Type 1 Diabetes in NOD Mice Unaffected by Mast Cell Deficiency

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ABSTRACT

Mast cells have been invoked as important players in immune responses associated with autoimmune diseases. Based on *in vitro* studies, or *in vivo* through the use of *Kit* mutant mice, mast cells have been suggested to play immunological roles in direct antigen presentation to both CD4⁺ and CD8⁺ T cells, in the regulation of T and dendritic cell migration to lymph nodes, and in Th1 versus Th2 polarization; all of which could significantly impact the immune response against self antigens in autoimmune disease, including type 1 diabetes (T1D). Until now, the role of mast cells in the onset and incidence of T1D has only been indirectly tested through the use of low-specificity mast cell inhibitors and activators, and published studies reported contrasting results. Our three laboratories have generated independently two strains of mast cell-deficient NOD mice, NOD.*Cpa3^{Cre/+}* (Heidelberg), and NOD.*Kit^{W-sh/W-sh* (Leuven and Boston), to address} the effects of mast cell deficiency on the development of T1D in the NOD strain. Our collective data demonstrate that both incidence and progression of T1D in NOD mice are independent of mast cells. Moreover, analysis of pancreatic lymph node cells indicated that lack of mast cells has no discernible effect on the autoimmune response, which involves both innate and adaptive immune components. Our results demonstrate that mast cells are not involved in T1D in the NOD strain, making their role in this process nonessential and excluding them as potential therapeutic targets.

INTRODUCTION

Mast cells are innate immune cells that are the main effectors in IgE-mediated allergic inflammation. In response to cross-linking of the high affinity IgE receptor FceRI, mast cells release preformed molecules stored in granules, such as histamine, proteoglycans and proteases that can contribute to allergic inflammation and to anaphylactic shock (1). In addition to this well-established role in IgE-mediated allergic diseases, mast cells, due to the plethora of factors that they can produce, are believed to take part in, and modulate, many immune responses, including autoimmunity. Based on either correlative evidence, for instance the presence of mast cells in inflamed tissues, or through the use of mast celldeficient *Kit* mutant mice and mast cell-inhibitors, mast cells have been suggested to play roles in several autoimmune diseases that include thyroid eye disease (2), bullous pemphigoid (3), pemphigus vulgaris (4), rheumatoid arthritis (5), multiple sclerosis (6), systemic sclerosis (7, 8), Guillain-Barre syndrome (9) and, notably, type 1 diabetes (T1D) (10-13) (reviewed in (14, 15). However, recent experiments in newly developed Kitindependent mast cell-deficient mice did not corroborate the proposed roles for mast cells, at least in models of rheumatoid arthritis and multiple sclerosis (16). The observed discrepancies call for a re-evaluation of mast cell functions in models more specific than the traditional *Kit* mutants (reviewed in (17, 18)).

T1D, which affects approximately 37 of every 100,000 children ages 14 or younger in countries with the highest incidence (19), results from an autoimmune attack on insulin-producing β cells by both cellular and humoral components of the immune system. In humans, the susceptibility for T1D is linked to the histocompatibility leukocyte antigen (HLA) locus (20). Akin to humans, in the universally utilized non-

obese diabetic (NOD) mouse model of T1D, the highest genetic contributor to disease susceptibility maps to the major histocompatibility complex (21, 22). In view of the T cell-dependency of this disease, and the putative roles of mast cells in the control of T cell responses, which may occur via direct antigen presentation to either CD4⁺ or CD8⁺ T cells (23-25), by induction of T cell migration to lymph nodes (26, 27), by control of dendritic cell activation and migration to lymph nodes (28-31), or by control of the Th1 versus Th2 skewing (32-35), it has been postulated that mast cells are likely important players in the development of T1D (14, 15, 36).

Earlier studies have reported an association between mast cells and T1D by showing upregulation of mast cell genes in the pancreatic lymph nodes of the BioBreeding (BB) $DR^{hp/hyp}$ rat (13), or by delaying disease onset in the BB rat following disodium cromoglycate (DSCG)-mediated mast cell-stabilization (10). In contrast, a third study found that activation, rather than inhibition, of mast cells with anti-FceRI antibody injections in NOD mice led to delayed onset of T1D (11). Thus, the existing data are either correlative or conflicting, and hence the potential role of mast cells in the incidence and progression of T1D remains largely unknown. Here, we report the analyses of two different mast cell-deficient mouse models (*NOD.Cpa3Cre/+* and *NOD.KitW-sh/W-sh*), which were independently generated at three different institutions (German Cancer Research Center, Harvard Medical School, and VIB) to evaluate the effects of mast cell deficiency on the incidence and progression of T1D in NOD mice are unaffected by mast cell deficiency.

RESEARCH DESIGN AND METHODS

Generation of *NOD.Cpa3^{Cre/+}* and *NOD.Kit^{W-sh/W-sh* mice. *Carboxypeptidase A3* $(Cpa3)^{Cre/+}$ mice have been previously described (16). These mice are wild type for *Kit*, lack mast cells in all tissues analyzed, and have no other known defect in the hematopoietic system, with the exception of a reduction in basophils. To obtain *NOD.Cpa3^{Cre/+}* mice, *Cpa3^{Cre/+}* mice were backcrossed into the *NOD/ShiLtJ* background (Jax stock number: 001976) for at least 12 generations. *Kit^{W-sh/W-sh}* ("sash") mice (37) were backcrossed into the *NOD/ShiLtJ* background for 11 or 12 generations to generate two independent lines of *NOD.Kit^{W-sh/W-sh* mutants.}}

Determination of disease status. Male and female *NOD.Cpa3^{Cre/+}*, their NOD.*Cpa3^{+/+}* littermate controls, and *NOD/ShiLtJ* controls, all housed in the same animal facility (DKFZ, Heidelberg, Germany), were monitored for the development of diabetes starting from 10 weeks of age by weekly assessing blood glucose levels via tail-vein bleeding using a ONETOUCH Ultra glucometer (LifeScan, Inc; Milipitas, CA). Female *NOD.KitW-sh/W-sh*, *NOD. KitW-sh/+* and *NOD.Kit+/+* controls were monitored for the development of diabetes starting at 10 weeks of age. Two independent studies were performed with *NOD.KitW-sh/W-sh*, one at the VIB in Leuven, Belgium, and one at Harvard Medical School in Boston, Massachusetts. In all studies, mice with glucose concentrations >250 mg/dL on 2 consecutive days were considered diabetic, and were immediately killed by CO₂ asphyxiation.

Insulitis scoring and pancreatic toluidine blue staining. Pancreata from 15-week old (Heidelberg) or 10-week old (Boston) mice were isolated and fixed in 10% formalin

(Sigma-Aldrich; St Louis, MO) overnight. Subsequently tissues were paraffin embedded and cut into 5 μ M sections. Serial paraffin sections were stained by hematoxylin and eosin (H&E) coupled with aldehyde Fuchsin as previously described (38). Images of islets were taken using a Zeiss Axioplan light microscope coupled with an Axiocam ICc3 color camera at 200X, or 630X magnifications. For the *NOD.Cpa3Cre/+* mice (Heidelberg), the scoring was performed using the following scale (Supplementary Fig. 1: A) 0 = no insulitis; B) 1 = peri-islet insulitis; C) 2 = intermediate insulitis; D) 3 = intraislet insulitis; and F) 4 = complete islet insulitis) in a blinded manner. For the *NOD.KitWsh/W-sh* mice (Boston), scoring was divided into three categories: Insulitis, peri-insulitis and no insulitis. Pancreas and ear paraffin sections from *NOD.Cpa3^{Cre/+}*, *NOD.Cpa3^{+/+}*, *NOD.KitW-sh/W-sh* and *NOD.Kit*^{+/+} mice were stained with a solution of 0.1% (w/m) toluidine blue (Sigma-Aldrich; St Louis, MO), and analyzed by light microscopy for the presence of metachromatic mast cells.

Pancreatic lymph node immune cell analysis. Pancreatic lymph nodes from 15-week old mice were isolated, finely minced and mechanically sieved through a 40μM mesh to isolate immune cells. Isolated single cells were stained for flow cytometry as previously described (39). The following antibodies were used: CD45 A700 (30-F11; eBioscience), CD117 (Kit) APC (2B8; BD Pharmingen), IgE FITC (R35-72; BD Pharmingen), CD19 PerCP-Cy5.5 (1D3; BD Pharmingen), CD3 PerCP-Cy5.5 (145-2C11; BioLegend), CD4 APC (RM4.5; eBioscience), CD8 PE-Cy7 (53-6.7; eBioscience), CD44 FITC (IM7; BD Pharmingen), CD62L Vio605 (MEL-14; BioLegend) and FOXP3 PE (FJK-16S; eBioscience). SYTOX blue (Life Technologies) was utilized for dead cell exclusion. All

samples were measured using a BD LSRFortessa flow cytometer (BD Heidelberg) and analyzed using Flowjo X software.

Statistical analyses. Progression to diabetes and diabetes incidence were calculated using the product limit method (Kaplan Meier analysis). Statistical significance between a pair of curves was determined by the log-rank (Mantel-Cox) test using Prism 5.0 software. Flow cytometry data were analyzed with two-tailed student t test. P values \leq 0.05 were considered statistically significant.

RESULTS

Analysis of mast cells in pancreas and pancreatic lymph nodes in normal and mast cell-deficient NOD mice

As mast cell deficient models, we used $Cpa3^{Cre/+}$ mice which lack mast cells and are wild type for *Kit*, and *KitW-sh/W-sh* mice which are double-deficient for mast cells and *Kit. Cpa3^{Cre/+}* mice are targeted knockin mice that bear Cre recombinase in the *Cpa3* locus. Cre expression under the control of the endogenous *Cpa3* gene leads to genotoxic ablation of mast cells through a Trp53-dependent mechanism (16, 40, 41). *Cpa3^{Cre/+}* mice are devoid of mucosal and connective tissue mast cells under steady state and remain so under various challenges (16, 40, 41). In *KitW-sh/W-sh* mice, an approximately 3.1 Mb genomic inversion upstream of the *Kit* locus disrupts the physiological regulation of *Kit* transcription that results in mast cell deficiency (42, 43). In addition, *KitW-sh/W-sh* have further Kit-related and Kit-unrelated defects (43). *Cpa3^{Cre/+}* or *KitW-sh/W-sh* mice were backcrossed onto the NOD background to obtain mast cell-deficient NOD mouse lines (see Research Design and Methods section).

To confirm that $NOD.Cpa3^{Cre/+}$ mice remained mast cell-deficient during the progression of insulitis and diabetes, we examined $NOD.Cpa3^{Cre/+}$ and $NOD.Cpa3^{+/+}$ mice for the presence of mast cells in islets, ears, pancreatic lymph nodes and the peritoneal cavity (Fig. 1A-H). Histological analysis of pancreatic islets from 15-week old female mice, a time point when autoimmune pathology is progressing, showed complete lack of mast cells in the islet-immune infiltrates of both $NOD.Cpa3^{+/+}$ and $NOD.Cpa3^{Cre/+}$ mice, indicating that even in mast cell-proficient mice ($NOD.Cpa3^{+/+}$)

mast cells do not infiltrate the pancreatic islets. This was substantiated by an independently performed flow cytometric analysis of islets from 10-week old NOD/ShiLtJ mice (Supplementary Fig. 2). To substantiate the mast cell-deficient phenotype by histological analysis, paraffin-embedded ear sections were stained with toluidine blue; these showed a large number of mast cells delineating the epidermis (larger magnification image depicts mast cells) in the NOD. $Cpa3^{+/+}$ mice (Fig. 1C-D) and complete absence of mast cells in the sections of $NOD.Cpa3^{Cre/+}$ mice (Fig. 1E). Flow cytometric analysis of pancreatic lymph node cells showed the presence of mast cells in NOD. $Cpa3^{+/+}$ mice supporting previous reports (Fig. 1F) (10, 13). However, these cells were absent in NOD.Cpa3^{Cre/+} mice (Fig. 1G). Moreover, flow cytometric analysis of peritoneal lavage cells revealed that approximately 4% of all CD45⁺ cells in the peritoneal cavity of $NOD.Cpa3^{+/+}$ mice were mast cells (Fig. 1H), while mast cells in the peritoneal cavity layage of $NOD.Cpa3^{Cre/+}$ mice were undetectable (Fig. 11). Toluidine blue staining of ear paraffin sections confirmed mast cell-deficiency in *NOD.Kit*^{*W-sh/W-sh*} mice (Supplementary Fig. 3). Collectively, in *NOD.Cpa3*^{+/+} but not in NOD.Cpa3^{Cre/+} mice, mast cells are present in the skin, peritoneal cavity and in draining pancreatic lymph nodes but mast cells were not found in the islet immune infiltrates.

Incidence and progression of T1D in mast cell-deficient mice

NOD.Cpa3^{Cre/+} mice, their *NOD.Cpa3^{+/+}* littermate controls and the original *NOD/ShiLtJ* mice, all housed in the same animal facility, were monitored for diabetes

starting at 10 weeks of age. In female mice, there was no statistically significant difference in the incidence (~70 %), or median onset time (21 vs. 19 weeks) between NOD. $Cpa3^{Cre/+}$ mice and their NOD. $Cpa3^{+/+}$ littermates. A similar incidence and onset time (20 weeks) was also observed for the NOD/ShiLtJ female mice (Fig. 2A). A parallel study was conducted for a cohort of male mice and again no differences were found between NOD.Cpa3^{Cre/+}, NOD.Cpa3^{+/+} and NOD/ShiLtJ mice (Fig. 2B). Comparing male and female mice, the sex differences (44) in median onset of disease were similar for NOD. Cpa3^{Cre/+} (21 versus ~35 weeks, P = 0.0015), NOD. Cpa3^{+/+} (19 vs. 36 weeks, P = 0.0005) and NOD/ShiLtJ mice (20 vs. 35 weeks, P=0.0149). In keeping with analyses of *NOD.Cpa3^{Cre/+}* mice, female NOD mice on the *Kit* mutant background generated in Leuven (Fig. 2C) and in Boston (Fig. 2D) showed no significant differences time comparing NOD.Kit^{W-sh/W-sh} onset incidence or in diabetes and *NOD.Kit*^{+/+}littermates.

Insulitis assessment

Histological analyses were performed on serial paraffin sections of pancreata stained by H&E together with aldehyde Fuchsin to assess the level of insulitis in 15-week old female and male $NOD.Cpa3^{Cre/+}$, $NOD.Cpa3^{+/+}$ and NOD/ShiLtJ mice. We scored insulitis in islets from serial sections of each pancreas. Numbers of islets were counted on 5 serial sections, each 5µm in thickness and with 25µm distance between each section. Based on these pancreas tissue samples numbers of islets per mouse did not differ between genotypes (Supplementary Fig. 1F). Quantification of insulitis showed no differences in scores comparing female (Fig. 3A) and male (Fig. 3B) $NOD.Cpa3^{Cre/+}$,

NOD.*Cpa3*^{+/+} and *NOD/ShiLtJ* mice. Moreover, the degree of islet insulitis was comparable in female *NOD*.*KitW-sh/W-sh* and *NOD*.*Kit*^{+/+}littermates (Supplementary Fig. 4).

In summary, these experiments establish that the incidence, progression and histopathological degree of insulitis during diabetes development are unaffected by the presence or absence of mast cells in the NOD mouse.

Analysis of the immune cell populations and T cell activation in mast cell-deficient NOD mice

Pancreatic lymph nodes (PLN) from *NOD.Cpa3*^{+/+} and *NOD.Cpa3*^{Cre/+} mice were collected, subsequently cells were counted and analyzed by flow cytometry. Absolute overall cell numbers (2.8 x 10⁶ ± 1.0 [mean ± SD] for *NOD.Cpa3*^{+/+} [n = 4] vs 2.6 x $10^{6} \pm 0.5$ for *NOD.Cpa3*^{Cre/+} [n = 3] mice), absolute numbers of CD45⁺ cells (1.8 x $10^{6} \pm 1.1$ for *NOD.Cpa3*^{+/+} vs 2.0 x $10^{6} \pm 0.5$ for *NOD.Cpa3*^{Cre/+} mice), percent CD3⁺ T cells (78 ± 11 for *NOD.Cpa3*^{+/+} vs 76 ± 6 for *NOD.Cpa3*^{Cre/+} mice), and absolute numbers of CD3⁺ T cells (1.8 x $10^{6} \pm 1.1$ for *NOD.Cpa3*^{Cre/+} mice), were comparable in mast cell-sufficient and mast cell-deficient NOD mice (Supplementary Fig. 5). In a larger cohort of *NOD.Cpa3*^{+/+} (n = 8) and *NOD.Cpa3*^{Cre/+} (n = 9) mice, we determined in the PLN the relative proportions of T cells, B cells, and dendritic cells (Fig. 4A-C), all of which were similar in both genotypes. In accordance to earlier analysis (16), proportions of CD4⁺ (Fig. 4D) and

CD8⁺ (Fig. 4E) cells among total T cells, and the fraction of T regulatory cells per total CD4⁺ T cells (Fig. 4F) were all not affected by mast cell deficiency.

Immune-driven effector cell differentiation of conventional T cells was assessed by expression analysis for CD62L and CD44, and T cells were classified into three categories: naïve cells (CD62L⁺CD44⁻), central memory cells (CD62L⁺CD44⁺) and effector memory cells (CD44⁺CD62L⁻) (reviewed in (45). In the CD4 T cell population (Fig. 4G), percentages of naïve, central memory and effector memory subsets were comparable in *NOD.Cpa3^{Cre/+}* mice and *NOD.Cpa3^{+/+}* mice.

Collectively, all analyzed immunological parameters were similar comparing mast cell-deficient and mast cell-proficient NOD mice. This is in full agreement with the lack of evidence for a role of mast cells on incidence, progression or degree of insulitis during diabetes development in the NOD strain.

DISCUSSION

Many years of work by several groups leading to the current study have postulated that mast cells are important players in the initiation and progression of autoimmune diseases (14, 15, 36, 46, 47). In most cases, these studies have been performed using non-specific mast cell "stabilizers" (48), or Kit mutant mice that, although mast cell-deficient, have other immune (18) and relevant non-immune abnormalities, which include, notably the recent discovery of the role of *Kit* in pancreatic β cell function (49). Taking into account the pleiotropic abnormalities in *Kit* mutant mice it is conceivable that several roles that have been attributed to mast cells are not mast cell-specific, but instead caused by the Kit mutations. To circumvent this problem, our group (16) and others (50, 51) have generated Kit-independent mast cell-deficient mice; experiments in these mice have already challenged the role of mast cells in antibody-induced autoimmune arthritis and experimental autoimmune encephalomyelitis (16). The role of mast cells in the autoimmune destruction of β cells, or T1D, is not well understood. In fact, although highly speculated (14, 15, 36), the effect of mast cell deficiency on T1D had never been directly assessed genetically in vivo, with the exception of an abstract suggesting that NOD.Kit^{W/Wv} mice failed to develop type 1 diabetes (Julianne Hatfield, Mary Quirion, Kavitha Rao, and Melissa Brown. J Immunol 184:135.39; 2010). Indirectly, one study tested the effect of mast cell inhibition using the mast cell-stabilizer DSCG, and found that this significantly delayed the onset of T1D (10); however, the specificity and function of this drug is controversial (48). In contrast, a second study activated mast cells and basophils via anti-FccR1 antibody treatment, and reported that this treatment delayed

the onset of T1D (11). It is difficult to interpret these opposing results, and therefore the role of mast cells on T1D remained at best controversial.

In the present study, we assessed the potential effects of mast cell deficiency on T1D, using both a traditional Kit mutant ($Kit^{W-sh/W-sh}$) and the more recent Kitindependent mast cell-deficient Cpa3Cre/+ strain. Both strains were backcrossed to the NOD background until the new lines developed diabetes with similar onset times and rates as the NOD/ShiLtJ mice housed under identical conditions. We present data from independent studies conducted in three different institutions, all of which showed that lack of mast cells did not affect the incidence and progression of T1D in mast cell deficient NOD strains. It was critical to show that NOD.Cpa3Cre/+ mice remained mast cell-deficient during initiation and progression of the autoimmune attack. Both in the peritoneal cavity and in the relevant pancreatic lymph node, there was a complete absence of mast cells in these NOD mice. Of note, the absence of mast cells in the islets of *NOD/ShiLtJ* mice, or *NOD.Cpa3*^{+/+} mice at stages when they were infiltrated by other immune cells strongly suggest that mast cells cannot be directly involved in islet immunopathology. However, the presence of mast cells in secondary lymphoid organs, including the pancreatic lymph node, in mast cell-proficient NOD mice may reflect an active role of mast cells in the immunomodulation of the autoimmune response leading to T1D. After establishing mast cell deficiency in $Cpa3^{Cre/+}$ mice on the NOD background, we assessed the onset and severity of diabetes, and found that the presence or absence of mast cells had no impact on the development of T1D. Furthermore, evaluations of T1D in NOD.KitW-sh/W-sh mice in Leuven and in Boston showed a similar outcome regardless of the Kit genotype (wild type or mutant), and hence

irrespective of the presence of mast cells. Moreover, we compared immunological parameters associated with the autoimmune response in $NOD.Cpa3^{Cre/+}$ mice and their littermate controls, and found comparable cellularity of lymphocyte and DC subsets, and in T helper cell activation.

Collectively, at least two certain conclusions can be drawn from this study: firstly, the T1D autoimmune response in NOD mice is mast cell-independent, as well as independent from defects associated with hypomorphic mutations in Kit. Secondly, our study, along with earlier studies (16), put into question the suggested ability of mast cells to control or modulate autoimmunity-promoting adaptive immune response (14, 46). Mast cell deficiency did not affect the levels of conventional or regulatory T cells, B cells, or dendritic cells in the pancreatic lymph nodes; moreover, T cell activation, much of which had previously been suggested to be associated with mast cell function, was also unaffected by mast cell deficiency (23-25, 28-35). Obviously, we refrain from extrapolating negative data obtained in experiments on arthritis, EAE (16), and T1D (this study) to the potential roles of mast cells in other autoimmune responses, or adaptive immunity in general. However, as discussed recently (17, 52), it remains important to reevaluate mast cell functions, which had previously been suggested based on in vitro experiments, on the use of inhibitors or mast cell stabilizers, or on the use of *Kit* mutants, by turning to more conclusive in vivo mouse models for mast cell deficiency.

Although our results clearly show that mast cell deficiency does not affect T1D development in NOD mice, our results suggest, but do not prove, that mast cells are unimportant in rat and human T1D. In the BioBreeding (BB) $DR^{lyp/lyp}$ rat, upregulation of mast cell genes in the pancreatic lymph nodes suggest that mast cell numbers or their

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activity increase during the disease (13), and in this model disease onset was delayed by mast cell-stabilization (10). A link between T1D progression and mast cell activation has also more generally been invoked in parasite infections or during asthma which might be negatively correlated to T1D development (11, 36, 53). Further experiments should test these ideas by examining the prevalence of T1D in patients with mastocytosis or by studying the dependency on mast cells of helminth-mediated T1D inhibition. As it stands, mast cells are likely to play important evolutionarily conserved roles in immunity beyond their notorious role in allergic diseases. While beneficial mast cells roles are emerging in venom degradation (54, 55), and in IgE-mediated protection from lethal doses of venom (56, 57), the possible involvement of mast cells in the regulation of innate and adaptive immune responses, and their role as effector cells remains enigmatic.

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DAG designed and performed experiments (Heidelberg), analyzed the data and wrote the manuscript; WF (Boston) and SS (Leuven) designed and performed experiments; TBF contributed to experiment design, assisted with experiments and edited the manuscript; AOL and YL assisted with experiments; AL, DM and HRR conceived the studies, contributed to experiment design and edited the manuscript.

HRR is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

Figure 1. Establishing mast cell deficiency in *NOD.Cpa3^{Cre/+}* mice *NOD.Cpa3^{Cre/+}* and their *NOD.Cpa3^{+/+}* littermate controls were used to assess MCdeficiency in pancreas, ear, pancreatic lymph node (PLN) and peritoneal cavity. Pancreata were removed and paraffin sections were stained with toluidine blue to test for the presence of mast cells. Representative images are shown for *NOD.Cpa3^{+/+}* (A) and *NOD.Cpa3^{Cre/+}* (B) mice (n = 5 per group). Toluidine blue- stained ear sections from *NOD.Cpa3^{+/+}* (C) (200X magnification) (D) (630X magnification) and *NOD.Cpa3^{Cre/+}* (E) mice. Single cell suspensions from the PLN were analyzed by flow cytometry. Mast cells were identified as CD117 (Kit)⁺IgE⁺ cells. Data are representative for *NOD.Cpa3^{+/+}* (F) (n = 10), and *NOD.Cpa3^{Cre/+}* (G) (n = 9) mice. Peritoneal lavage cells were analyzed by flow cytometry. Mast cells were identified as described above. H). Data are representative for *NOD.Cpa3^{+/+}* (G) (n = 8), and *NOD.Cpa3^{Cre/+}* (I) (n = 6) mice.

Figure 2. Diabetes progression in *NOD*.*Cpa3*^{*Cre/+*} and *NOD*.*Kit*^{*W*-sh/W-sh mice *NOD*.*Cpa3*^{*Cre/+*} and *NOD*.*Kit*^{*W*-sh/W-sh mice were monitored weekly for diabetes (glucose threshold > 250 mg/dL) starting at 10 weeks of age. Percent diabetes incidence in all analyzed female (A) and male (B) *NOD*.*Cpa3*^{*Cre/+*} mice, and the indicated control genotypes, in the experiments conducted in Heidelberg. Percent diabetes incidence curve for female *NOD*.*Kit*^{*W*-sh/W-sh} and the indicated control genotypes in the experiments conducted in Leuven (C). Percent diabetes incidence curve for female *NOD*.*Kit*^{*W*-sh/W-sh} and the indicated control genotype in the experiments conducted in Boston (D). Numbers (n) for each group are indicated in the graph legends for each genotype.}}

Figure 3. Assessment of islet insulitis in mast cell-deficient mice

Pancreata from 15-week old female and male *NOD.Cpa3^{Cre/+}* and *NOD.Cpa3^{+/+}* mice were formalin fixed, paraffin embedded and stained with H&E plus aldehyde fuchsin to evaluate islet insulitis (see Supplementary Fig. 1 for scoring). Quantification of islet

insultis between of female (A) and male (B) *NOD*.*Cpa3*^{*Cre/+*}, *NOD*.*Cpa3*^{+/+} and *NOD*/*ShiLtJ* mice. A total of 17 to 111 islets per group were scored (n = 5 mice per group).

Figure 4. Immunological analysis of pancreatic lymph nodes Single cell suspensions of PLNs from 15-week old *NOD.Cpa3^{Cre/+}* and *NOD.Cpa3^{+/+}* mice were analyzed by flow cytometry. Displayed are percent CD3⁺ T cells (A), CD19⁺ B cells (B) and CD11c⁺ dendritic cells (C) per total CD45⁺ cells, percent CD4⁺ (D), and CD8⁺ (E) T cells per total T cells, percent Foxp3⁺regulatory T cells per total CD4+ T cells(F). Fractions of naïve cells (CD62L⁺CD44⁻), central memory (CD62L⁺CD44⁺) and effector memory (CD44⁺CD62L⁺) are shown among Foxp3⁻CD4⁺ T cells. In all panels, each symbol represents an individual mouse.



Gutierrez et al. Figure 1

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Gutierrez et al. Figure 2

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Gutierrez et al. Figure 3

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Insulitis Scoring

Pancreata from 15-week old female and male NOD.*Cpa3^{Cre/+}* and NOD.*Cpa3^{+/+}* mice were formalin fixed, paraffin embedded and stained with H&E plus aldehyde fuchsin to evaluate islet insulitis. Representations of the different insulitis scores are shown: score 0 = no insulitis (A); score 1 = peri-islet insulitis (B); score 2 = intermediate insulitis (C), score 3 = intra-islet insulitis (D), and score 4 = complete islet insulitis (E). <u>Total number of islets scored per genotype</u> (F). The genotypes were unknown to the scoring person,to ensure blind evaluation.

Supplementary Figure 2. Analysis for mast cells in peritoneal cavity, spleen, and pancreas in NOD mice

Flow cytometric analyses of mast cells in peritoneal cavity, spleen, and pancreas from B6 (left column) or NOD (right column) mice. CD45⁺ cells were stained for Kit and FccRI to identify mast cells.

Supplementary Figure 3. Mast cell deficiency in NOD.*KitW-sh/W-sh* mice Toluidine blue staining of ear sections (100x) of NOD.*Kit*^{+/+} (left) and NOD.*KitW-sh/W-sh* mice (right). Metachromatic mast cells were only found in NOD.*Kit*^{+/+} mice.

Supplementary Figure 4. Insulitis scores

Pancreata were excised at 10 weeks of age, and insulitis visualized by hematoxylin and eosin staining of paraffin sections counting all islets with a 3-step sectioning of the pancreas each 150 μ m apart. Scores were designated as insulitis (red), periinsulitis (dark blue), or no insulitis (light blue). Each bar represents the score distribution in one individual mouse (n = 5 for each group). Student's *t* test P value = 0.6394

Supplementary Figure 5. PLNs from 1<u>3</u>-week old NOD.*Cpa3^{Cre/+}* and NOD.*Cpa3^{+/+}* mice were collected and single cell suspensions were analyzed by

flow cytometry. A) Absolute number of live cells per PLN. B) Absolute number of

CD45⁺ cells per PLN<u>. C) Percent of CD3⁺ T cells of total CD45⁺ cells per PLN. D)</u> Absolute number of CD3⁺ cells per PLN. Each dot in the graph represents one mouse.



Gutierrez et al. Supp. Figure 1

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Gutierrez et al. Supp. Figure 3

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Gutierrez et al. Supp. Figure 4

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Gutierrez et al. Supp. Figure 5

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