



# 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  $\beta$  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 II 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<sup>o</sup>) NOD mice. While remaining immunocompetent, Tssp<sup>o</sup> NOD mice were protected from diabetes and severe insulinitis. Diabetes resistance of Tssp<sup>o</sup> 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  $\beta$  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 insulinitis 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-DQ<sup>2</sup>/DQ<sup>8</sup> and I-A<sup>s7</sup> contain a substitution of the Asp57 amino acid of the  $\beta$  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-A<sup>s7</sup> molecule is associated with an increased frequency of autoreactive CD4 T cells (5).

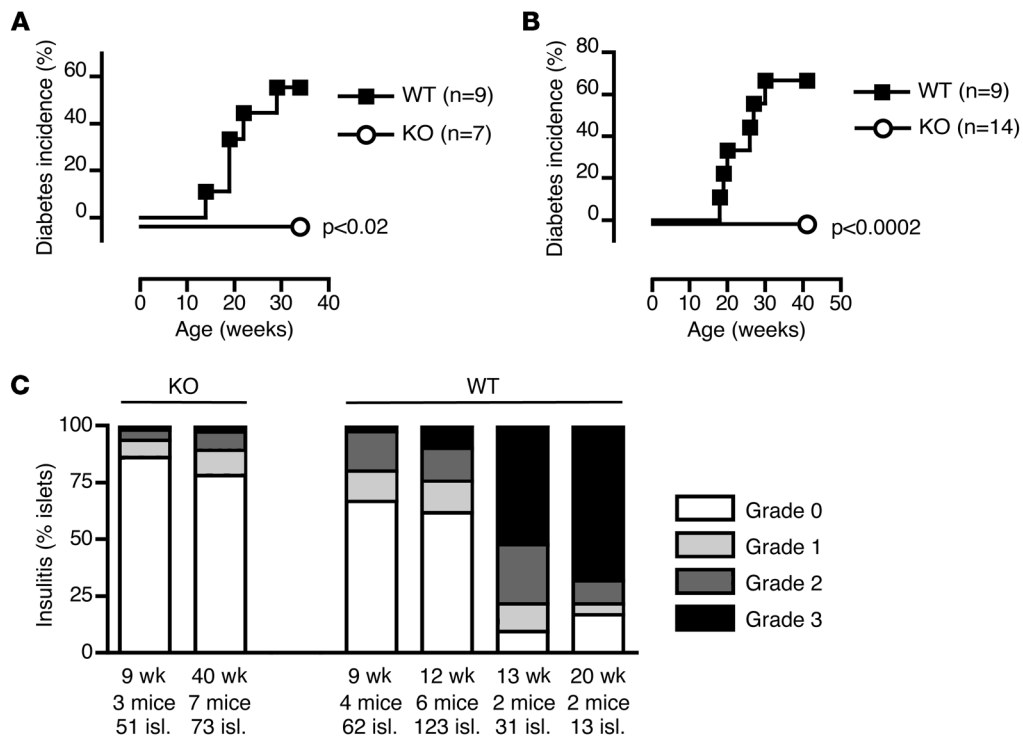
*PRSS16* was initially described as a gene of the extended HLA region linked to a diabetes susceptibility locus in humans (6, 7). *PRSS16* encodes a serine protease, thymus-specific serine protease (TSSP), that belongs to the family of lysosomal Pro-Xaa serine exopeptidases that includes lysosomal prolyl 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<sup>o</sup>) 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-A<sup>b</sup>-restricted TCR transgenes (OT-II and Marilyn) is impaired in Tssp<sup>o</sup> 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 (Ags) 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 Ags (16). Diabetogenic T cells target different islet Ags, including insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated protein 2 (IA-2), phogrin (IA-2 $\beta$ ), 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.

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**Figure 1**

Tssp<sup>o</sup> NOD mice are protected from spontaneous diabetes and insulinitis. Diabetes incidence in Tssp<sup>o</sup> (KO) and WT (A) N6F2 and (B) N13F2 female NOD mice (*n* indicates the number of mice). (C) Pancreatic sections from normoglycemic Tssp<sup>o</sup> (9 and 40 weeks old) and WT (9–20 weeks of age) N13F2 female NOD mice were scored blind for insulinitis (grade 0, healthy islets; grade 1, focal peri-insulinitis; grade 2, circular peri-insulinitis; grade 3, insulinitis). The number of mice and islets (isl.) scored for each age group is indicated. For statistical analysis, WT and KO mice were compared, and *P* values are shown.

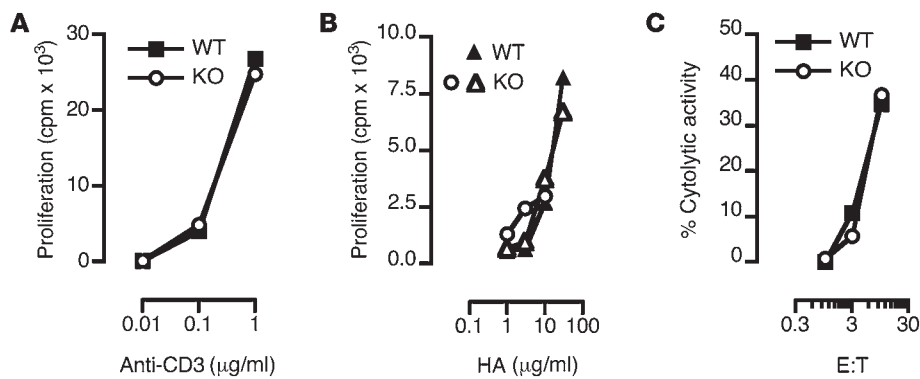
**Results**

*Tssp<sup>o</sup> NOD mice are resistant to insulinitis and diabetes.* To evaluate the role of Tssp in the development of autoimmune diabetes, we generated Tssp<sup>o</sup> NOD mice by backcrossing *Prss16*<sup>-/-</sup> 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*<sup>+/-</sup> mice were intercrossed at the sixth (N6F2), tenth (N10F2), and thirteenth (N33F2) backcross generation to generate Tssp<sup>o</sup> 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<sup>o</sup> NOD littermates were completely protected from disease (Figure 1A). Similarly, Tssp<sup>o</sup> 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 insulinitis, we found that while nondiabetic WT control mice showed an age-dependent progressive insulinitis characterized by peri-, marked, or full insulinitis, Tssp<sup>o</sup> mice showed only limited infiltration even at 40 weeks of age (Figure 1C).

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

*Tssp<sup>o</sup> NOD mice are immunocompetent.* In agreement with previous reports (10, 11), Tssp<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> NOD mice. Moreover, there was no alteration of the frequency of γδ T cells and NK and NKT cells in Tssp<sup>o</sup> NOD mice (data not shown).

In the periphery, the T cell populations of lymphoid organs were also normal in Tssp<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> NOD mice (Figure 2C). Similarly, the *in vitro* proliferation induced by anti-CD3 stimulation and the *in vitro* differentiation of Tssp<sup>o</sup> 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<sup>o</sup> NOD mice, further indicating that activation and differentiation of CD4 T cells into

**Figure 2**

Functional characterization of CD8 T cells from Tssp<sup>o</sup> and control NOD mice. (A) Proliferative response of purified CD8 LN T cells isolated from WT and Tssp<sup>o</sup> NOD mice after stimulation with anti-CD3/CD28 Abs. Syngeneic responses (<1,000 cpm) are subtracted. (B) In vitro recall response of CD8 T cells isolated from WT and Tssp<sup>o</sup> NOD mice immunized subcutaneously with HA<sub>512–520</sub> peptides emulsified in CFA. The peptide concentration used for in vitro challenge is shown. Syngeneic responses (<1,000 cpm) are subtracted. (C) Naive CD8 T cells isolated from WT and Tssp<sup>o</sup> NOD mice were cultured in the presence of allogenic C57BL/6 stimulators. The cytolytic activity in the presence of EL4 (C57BL/6) thymoma cells is shown. E:T, effector-to-target ratio. Each symbol shape corresponds to 1 individual mouse. The data are representative of at least 3 experiments.

effector cells and B cell activation were unaltered in Tssp<sup>o</sup> mice (Figure 3C). Collectively, these different results show that Tssp<sup>o</sup> NOD mice do not demonstrate major immune dysfunction and are thus globally immunocompetent.

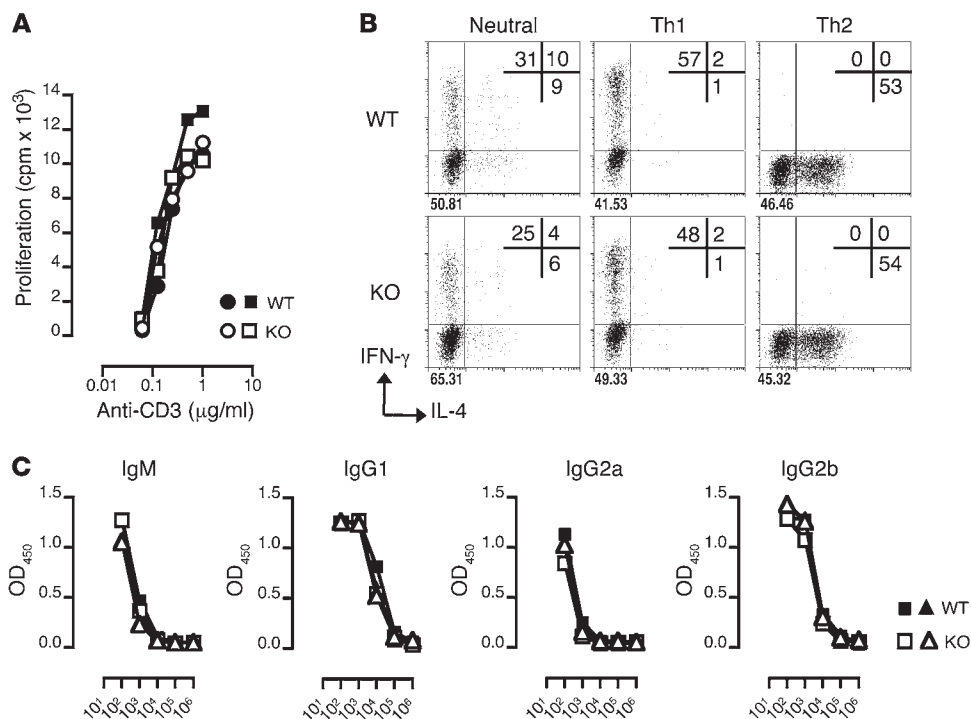
*Tssp<sup>o</sup> NOD mice are not intrinsically resistant to T1D development.* To decipher the mechanism of diabetes resistance of Tssp<sup>o</sup> NOD mice, we first determined whether Tssp deficiency might interfere with the access of lymphocytes to pancreatic islets or with the susceptibility of the mutant pancreata to immune attack. We therefore adoptively transferred splenocytes from prediabetic, WT control NOD female mice into Tssp<sup>o</sup> NOD/SCID mice. Under these conditions, we also evaluated the ability of the Tssp<sup>o</sup> 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<sup>o</sup> NOD mice are therefore not intrinsically resistant to autoimmune diabetes, suggesting that the diabetes resistance of Tssp<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> NOD mice reflects a property of their immune system.

*Diabetes resistance of Tssp<sup>o</sup> 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<sup>+</sup> 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  $\beta$  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<sup>o</sup> NOD mice, since peripheral DCs did not express Tssp and since Tssp<sup>o</sup> DCs in Tssp<sup>o</sup> 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 capacity of Tssp<sup>o</sup> 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<sup>o</sup>), 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<sup>o</sup> NOD mice and suggest a role for T cells in the diabetes resistance of Tssp<sup>o</sup> NOD mice. Indeed, NOD/SCID mice reconstituted with Tssp<sup>o</sup> T cells together with WT B cells showed a reduced disease progression that was comparable to that observed after transfer of total Tssp<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> splenocytes (Figure 4D). Collectively, these results clearly indicate that CD4 T cells are mainly responsible

**Figure 3**

Functional characterization of naive CD4 T cells and B cells from Tssp<sup>o</sup> and WT control NOD mice. **(A)** Proliferative response of purified CD4 LN T cells isolated from WT and Tssp<sup>o</sup> NOD mice after stimulation with anti-CD3/CD28 Abs in the presence of irradiated syngeneic splenocytes. One experiment with 2 individual mice (represented by a circle and a rectangle) of each genotype is shown. Syngeneic responses (<1,000 cpm) are subtracted. **(B)** In vitro differentiation of purified naive CD4 T cells isolated from WT or Tssp<sup>o</sup> NOD mice into Th1 and Th2 effector subsets upon anti-CD3/CD28-mediated stimulation performed under neutral and polarizing culture conditions, as indicated. The percentage of cells within each quadrant is indicated. **(C)** Ig primary response of Tssp<sup>o</sup> and WT control NOD mice to KLH. Mice were immunized with alum-mixed KLH, and sera were collected at day 10 prior to ELISA for KLH-specific IgG1, IgG2a, IgG2b, and IgM Abs. Preimmune sera were all negative in the assay (data not shown). The serum dilutions are shown on the x axis. One experiment with 2 individual mice (represented by a triangle and a rectangle) of each genotype is shown.

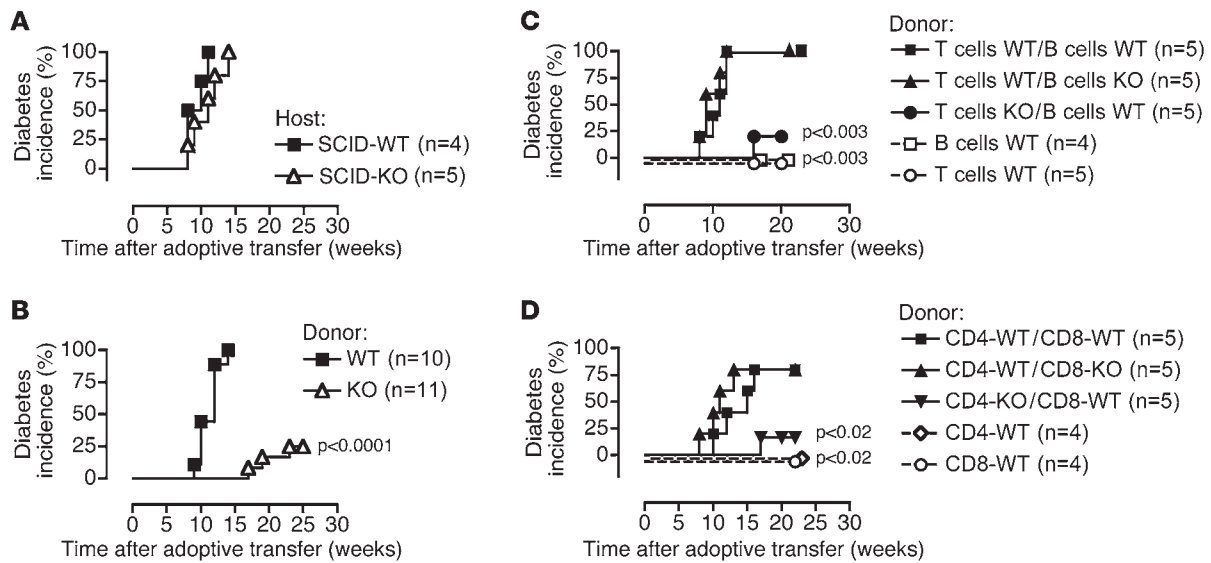
for the lowered capacity of Tssp<sup>o</sup> splenocytes to cause diabetes in NOD/SCID hosts, further suggesting that diabetes resistance of Tssp<sup>o</sup> NOD mice is a property of the CD4 T cell subset.

*Tssp<sup>o</sup> NOD mice do not show exacerbated Treg function.* Among CD4 T cells, CD25<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> 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<sup>o</sup> NOD mice. We found that the frequency and number of CD25<sup>+</sup>Foxp3<sup>+</sup>CD4<sup>+</sup> Tregs was comparable in all lymphoid organs of WT and Tssp<sup>o</sup> NOD mice (Supplemental Figure 6). We further examined the regulatory potential of Tssp<sup>o</sup> lymphocytes by mixing Tssp<sup>o</sup> and WT splenocytes in the NOD/SCID transfer system, reasoning that if Tssp<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> Tregs, since the representation of WT and Tssp<sup>o</sup> Tregs remained comparable 8 weeks after adoptive transfer (Figure 5B). To more directly assess the regulatory activity of Tssp<sup>o</sup> CD25<sup>+</sup>CD4<sup>+</sup> Tregs, we depleted the spleen population of prediabetic

WT NOD mice of CD25<sup>+</sup> T cells and complemented this population with the same number of CD25<sup>+</sup>CD4<sup>+</sup> Tregs isolated from age-matched WT or Tssp<sup>o</sup> NOD mice. We found that the addition of WT or Tssp<sup>o</sup> CD25<sup>+</sup>CD4<sup>+</sup> Tregs was equally able to significantly delay diabetes onset in this experimental system (Figure 5C). Collectively, these results show that Tssp<sup>o</sup> splenocytes and Tregs do not express an exacerbated T suppressor activity.

Finally, when diabetes was induced by administration of cyclophosphamide (CY), Tssp<sup>o</sup> 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<sup>+</sup>CD4<sup>+</sup> T cells (29), the reduced susceptibility of Tssp<sup>o</sup> 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<sup>o</sup> NOD mice is unlikely to be due to exacerbated immunosuppression mediated by CD4 Tregs.

*Tssp<sup>o</sup> NOD mice respond to some but not all islet Ags.* The above results suggested that the diabetes resistance of Tssp<sup>o</sup> 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<sup>o</sup> and



**Figure 4**

Diabetes resistance of *Tssp*<sup>o</sup> NOD mice is a property of their mature CD4 T cell subset. (A) Diabetes incidence in NOD/SCID (SCID-WT) and *Tssp*<sup>o</sup> NOD/SCID (SCID-KO) mice after injection of splenocytes from prediabetic NOD females. (B) Diabetes incidence in NOD/SCID female mice after injection of  $10 \times 10^6$  to  $15 \times 10^6$  splenocytes isolated from 6-week-old N10F2 *Tssp*<sup>o</sup> or WT control NOD female donors. (C) Splenocytes from 6-week-old N10F2 *Tssp*<sup>o</sup> or WT control NOD females were depleted of B cells (referred to as T cells) prior to complementation with either *Tssp*<sup>o</sup> or WT-purified B cells.  $15 \times 10^6$  cells were transferred to NOD/SCID females. (D) Splenocytes from 5-week-old N10F2 WT NOD females were depleted of either CD4 or CD8 T cells and complemented with purified *Tssp*<sup>o</sup> cells or WT CD4 cells or CD8 T cells, as indicated.  $9 \times 10^6$  cells were i.v. injected into NOD/SCID females. CD4-WT and CD8-WT correspond to noncomplemented CD8 or CD4 T cell-depleted WT splenocytes, respectively. One representative experiment out of 2 performed is shown. For statistical analysis, the different groups were compared with WT control mice, and significant *P* values are shown.

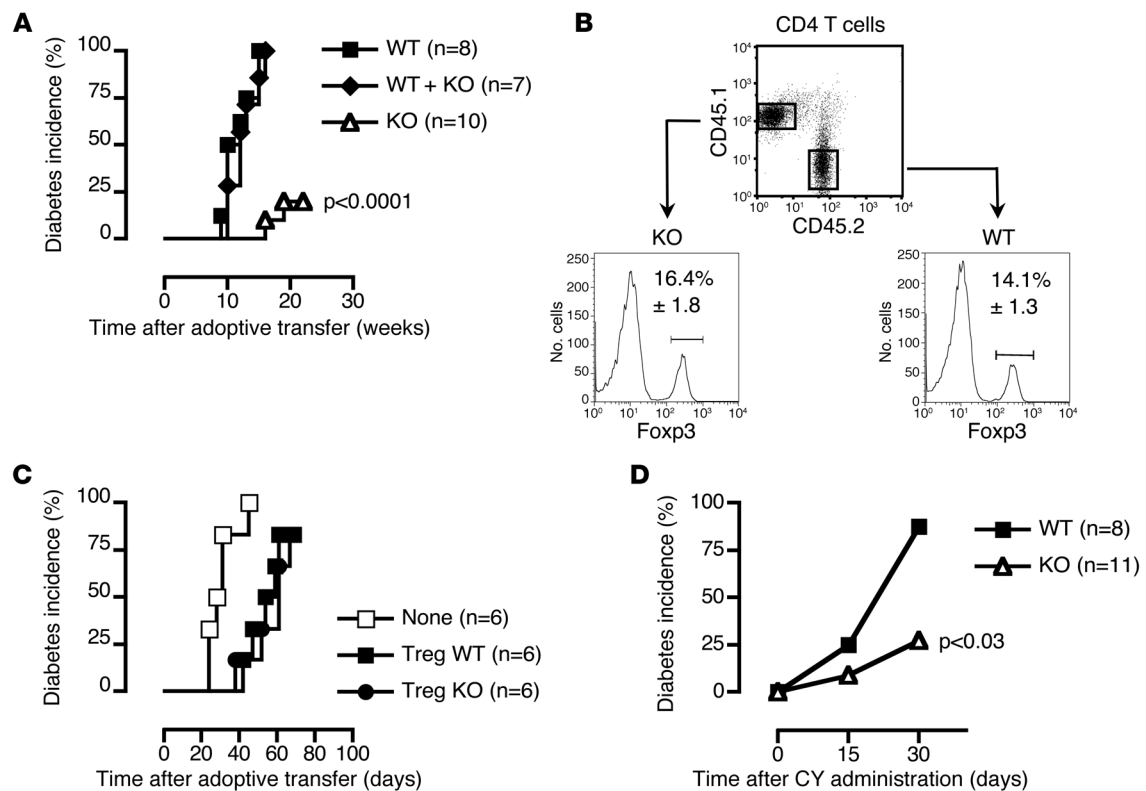
WT control NOD mice to the immunodominant peptide of known islet autoantigens suspected to play a role in diabetes development (16). Mice were immunized with the corresponding Ag in CFA, and their recall response was analyzed in vitro. Quite unexpectedly in light of the foremost role of insulin in diabetes development (31, 32), we found that the frequency of IL-2-producing CD4 T cells specific for Ins<sub>9-23</sub> and Ins<sub>49-66</sub> was comparable in *Tssp*<sup>o</sup> and WT NOD mice (Figure 6). Likewise WT and *Tssp*<sup>o</sup> NOD mice responded similarly to the GAD<sub>206-220</sub> peptide and the known IGRP immunodominant epitopes (Figure 6). However, while all WT NOD mice responded to the IA-2β<sub>755-777</sub> epitope of phogrin, a transmembrane protein found in the secretory granules of pancreatic islet cells, only 1 out of 5 *Tssp*<sup>o</sup> NOD mice analyzed responded to that epitope and at a lower level than that of control WT mice (Figure 6). *Tssp*<sup>o</sup> 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-2β<sub>755-777</sub> 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*<sup>o</sup> or WT NOD/SCID mice (33, 34). Due to a defect in DNA repair enzymes, NOD/SCID hosts were only mildly irradiated. The thymus of *Tssp*<sup>o</sup> NOD/SCID hosts therefore also contained host-derived *Tssp*<sup>o</sup> DCs that may, in addition to *Tssp*<sup>o</sup> TECs, contribute to any alteration of the intrathymic differentiation of the transduced thymocytes. Due to the SCID mutation of the donor and host BM cells, generation of double-positive (DP) and single-positive (SP) thymo-

cytes was not observed when donor BM cells were transduced with empty vectors (data not shown). Hence, expression of the retrovirally encoded TCR is necessary for differentiation into DP and CD4-SP thymocytes.

We first examined the development of a GAD<sub>206-220</sub>-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*<sup>o</sup> 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-2β<sub>755-777</sub> epitope was impaired in *Tssp*<sup>o</sup> 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*<sup>o</sup> 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

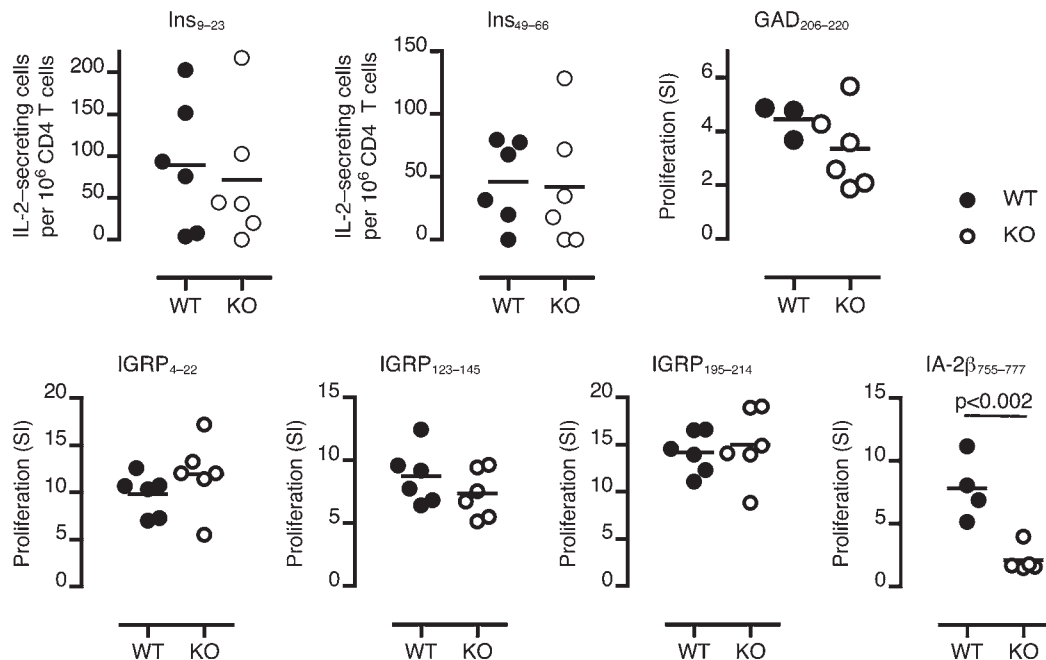
**Figure 5**

Diabetes resistance of  $Tssp^{\circ}$  NOD mice is not due to exacerbated T cell–dependent immunosuppression. **(A)** Diabetes incidence in NOD/SCID female mice after transfer of  $15 \times 10^6$  splenocytes isolated from young  $Tssp^{\circ}$  or WT NOD female donors or a 1:1 ratio of both cell types ( $15 \times 10^6$  total cells). For statistical analysis, the different groups were compared with the WT control group, and significant  $P$  values are shown. **(B)** CD45.1  $Tssp^{\circ}$  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 ( $\pm$  SD) of Foxp3<sup>+</sup> T cells between CD4<sup>+</sup>CD45.1<sup>+</sup> (KO) or CD4<sup>+</sup>CD45.2<sup>+</sup> (WT) splenic T cells ( $n = 3$ ) is shown. **(C)** Diabetes incidence in NOD/SCID female mice after transfer of CD25<sup>+</sup> cell–depleted splenocytes isolated from 10-week-old NOD female donors either alone (none) or together with purified CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from 6- to 8-week-old WT control (Treg WT) or  $Tssp^{\circ}$  (Treg KO) NOD mice. A total of  $5 \times 10^6$  cells, containing 14% CD4<sup>+</sup>CD25<sup>+</sup> Tregs was injected. Addition of CD4<sup>+</sup>CD25<sup>+</sup> Tregs significantly delayed diabetes onset ( $P < 0.002$ ). **(D)** Diabetes incidence of WT and  $Tssp^{\circ}$  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.

targeted disruption of IA-2 $\beta$  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 transgenic mice. Since  $Tssp$  is expressed by cTECs and thymic DCs, we assessed the role of both cell types in the development of the NY4.1 TCR using mixed BM chimeras. To examine the role of  $Tssp^{\circ}$  radio-resistant TECs in the differentiation of NY4.1 CD4 T cells, we reconstituted lethally irradiated  $Tssp^{\circ}$  TCR $\alpha$ -deficient NOD mice (NOD-Ca<sup>o</sup> mice) and, as control, WT NOD-Ca<sup>o</sup> hosts with a 1:1 mix of BM cells from NY4.1 transgenic mice and NOD/SCID BM cells (NY4.1 + WT  $\rightarrow$  KO or NY4.1 + WT  $\rightarrow$  WT, respectively). Conversely, to evaluate the possible role of thymic DCs in deleting developing NY4.1 thymocytes, we reconstituted lethally irradiated NOD-Ca<sup>o</sup> hosts with a 1:1 mix of BM cells from NY4.1 transgenic mice and  $Tssp^{\circ}$  NOD/SCID BM cells (NY4.1 + KO  $\rightarrow$  WT) as a source of  $Tssp^{\circ}$  DCs. Lack of  $Tssp$  expression by TECs did not lead to significant alteration in the percentage of DP and CD4-SP thymocytes or in the expression level of the transgenic  $\beta$  chain ( $V\beta 11$ ) by these 2 subpopulations (Figure 8A). In NY4.1  $\rightarrow$  KO chi-

meras, however, the total number of thymocytes and consequently the total number of  $V\beta 11^+$ DP and  $V\beta 11^+$ CD4-SP thymocytes were dramatically reduced as compared with those found in NY4.1  $\rightarrow$  WT chimeras (Figure 8B). Highly reminiscent of reinforced negative selection (39–42), this decrease in cellularity strongly suggests that  $Tssp^{\circ}$  TECs and more likely cTECs cause, either directly or indirectly, the marked deletion of developing NY4.1 thymocytes. Likewise, the development of NY4.1-expressing CD4 T cells was impaired in chimeras in which only thymic DCs were  $Tssp^{\circ}$  (Figure 8, NY4.1 + KO  $\rightarrow$  WT). In this case, the total cellularity was also decreased (albeit not as severely as in NY4.1  $\rightarrow$  KO chimeras) and the number of DP thymocytes and percentage and number of  $V\beta 11^+$  DP thymocytes was reduced (Figure 8 and data not shown). Impaired transition to mature CD4-SP cells in NY4.1 + KO  $\rightarrow$  WT chimeras was further highlighted by the decreased number of CD4-SP cells and  $V\beta 11^+$  CD4-SP cells (Figure 8B and not shown).  $Tssp^{\circ}$  DCs therefore induce negative selection of developing NY4.1 CD4 T cells.

These different results show that  $Tssp$  expression in the thymus is necessary for the development of the NY4.1 and IA18 but not PA19 TCRs. They also show that  $Tssp^{\circ}$  cTECs and DCs present



**Figure 6**

Deficient response of mature CD4 T cells from Tssp<sup>o</sup> NOD mice to IA-2 $\beta$ <sub>755-777</sub> autoantigen. In vitro recall response of CD4 T cells isolated from skin draining LNs of WT and Tssp<sup>o</sup> NOD mice immunized subcutaneously with the indicated peptides. The frequency of IL-2-producing CD4 T cells specific for the Ins<sub>9-23</sub> and Ins<sub>49-66</sub> peptides was determined by ELISPOT analysis after stimulation with 30  $\mu$ g/ml peptide. Otherwise, the proliferative response induced by stimulating purified CD4 T cells with the relevant peptide at a concentration of 30  $\mu$ g/ml is expressed as stimulation index (SI). Each symbol corresponds to 1 individual mouse, analyzed in 2 to 3 independent experiments. The black lines correspond to the mean value of WT or Tssp<sup>o</sup> NOD mice. Significant P values are shown.

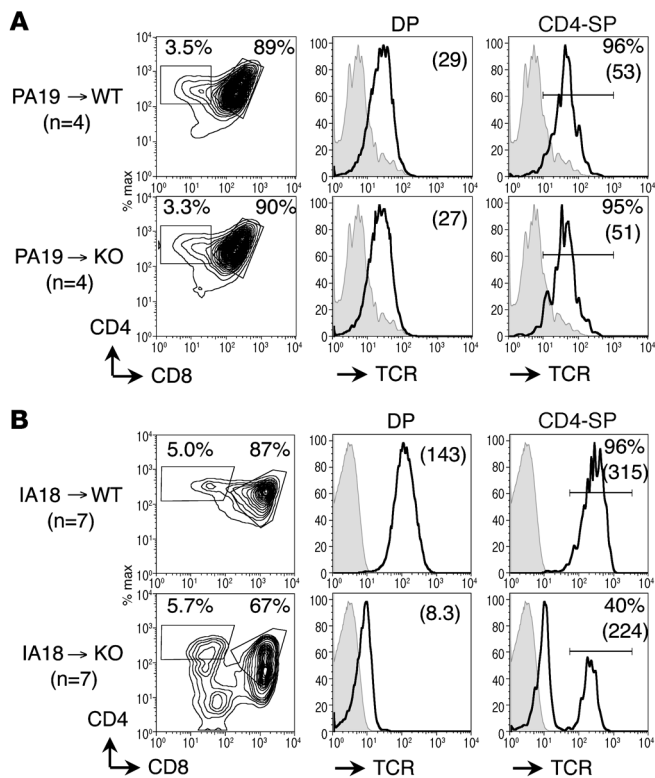
an NY4.1 ligand that can induce negative selection of thymocytes expressing this TCR, further suggesting that Tssp, in WT mice, impairs the presentation of this ligand.

*Diabetes resistance of Tssp<sup>o</sup> 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<sup>o</sup> NOD mice, suggesting that, likewise, the diabetes resistance of Tssp<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> NOD mice (Figure 4A), this group of BM chimeras permits the evaluation of the contribution of Tssp<sup>o</sup> thymic epithelium to the diabetes resistance of Tssp<sup>o</sup> mice. Though reconstitution of the peripheral CD4 and CD8 compartments was comparable for the different BM chimeras, diabetes incidence was significantly reduced in Tssp<sup>o</sup> 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<sup>o</sup> T cells but instead can be acquired by WT T cells upon maturation in a Tssp<sup>o</sup>, 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<sup>o</sup> NOD mice, we generated the reverse chimeras, in which Tssp<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> NOD mice are protected from severe insulinitis and from diabetes, suggesting that protection affects a critical event in disease initiation. We further showed that the diabetes resistance of Tssp<sup>o</sup> 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<sup>o</sup> mice likely results from defective selection of CD4 T cells specific for autoantigen(s) essential for diabetes initiation/development.

**Figure 7**

Thymic expression of Tssp is necessary for the differentiation of IA-2 $\beta$ <sub>755-777</sub>-specific CD4 T cells. NOD/SCID BM cells were transduced with retrovirus encoding rearranged TCR $\alpha$  and TCR $\beta$  chains, along with EGFP. The same transduced BM cells were transferred into NOD/SCID (WT) or Tssp<sup>o</sup> NOD/SCID (KO) recipients. Only mice with more than 20% EGFP<sup>+</sup> thymocytes were considered. The CD4 and CD8 profile of transduced (EGFP<sup>+</sup>) total thymocytes and the TCR profile for electronically gated EGFP<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> (DP) and mature EGFP<sup>+</sup>CD4<sup>+</sup> (CD4-SP) thymocytes are shown. The percentage of DP and CD4-SP thymocytes is indicated in the dot plots and in the histograms, together with the percentage and (in parenthesis) the TCR MFI of TCR<sup>+</sup> DP and CD4-SP thymocytes. Shaded histograms correspond to unstained controls. The development of thymocytes bearing (A) the GAD<sub>206-220</sub>-specific PA19.5E11 (PA19) TCR and (B) the IA-2 $\beta$ <sub>755-777</sub>-specific IA18 TCR is shown. One representative mouse from 1 experiment out of (A) 2 or (B) 3 performed is shown. Additional mice are depicted in Supplemental Figure 7. PA19 → WT refers to injection of PA19-transduced NOD/SCID BM cells into Tssp-sufficient hosts; PA19 → KO refers to injection of PA19-transduced NOD/SCID BM cells into Tssp<sup>o</sup> hosts; IA18 → WT refers to injection of IA18-transduced NOD/SCID BM cells into Tssp-sufficient hosts; IA18 → KO refers to injection of IA18-transduced NOD/SCID BM cells into Tssp<sup>o</sup> hosts.

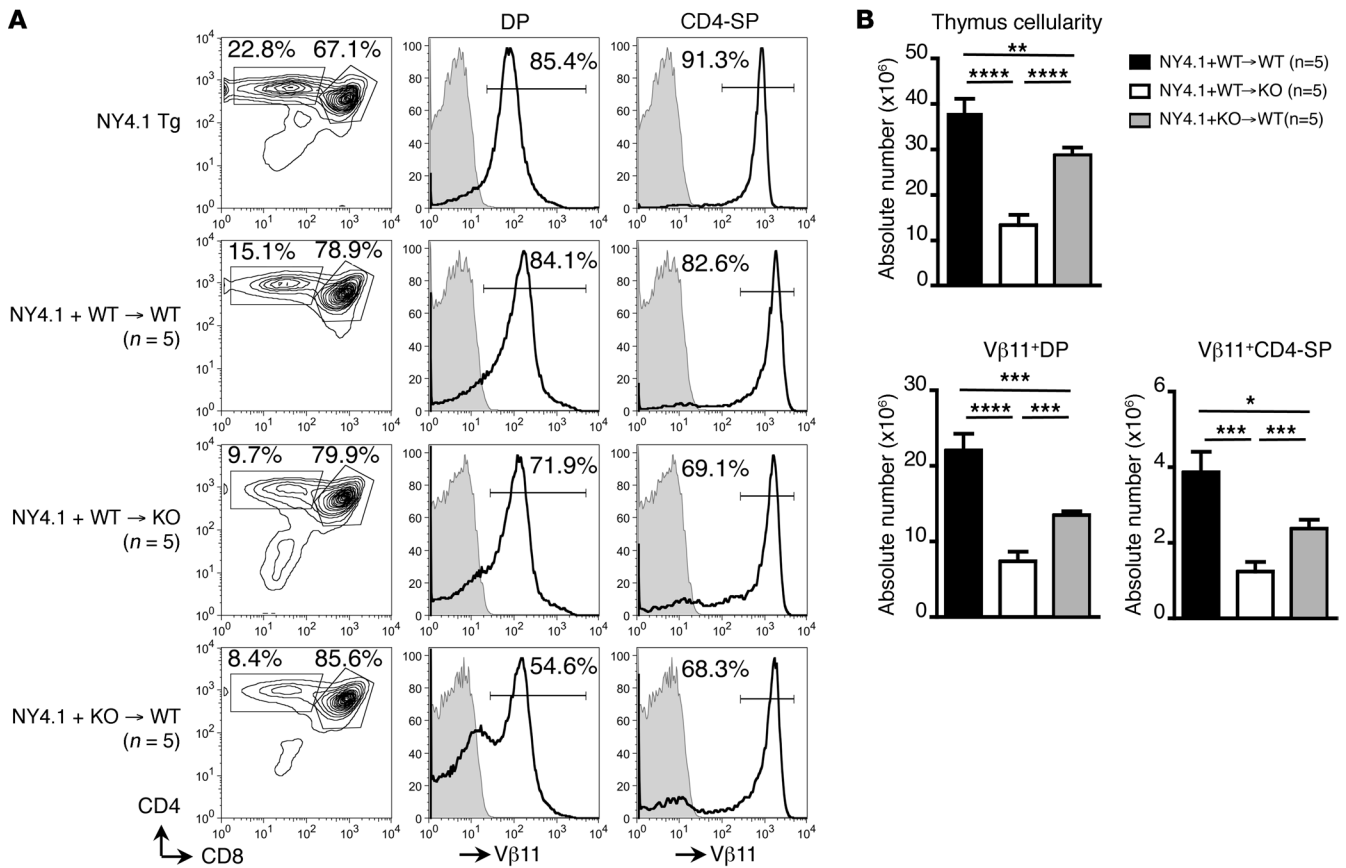
In support of our hypothesis of a defective selection of some islet-reactive CD4 T cells, we found that Tssp<sup>o</sup> NOD mice are tolerant to IA-2 $\beta$ <sub>755-777</sub>. We further showed that the development of IA-2 $\beta$ <sub>755-777</sub>-specific CD4 T cells and CD4 T cells expressing 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<sup>o</sup> NOD mice the islet-reactive CD4 T cell repertoire is purged of some specificities that are essential for diabetes development. Diabetes resistance of Tssp<sup>o</sup> 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<sup>o</sup> NOD mice to several islet Ags that are targeted by diabetogenic CD4 T cell clones and found normal responses to the immunodominant GAD<sub>206-220</sub> epitopes and the 3 known IGRP epitopes and normal frequency of insulin-specific CD4 T cells in both naive and primed Tssp<sup>o</sup> NOD mice. Instead, we found only some specific holes in the autoreactive CD4 T cell repertoire of Tssp<sup>o</sup> 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-2 $\beta$  were detected in the NOD mouse strain, and IA-2 $\beta$ <sub>755-777</sub>-specific T cell clones can induce diabetes upon transfer into NOD/SCID mice, suggesting that IA-2 $\beta$  may be an autoantigen important for diabetes development (35, 36). IA-2 $\beta$  is, however, unlikely an essential autoantigen, since NOD mice with targeted disruption of IA-2 $\beta$  do not show significant protection from diabetes (37). Furthermore, NOD/SCID retrogenic mice expressing the IA-2 $\beta$ <sub>755-777</sub>-specific (IA18) TCR show some level of insulinitis 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<sup>o</sup> NOD mice will likely permit the identification and characterization of novel islet Ags that are important in the pathogenesis of diabetes.

While all Tssp<sup>o</sup> NOD mice remained free of diabetes, we found that 20%–30% of NOD/SCID mouse recipients of Tssp<sup>o</sup> 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<sup>o</sup> NOD mice. Hence, Tssp<sup>o</sup> 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<sup>o</sup> splenocytes into NOD/SCID mice is not surprising, since Tssp<sup>o</sup> NOD mice have some islet-reactive T cells and since the homeostatic proliferation induced by T cell transfer into lymphopenic 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<sup>o</sup> 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



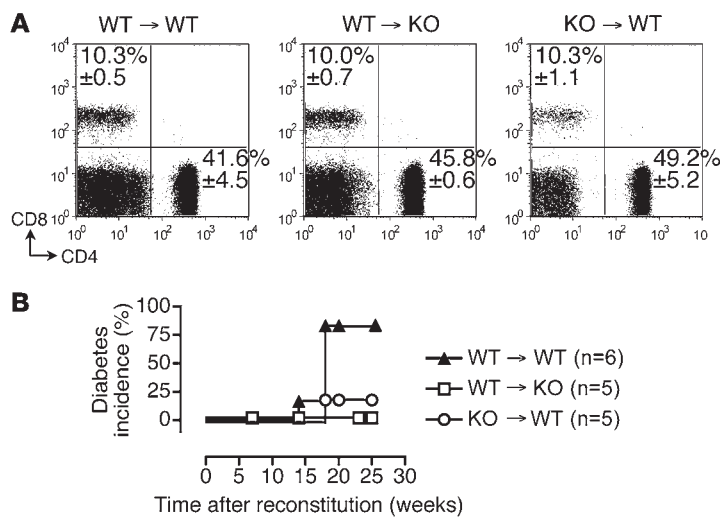


**Figure 8** Impaired thymic development of CD4 T cells expressing the diabetogenic NY4.1 TCR in Tssp<sup>o</sup> NOD mice. A 1:1 mix of BM cells from NY4.1 TCR transgenic NOD mice and WT NOD/SCID BM cells (NY4.1 + WT) or Tssp<sup>o</sup> NOD/SCID BM cells (NY4.1 + KO) were i.v. injected into lethally irradiated NOD-Ca<sup>o</sup> (WT) or Tssp<sup>o</sup> NOD-Ca<sup>o</sup> 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.

irradiated Tssp<sup>o</sup> 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<sup>o</sup> 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<sup>o</sup> TECs. We also show that Tssp<sup>o</sup> hematopoietic cells can induce the deletion of thymocytes expressing the NY4.1 TCR and contribute to disease resistance in BM chimeras, suggesting that Tssp<sup>o</sup> 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<sup>o</sup> 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 9**

Diabetes resistance is imposed during thymic differentiation. WT and Tssp<sup>o</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>o</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.

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 insulinitis.** NOD/LtJ and NOD/SCID (NOD.CB17-Prkdc<sup>cid/J</sup>) mice were purchased from the Charles Rivers Laboratories. NOD-Ca<sup>o</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>o</sup> B6 mice (Prss16<sup>miMal</sup> mice) (11) were backcrossed to NOD/LtJ for up to 13 generations, and Tssp<sup>o</sup> NOD, NOD/SCID, NOD-Ca<sup>o</sup>, and WT control mice were generated as described in the Supplemental Methods section. Mice were considered diabetic when blood glucose levels exceed 250 mg/dl for 2 consecutive weeks, the first positive measurement defining disease onset. Histology sections were scored blindly. For CY-induced diabetes, 10- to 12-week-old male mice were injected intraperitoneally with 250 mg/ml CY (Sigma-Aldrich), and diabetes incidence was examined after 2 weeks. A second CY injection was performed in case of normal glycosuria.

All experiments involving animals were performed in accordance with the institutional guidelines of INSERM, and mouse experimental protocols were approved by the local ethics committee of Midi-Pyrénées, Toulouse, France.

**T cell proliferation assays.** Mice were immunized by subcutaneous injections into the footpad and tail base of 20  $\mu$ g HPLC-purified (>95% purity) synthetic peptides (GeneCust) emulsified in CFA (Sigma-Aldrich). Sequences of the peptides used in this study are shown 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 $\alpha$  (H58.55.8) or anti-CD4 (H129-19-6) mAbs together with anti-Fc $\gamma$ Rb (2.4G2), anti-CD11b (M1/70), and anti-B220 (RA3-6B2) mAbs and anti-rat IgG-coated magnetic beads (Dynabeads, Invitrogen). For polyclonal activation,  $2 \times 10^4$  to  $4 \times 10^4$  purified CD4 or CD8 T cells were stimulated with titrated doses of anti-CD3 and 1  $\mu$ g/ml of anti-CD28 mAb along with  $10^6$  irradiated (20 Gy) NOD splenocytes. For Ag responses,  $4 \times 10^5$  purified CD4 or CD8 T cells were stimulated in the presence of the appropriate Ag along with  $10^6$  irradiated (20 Gy) NOD splenocytes. After 3 days, the cultures were pulsed with 1  $\mu$ Ci/well of <sup>3</sup>H-thymidine (Amersham).

**T cell cytotoxicity assay.** LNs cells from WT or Tssp<sup>o</sup> NOD mice ( $4 \times 10^6$  cells/well) were cultured in the presence of  $2 \times 10^6$  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 polarization assay.** Purified CD4 T cells were stimulated with immobilized anti-CD3 (10  $\mu$ g/ml) and soluble anti-CD28 (1  $\mu$ g/ml) mAbs alone or in the presence of 3.5 ng/ml IL-12 and 10  $\mu$ g/ml anti-IL-4 Ab (11B11) for Th1 cell differentiation or 10 ng/ml IL-4 and 10  $\mu$ g/ml anti-IFN- $\gamma$  Ab (XMG1.2) for Th2 cell differentiation, as described previously (49). Intracellular staining for IL-4 and IFN- $\gamma$  were performed after 4 hours of stimulation with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 4  $\mu$ M Monensin, as previously described (49).

**Ab titer.** Intraperitoneal immunizations were performed with KLH (Sigma-Aldrich) mixed with Imject Alum (Pierce) (50  $\mu$ g/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  $\mu$ g/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.



**Adoptive transfer and BM chimeras.** For adoptive transfer, recipient mice were i.v. injected with  $10 \times 10^6$  to  $15 \times 10^6$  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<sup>+</sup> 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, CD8 $\alpha$ , Thy-1, and B220 (eBioscience) were used with anti-rat IgG-coated magnetic beads in negative selection procedures.

To analyze the suppressive activity of Tssp<sup>o</sup> and WT Tregs, WT splenocytes were depleted of CD25<sup>+</sup> T cells using PE-anti-CD25 and anti-PE-microbeads (Miltenyi Biotech). For CD4<sup>+</sup>CD25<sup>+</sup> Treg isolation, CD4 T cells were purified by negative selection as describe above and CD25<sup>+</sup> T cells were further isolated using PE-anti-CD25 and anti-PE-microbeads.  $5 \times 10^6$  CD25-depleted splenocytes were i.v. injected either alone or together with  $7 \times 10^5$  purified Tregs (corresponding to the relative frequency of Tregs in the spleen population) into NOD Ca<sup>o</sup> mice.

For BM chimeras, 6- to 8-week-old female recipient mice were irradiated with 10 Gy 6 hours prior to i.v. injection of  $4 \times 10^6$  T cell-depleted BM cells. For the NY4.1 chimeras, host mice were irradiated with 9.5 Gy the day before reconstitution with  $5 \times 10^6$  BM cells from NY4.1 transgenic mice together with  $5 \times 10^6$  NOD/SCID or Tssp<sup>o</sup> 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-CD8 $\alpha$  (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, CD8 $\alpha$ , and CD25 or CD4, CD45.1, and CD45.2 prior to intracellular staining with allophycocyanin-conjugated FJK-16s 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.

**Retrogenic mice.** TCR retrogenic mice were generated by injection of retrovirally transduced BM cells into WT or Tssp<sup>o</sup> 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 ng/ml IL-3, 50 ng/ml IL-6, and 50 ng/ml SCF and infected with retroviral particles. Transduced cells were maintained in culture for an additional 48 hours, and  $1 \times 10^6$  transduced BM cells were i.v. injected. The different retroviruses were generated by transfection of PlatE (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-C $\beta$ -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.

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