sup>5</sup> purified CD4 or CD8 T cells were stimulated in the presence of the appropriate Ag along with 10<sup>6</sup> irradiated (20 Gy) NOD splenocytes. After 3 days, the cultures were pulsed with 1 μCi/well of <sup>3</sup>H-thymidine (Amersham).

T cell cytotoxicity assay. LN cells from WT or Tssp<sup>+</sup> NOD mice (4×10<sup>6</sup> cells/well) were cultured in the presence of 2×10<sup>4</sup> irradiated allogeneic C57BL/6 splenocytes per well for 5 days in the presence of 10 U/ml IL-2 in a 24-well plate and used in a cytotoxicity assay against the EL4 (C57BL/6) thymoma cells. Cytolytic activity was measured using the CytoTox 96 colorimetric assay (Promega).

T cell proliferation assay. Purified CD4 T cells were stimulated with immobilized anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) mAbs alone or in the presence of 3.5 ng/ml IL-12 and 10 μg/ml anti-IL-4 Ab (1B11) for Th1 cell differentiation or 10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ Ab (XMG1.2) for Th2 cell differentiation, as described previously (49). Intracellular staining for IL-4 and IFN-γ were performed after 4 hours of stimulation with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 4 μM Momensin, as previously described (49).

Ab titer. Intraperitoneal immunizations were performed with KLH (Sigma-Aldrich) mixed with Imject Alum (Pierce) (50 μg/ml per mouse). Sera were collected prior to and 10 days after immunization and frozen. A KLH-specific ELISA was run using microplates coated with 20 μg/ml KLH (Nunc); biotin-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgM (SouthernBiotech); and avidin-conjugated HRP and TMB substrate solution (BD Biosciences) according to standard procedures.

Figure 9

Diabetes resistance is imposed during thymic differentiation. WT and Tssp<sup>+</sup> female NOD mice were lethally irradiated prior to reconstitution with T cell–depleted BM cells prepared from 8-week-old WT and Tssp<sup>+</sup> NOD female donors, as indicated in the figure legend (BM→host). (A) FACS analysis of the peripheral blood CD4 and CD8 profile is shown. The percentage (mean±SD) of CD4 (top left quadrant) and CD8 T cells (bottom right quadrant) is indicated (n=3). (B) Diabetes incidence in the indicated BM chimeras is shown. Diabetes incidence in WT→KO chimeras (P<0.03) and KO→WT chimeras (P<0.05) is significantly different from that of WT→WT chimeras.
Adoptive transfer and BM chimeras. For adoptive transfer, recipient mice were iv. injected with 10 x 10^9 to 15 x 10^9 red blood cell-depleted spleen cells from 5- to 6-week-old female donors. In some experiments, chimeric splenocytes suspensions were prepared by complementing B220+ cell-depleted splenocytes with purified B cells. Splenocytes containing a chimeric T cell subset were prepared by depleting splenocytes of either CD4 or CD8 T cells prior to complementation with purified CD4 or CD8 T cells. For such preparations, functional grade Abs to CD4, CD8α, Thy-1, and B220 (biotin) were used with anti-rat IgG-coated magnetic beads in negative selection procedures.

To analyze the suppressive activity of Tssp+ and WT Tregs, WT spleenocytes were depleted of CD25+ T cells using PE-anti-CD25 and anti-PE-microbeads (Miltenyi Biotec). For CD4+CD25+ Treg isolation, CD4 T cells were purified by negative selection as described above and CD25+ T cells were further isolated using PE-anti-CD25 and anti-PE-microbeads. 5 x 10^6 CD25-depleted spleenocytes were iv. injected either alone or together with 7 x 10^6 purified Tregs (corresponding to the relative frequency of Tregs in the spleen population) into NOD Ca+ mice.

For BM chimeras, 6- to 8-week-old female recipient mice were irradiated with 10 Gy 6 hours prior to iv. injection of 4 x 10^8 T cell-depleted BM cells. For the NY4.1 chimeras, host mice were irradiated with 9.5 Gy the day before reconstitution with 5 x 10^6 BM cells with NY4.1 transgenic mice together with 5 x 10^6 NOD/SCID or Tssp+ NOD/SCID BM cells. T cell development was analyzed 4 weeks after reconstitution.

Flow cytometry. Cells were stained with a combination of FITC-, PE-, allophycocyanin-, and PerCP-Cy5.5-conjugated Abs. The anti-CD4 (clone RM4-5), anti-CD8α (clone 53-6.7), anti-CD25 (clone PC61), anti-CD45.1 (clone A20), and anti-CD45.2 (clone 104) were obtained from eBioscience. For Foxp3 staining, cells were stained for surface expression of CD4, CD8α, and CD25 or CD4, CD45.1, and CD45.2 prior to intracellular staining with allophycocyanin-conjugated FJK-16 mAb or rat IgG2a isotype control by using the anti-mouse/rat Foxp3 Staining Set (eBioscience) according to the manufacturer’s instructions. Stained cells were acquired on FACSCalibur (Becton Dickinson), and data were analyzed using FlowJo software (Treestar). Events were collected within a lymphoid gate based on forward-scatter and side-scatter profiles.

Retrospective mice. TCR retrogenic mice were generated by injection of retrovirally transduced BM cells into WT or Tssp+ NOD/SCID mice irradiated with 1 Gy, as previously described (33). In brief, BM-donor NOD/SCID mice were injected with 0.15 mg/g body weight of 5-fluorouracil. BM cells were recovered 48 hours later and cultured for 48 hours in DMEM medium supplemented with 20 mg/ml IL-3, 50 mg/ml IL-6, and 50 ng/ml SCF and infected with retrovirial particles. Transduced cells were maintained in culture for an additional 48 hours, and 1 x 10^6 transduced BM cells were iv. injected. The different retroviruses were generated by transfection of Plat-E (50) packaging line with the appropriate constructs (34), as previously described (51). Mice were analyzed 4–6 weeks after reconstitution, and thymocytes were stained with CD4, CD8, and TCR-Cβ-specific Abs.

Statistics. Diabetes incidence curves were analyzed using the log-rank test of Prism software. Two-tailed P values are shown values are shown in the corresponding figures. Unpaired 1-tailed Student’s t tests were used otherwise. P < 0.05 was considered significant.

Acknowledgments

We thank J.-C. Guery, J. van Meerwijk, L. Pelletier, and P. Romanelli for helpful discussion and critical reading of the manuscript; S. Garcia for advise on insulin scoring; and the personnel of the Institut Fédératif de Recherche 150 animal facility, flow cytometry facility, and histopathology facility for expert technical assistance. This work was supported in part by institutional grants from INSERM and CNRS and by grants from European Foundation for the study of Diabetes/Novo Nordisk Program in Diabetes Research and Juvenile Diabetes Research Foundation.

Received for publication April 12, 2010, and revised in accepted form February 23, 2011.

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