Dario A. Gutierrez,[1] Wenxian Fu,[2] Susann Schonefeldt,[3,4] Thorsten B. Feyerabend,[1]
Adriana Ortiz-Lopez,[2] Yulia Lampi,[3,4] Adrian Liston,[3,4] Diane Mathis,[2] and Hans-Reimer Rodewald[1]

Type 1 Diabetes in NOD Mice Unaffected by Mast Cell Deficiency

Diabetes 2014;63:3827–3834 | DOI: 10.2337/db14-0372

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-cell 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 nonobese diabetic (NOD) mice, *NOD.Cpa3*$^{Cre/+}$ (Heidelberg) and *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$ (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.

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 FcεRI, 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 cell–deficient *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 *Kit*-independent 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 reevaluation of mast cell functions in models more specific than the traditional *Kit* mutants (reviewed in 17,18).

[1]Division of Cellular Immunology, German Cancer Research Center, Heidelberg, Germany
[2]Division of Immunology, Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA
[3]Autoimmune Genetics Laboratory, VIB, Leuven, Belgium
[4]Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium

Corresponding authors: Hans-Reimer Rodewald, hr.rodewald@dkfz.de; Diane Mathis, diane_mathis@hms.harvard.edu; and Adrian Liston, adrian.liston@vib.be.

Received 4 March 2014 and accepted 5 June 2014.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db14-0372/-/DC1.

W.F. is currently affiliated with the Department of Pediatrics, University of California, San Diego, La Jolla, CA.

T1D, which affects ~37 of every 100,000 children aged 14 years 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 locus (20). Akin to humans, in the universally used nonobese 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 (PLNs) of the BioBreeding (BB) $DR^{lyp/lyp}$ rat (13) or by delaying disease onset in the BB rat following disodium cromoglycate–mediated mast cell stabilization (10). In contrast, a third study found that activation, rather than inhibition, of mast cells with anti-FcεRI 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.Cpa3*$^{Cre/+}$ and *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$), 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. Our data show that the progression or incidence of T1D in NOD mice are unaffected by mast cell deficiency.

RESEARCH DESIGN AND METHODS

Generation of *NOD.Cpa3*$^{Cre/+}$ and *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$ 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*$^{W\text{-}sh/W\text{-}sh}$ ("sash") mice (37) were backcrossed into the *NOD/ShiLtJ* background for 11 or 12 generations to generate two independent lines of *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$ 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 (German Cancer Research Center, 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., Milpitas, CA). Female *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$, *NOD.Kit*$^{W\text{-}sh/+}$, and *NOD.Kit*$^{+/+}$ controls were monitored for the development of diabetes starting at 10 weeks of age. Two independent studies were performed with *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$, one at the VIB in Leuven, Belgium, and one at Harvard Medical School, Boston, MA. In all studies, mice with glucose concentrations >250 mg/dL on 2 consecutive days were considered diabetic and were immediately killed by CO_2 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 μm 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 200× or 630× magnifications. For the *NOD.Cpa3*$^{Cre/+}$ mice (Heidelberg), the scoring was performed using the following scale in a blinded manner (Supplementary Fig. 1): *A*) 0, no insulitis; *B*) 1, peri-islet insulitis; *C*) 2, intermediate insulitis; *D*) 3, intra-islet insulitis; and *E*) 4, complete islet insulitis. For the *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$ 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.Kit*$^{W\text{-}sh/W\text{-}sh}$, and *NOD.Kit*$^{+/+}$ mice were stained with a solution of 0.1% (w/m) toluidine blue (Sigma-Aldrich) and analyzed by light microscopy for the presence of metachromatic mast cells.

PLN Immune Cell Analysis

PLNs from 15-week-old mice were isolated, finely minced, and mechanically sieved through a 40 μm mesh to isolate immune cells. Isolated single cells were stained for flow cytometry as previously described (39). The following antibodies were used: CD45 Alexa Fluor 700 (30-F11; eBioscience), CD117 (Kit) allophycocyanin (2B8; BD Pharmingen), IgE fluorescein isothiocyanate (R35–72; BD Pharmingen), CD19 PerCP-Cy5.5 (1D3; BD Pharmingen), CD3 PerCP-Cy5.5 (145–2C11; BioLegend), CD4 allophycocyanin (RM4.5; eBioscience), CD8 PE-Cy7 (53–6.7; eBioscience), CD44 fluorescein isothiocyanate (IM7; BD Pharmingen), CD62L Vio605 (MEL-14; BioLegend), and Foxp3 PE (FJK-16S; eBioscience). SYTOX blue (Life Technologies) was used for dead cell exclusion. All samples were measured using a BD LSRFortessa flow cytometer (BD Biosciences, 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 ≤0.05 were considered statistically significant.

RESULTS

Analysis of Mast Cells in Pancreas and PLNs 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 *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, which are double deficient for mast cells and *Kit*. *Cpa3*$^{Cre/+}$ mice are targeted knock-in 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 *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, an ~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, *Kit*$^{W\text{-}sh/W\text{-}sh}$ have further Kit-related and Kit-unrelated defects (43). *Cpa3*$^{Cre/+}$ or *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were backcrossed onto the NOD background to obtain mast cell–deficient NOD mouse lines (see RESEARCH DESIGN AND METHODS).

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, PLNs, and the peritoneal cavity (Fig. 1*A–I*). 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 (Fig. 1*A* and *B*). 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. 1*C* and *D*) and complete absence of mast cells in the sections of *NOD.Cpa3*$^{Cre/+}$ mice (Fig. 1*E*). Flow cytometric analysis of PLN cells showed the presence of mast cells in *NOD.Cpa3*$^{+/+}$ mice, supporting previous reports (Fig. 1*F*) (10,13). However, these cells were absent in *NOD.Cpa3*$^{Cre/+}$ mice (Fig. 1*G*). Moreover, flow cytometric analysis of peritoneal lavage cells revealed that ~4% of all CD45^{+} cells in the peritoneal cavity of *NOD.Cpa3*$^{+/+}$ mice were mast cells (Fig. 1*H*), while mast cells in the peritoneal cavity lavage of *NOD.Cpa3*$^{Cre/+}$ mice were undetectable (Fig. 1*I*). Toluidine blue staining of ear paraffin sections confirmed mast cell deficiency in *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Supplementary Fig. 3).

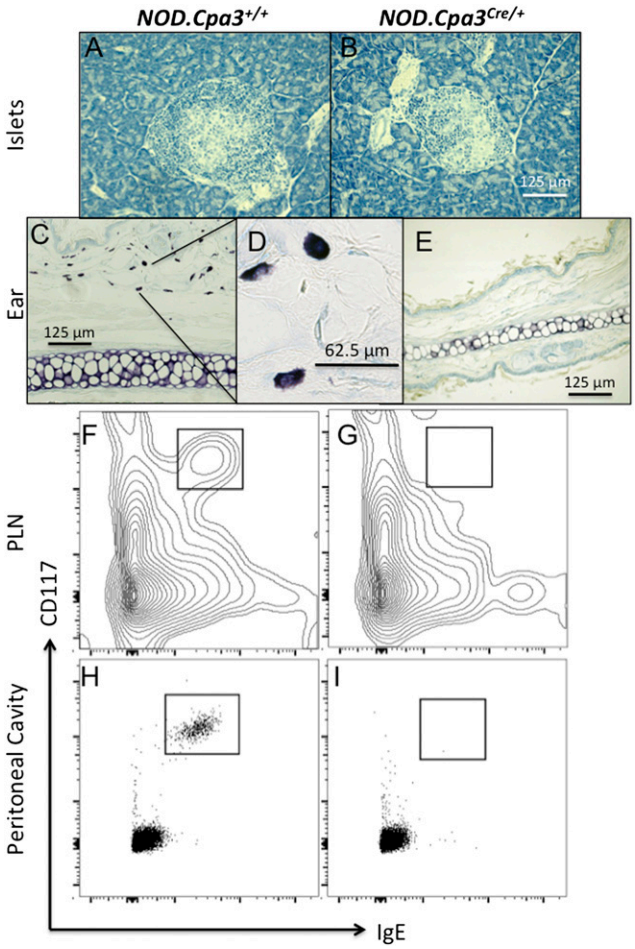

Figure 1—Establishing mast cell deficiency in *NOD.Cpa3*$^{Cre/+}$ mice. *NOD.Cpa3*$^{Cre/+}$ and their *NOD.Cpa3*$^{+/+}$ littermate controls were used to assess mast cell deficiency in pancreas, ear, PLN, and peritoneal cavity. Pancreata were removed, and paraffin sections were stained with toluidine 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*, 200× magnification; *D*, 630× 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.

Collectively, in *NOD.Cpa3*$^{+/+}$, but not in *NOD.Cpa3*$^{Cre/+}$ mice, mast cells are present in the skin, in the peritoneal cavity, and in draining PLNs, 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. 2*A*). 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. 2*B*). Comparing male and female mice, the sex differences (44) in median onset of disease were similar for *NOD.Cpa3*$^{Cre/+}$ (21 vs. ~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. 2*C*) and in Boston (Fig. 2*D*) showed no significant differences in diabetes incidence or onset time comparing *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$ 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. Numbers of islets were counted on five serial sections, each 5 μm in thickness and with 25 μm distance between each section. Relative numbers of islets per mouse did not differ between genotypes (Supplementary Fig. 1*F*). Quantification of insulitis showed no differences in scores comparing female (Fig. 3*A*) and male (Fig. 3*B*) *NOD.Cpa3*$^{Cre/+}$, *NOD.Cpa3*$^{+/+}$, and *NOD/ShiLtJ* mice. Moreover, the degree of islet insulitis was comparable

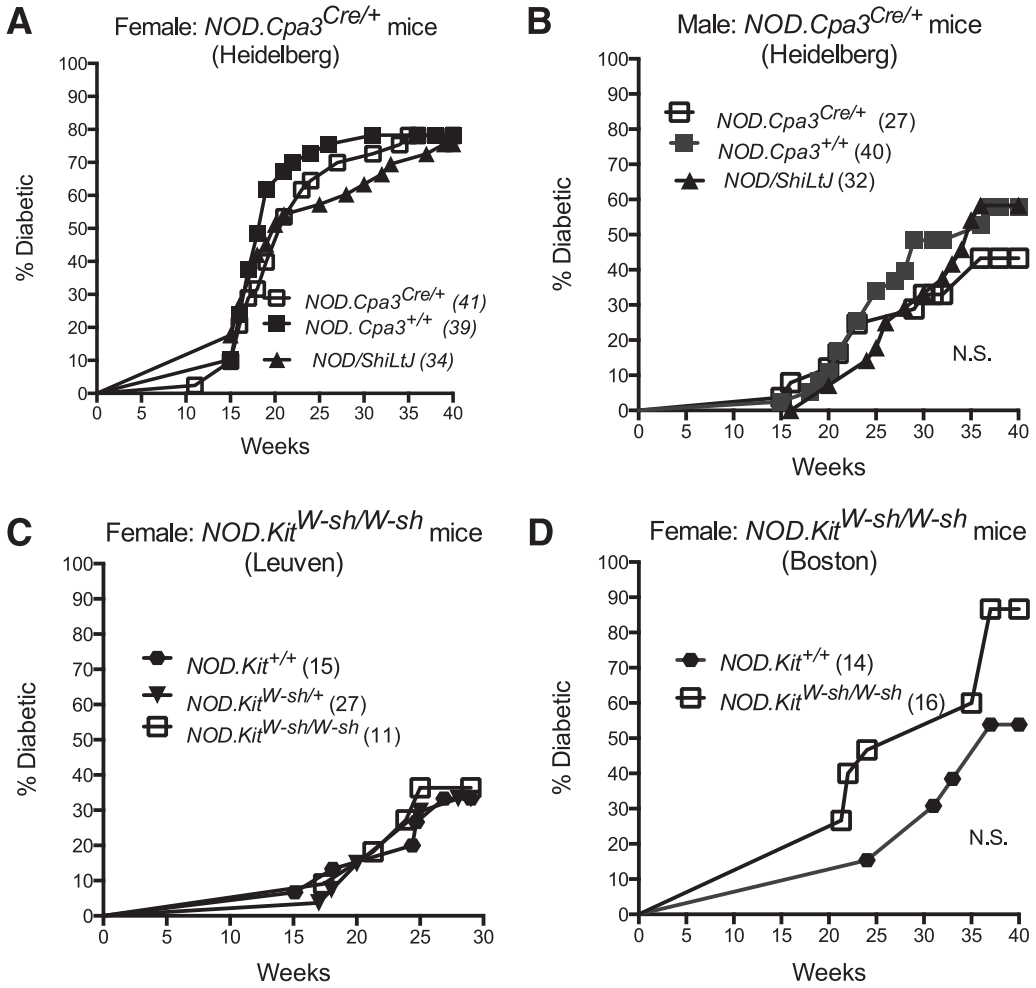

Figure 2—Diabetes progression in *NOD.Cpa3*$^{Cre/+}$ and *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. *NOD.Cpa3*$^{Cre/+}$ and *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were monitored weekly for diabetes (glucose threshold >250 mg/dL) starting at 10 weeks of age. Percent of diabetes incidence in all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

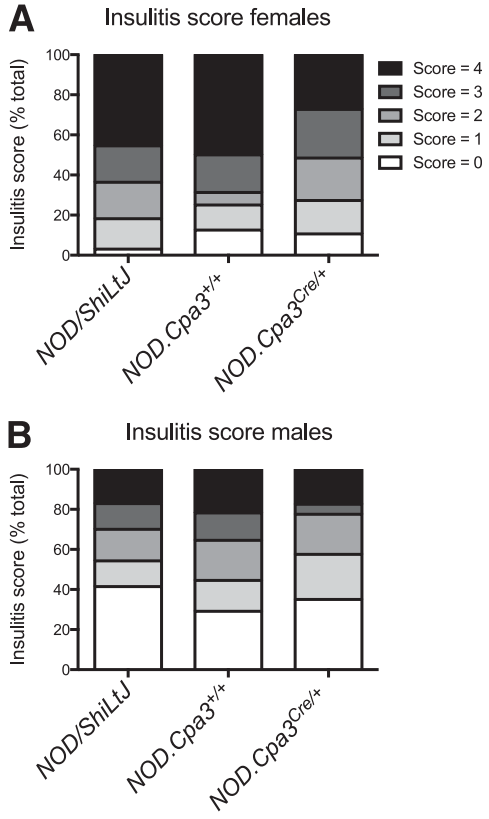

Figure 3—Assessment of islet insulitis in mast cell–deficient mice. Pancreata from 15-week-old female and male *NOD.Cpa3*$^{Cre/+}$ and *NOD.Cpa3*$^{+/+}$ mice were formalin fixed, paraffin embedded, and stained with H&E plus aldehyde fuchsin to evaluate islet insulitis (see Supplementary Fig. 1 for scoring). Quantification of islet insulitis between 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).

in female *NOD.Kit*$^{W\text{-}sh/W\text{-}sh}$ 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

PLNs from *NOD.Cpa3*$^{+/+}$ and *NOD.Cpa3*$^{Cre/+}$ mice were collected, and subsequently, cells were counted and analyzed by flow cytometry. Absolute overall cell numbers ($2.8 \times 10^6 \pm 1.0$ [mean ± SD] for *NOD.Cpa3*$^{+/+}$ [n = 4] vs. $2.6 \times 10^6 \pm 0.5$ for *NOD.Cpa3*$^{Cre/+}$ [n = 3] mice), absolute numbers of CD45$^+$ cells ($1.8 \times 10^6 \pm 1.1$ for *NOD.Cpa3*$^{+/+}$ vs. $2.0 \times 10^6 \pm 0.5$ for *NOD.Cpa3*$^{Cre/+}$ mice), percent of CD3$^+$ T cells (78 ± 11 for *NOD.Cpa3*$^{+/+}$ vs. 76 ± 6 for *NOD.Cpa3*$^{Cre/+}$ mice), and absolute numbers of CD3$^+$ T cells ($1.8 \times 10^6 \pm 1.1$ for *NOD.Cpa3*$^{+/+}$ vs. $2.0 \times 10^6 \pm 0.5$ 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 PLNs the relative proportions of T cells, B cells, and dendritic cells (Fig. 4*A–C*), all of which were similar in both genotypes. In accordance with earlier analysis (16), proportions of CD4$^+$ (Fig. 4*D*) and CD8$^+$ (Fig. 4*E*) cells among total T cells and the fraction of T regulatory cells per total CD4$^+$ T cells (Fig. 4*F*) 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 (CD62L$^-$CD44$^+$) (reviewed in 45). In the CD4 T-cell population (Fig. 4*G*), percentages of naïve, central memory, and effector memory subsets were comparable in *NOD.Cpa3*$^{Cre/+}$ 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 nonspecific mast cell "stabilizers" (48) or *Kit* mutant mice that, although mast cell–deficient, have other immune (18) and relevant nonimmune 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*$^{W/W^v}$ mice failed to develop T1D (52). Indirectly, one study tested the effect of mast cell inhibition using the mast cell stabilizer disodium cromoglycate 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-FcεRI 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.

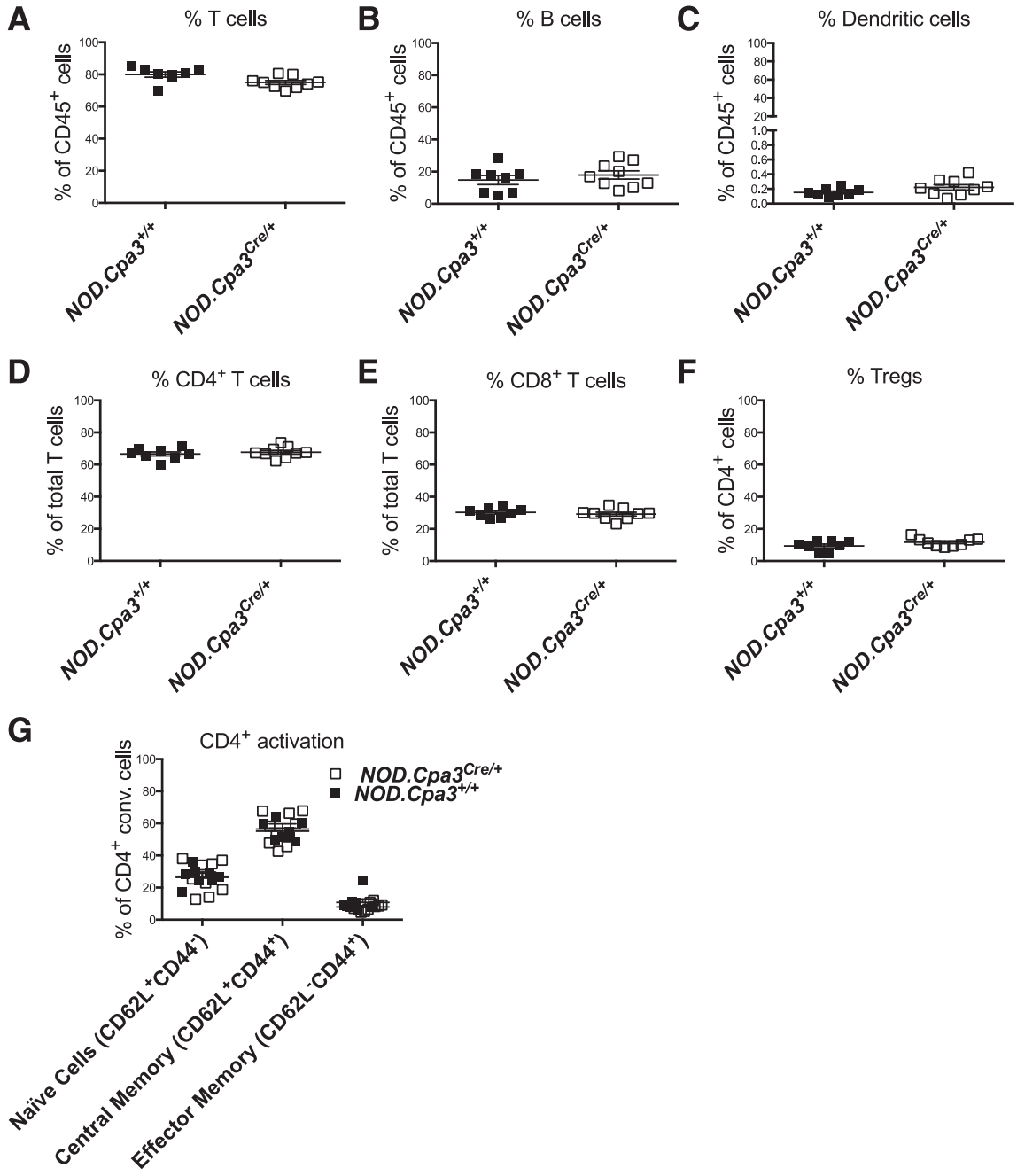

Figure 4—Immunological analysis of PLNs. Single-cell suspensions of PLNs from 15-week-old *NOD.Cpa3*$^{Cre/+}$ and *NOD.Cpa3*$^{+/+}$ mice were analyzed by flow cytometry. Displayed are percent of CD3$^+$ T cells (*A*), CD19$^+$ B cells (*B*), and CD11c$^+$ dendritic cells (*C*) per total CD45$^+$ cells;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

In the current study, we assessed the potential effects of mast cell deficiency on T1D, using both a traditional *Kit* mutant (*Kit*$^{W\text{-}sh/W\text{-}sh}$) and the more recent *Kit*-independent mast cell–deficient *Cpa3*$^{Cre/+}$ 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.Cpa3*$^{Cre/+}$ mice remained mast cell–deficient during initiation and progression of the autoimmune attack. Both in the peritoneal cavity and in the relevant PLNs, 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 suggests that mast cells cannot be directly involved in islet immunopathology. However, the presence of mast cells in secondary lymphoid organs, including the PLNs, 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.Kit*$^{W\text{-}sh/W\text{-}sh}$ 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 dendritic cell 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 PLNs; 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, experimental autoimmune encephalomyelitis (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 (17,53), 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 BB *DR*$^{lyp/lyp}$ rat, upregulation of mast cell genes in the PLNs suggest that mast cell numbers or their 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 (11,36,54). 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 cell roles have emerged in venom degradation (55,56) and in IgE-mediated protection from lethal doses of venom (57,58), the possible involvement of mast cells in the regulation of innate and adaptive immune responses and their roles as effector cells remain enigmatic.

Acknowledgments. We thank Sathya Muralidhar and Tabea Arnsperger (Division of Cellular Immunology, German Cancer Research Center, Heidelberg, Germany) for technical assistance in this project.

Funding. D.A.G. was a recipient of a postdoctoral fellowship from the German Cancer Research Center. H.-R.R. was supported by European Research Council advanced grant 233074 and Deutsche Forschungsgemeinschaft-Sonderforschungsbereiche 938-project L. A.L. was supported by a Career Development Award from JDRF. D.M. received funding from JDRF.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. D.A.G. designed and performed experiments, analyzed the data, and wrote the manuscript. W.F. and S.S. designed and performed experiments. T.B.F. contributed to experiment design, assisted with experiments, and edited the manuscript. A.O.-L. and Y.L. assisted with experiments. A.L., D.M., and H.-R.R. conceived the studies, contributed to experiment design, and edited the manuscript. H.-R.R. 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.

References

1. Grimbaldeston MA, Metz M, Yu M, Tsai M, Galli SJ. Effector and potential immunoregulatory roles of mast cells in IgE-associated acquired immune responses. Curr Opin Immunol 2006;18:751–760

2. Ludgate M, Baker G. Unlocking the immunological mechanisms of orbital inflammation in thyroid eye disease. Clin Exp Immunol 2002;127:193–198

3. Nelson KC, Zhao M, Schroeder PR, et al. Role of different pathways of the complement cascade in experimental bullous pemphigoid. J Clin Invest 2006; 116:2892–2900

4. Levi-Schaffer F, Klapholz L, Kupietzky A, Weinrauch L, Shalit M, Okon E. Increased numbers of mast cells in pemphigus vulgaris skin lesions. A histochemical study. Acta Derm Venereol 1991;71:269–271

5. Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D, Brenner MB. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. Science 2002;297:1689–1692

6. Secor VH, Secor WE, Gutekunst CA, Brown MA. Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis. J Exp Med 2000;191:813–822

7. Akimoto S, Ishikawa O, Igarashi Y, Kurosawa M, Miyachi Y. Dermal mast cells in scleroderma: their skin density, tryptase/chymase phenotypes and degranulation. Br J Dermatol 1998;138:399–406

8. Shiota N, Kakizoe E, Shimoura K, Tanaka T, Okunishi H. Effect of mast cell chymase inhibitor on the development of scleroderma in tight-skin mice. Br J Pharmacol 2005;145:424–431

9. Seeldrayers PA, Yasui D, Weiner HL, Johnson D. Treatment of experimental allergic neuritis with nedocromil sodium. J Neuroimmunol 1989;25:221–226

10. Geoffrey R, Jia S, Kwitek AE, et al. Evidence of a functional role for mast cells in the development of type 1 diabetes mellitus in the BioBreeding rat. J Immunol 2006;177:7275–7286

11. Hübner MP, Larson D, Torrero MN, et al. Anti-FcεRI antibody injections activate basophils and mast cells and delay Type 1 diabetes onset in NOD mice. Clin Immunol 2011;141:205–217
12. Jones SE, Gilbert RE, Kelly DJ. Tranilast reduces mesenteric vascular collagen deposition and chymase-positive mast cells in experimental diabetes. J Diabetes Complications 2004;18:309–315
13. Hessner MJ, Wang X, Meyer L, et al. Involvement of eotaxin, eosinophils, and pancreatic predisposition in development of type 1 diabetes mellitus in the BioBreeding rat. J Immunol 2004;173:6993–7002
14. Sayed BA, Christy A, Quirion MR, Brown MA. The master switch: the role of mast cells in autoimmunity and tolerance. Annu Rev Immunol 2008;26:705–739
15. Benoist C, Mathis D. Mast cells in autoimmune disease. Nature 2002;420:875–878
16. Feyerabend TB, Weiser A, Tietz A, et al. Cre-mediated cell ablation contests mast cell contribution in models of antibody- and T cell-mediated autoimmunity. Immunity 2011;35:832–844
17. Rodewald HR, Feyerabend TB. Widespread immunological functions of mast cells: fact or fiction? Immunity 2012;37:13–24
18. Reber LL, Marichal T, Galli SJ. New models for analyzing mast cell functions in vivo. Trends Immunol 2012;33:613–625
19. Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ. Epidemiology of type 1 diabetes. Endocrinol Metab Clin North Am 2010;39:481–497
20. Kukreja A, Maclaren NK. Autoimmunity and diabetes. J Clin Endocrinol Metab 1999;84:4371–4378
21. Wicker LS, Todd JA, Peterson LB. Genetic control of autoimmune diabetes in the NOD mouse. Annu Rev Immunol 1995;13:179–200
22. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. Annu Rev Immunol 2005;23:447–485
23. Fox CC, Jewell SD, Whitacre CC. Rat peritoneal mast cells present antigen to a PPD-specific T cell line. Cell Immunol 1994;158:253–264
24. Frandji P, Oskéritzian C, Cacaraci F, et al. Antigen-dependent stimulation by bone marrow-derived mast cells of MHC class II-restricted T cell hybridoma. J Immunol 1993;151:6318–6328
25. Stelekati E, Bahri R, D'Orlando O, et al. Mast cell-mediated antigen presentation regulates CD8+ T cell effector functions. Immunity 2009;31:665–676
26. Wang HW, Tedla N, Lloyd AR, Wakefield D, McNeil PH. Mast cell activation and migration to lymph nodes during induction of an immune response in mice. J Clin Invest 1998;102:1617–1626
27. McLachlan JB, Hart JP, Pizzo SV, et al. Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. Nat Immunol 2003;4:1199–1205
28. Jawdat DM, Albert EJ, Rowden G, Haidl ID, Marshall JS. IgE-mediated mast cell activation induces Langerhans cell migration in vivo. J Immunol 2004;173:5275–5282
29. Suto H, Nakae S, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cell-associated TNF promotes dendritic cell migration. J Immunol 2006;176:4102–4112
30. de Vries VC, Pino-Lagos K, Nowak EC, Bennett KA, Oliva C, Noelle RJ. Mast cells condition dendritic cells to mediate allograft tolerance. Immunity 2011;35:550–561
31. Jawdat DM, Rowden G, Marshall JS. Mast cells have a pivotal role in TNF-independent lymph node hypertrophy and the mobilization of Langerhans cells in response to bacterial peptidoglycan. J Immunol 2006;177:1755–1762
32. Jutel M, Watanabe T, Klunker S, et al. Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. Nature 2001;413:420–425
33. Jutel M, Klunker S, Akdis M, et al. Histamine upregulates Th1 and downregulates Th2 responses due to different patterns of surface histamine 1 and 2 receptor expression. Int Arch Allergy Immunol 2001;124:190–192
34. Caron G, Delneste Y, Roelandts E, et al. Histamine polarizes human dendritic cells into Th2 cell-promoting effector dendritic cells. J Immunol 2001;167:3682–3686
35. Kitawaki T, Kadowaki N, Sugimoto N, et al. IgE-activated mast cells in combination with pro-inflammatory factors induce Th2-promoting dendritic cells. Int Immunol 2006;18:1789–1799
36. Shi MA, Shi GP. Different roles of mast cells in obesity and diabetes: lessons from experimental animals and humans. Front Immunol 2012;3:7
37. Lyon MF, Glenister PH. A new allele sash (Wsh) at the W-locus and a spontaneous recessive lethal in mice. Genet Res 1982;39:315–322
38. Leiter EH. The NOD mouse: a model for insulin-dependent diabetes mellitus. Curr Protoc Immunol 2001;15.9:Unit 15.9
39. Rodewald HR, Ogawa M, Haller C, Waskow C, DiSanto JP. Pro-thymocyte expansion by c-kit and the common cytokine receptor gamma chain is essential for repertoire formation. Immunity 1997;6:265–272
40. Antsiferova M, Martin C, Huber M, et al. Mast cells are dispensable for normal and activin-promoted wound healing and skin carcinogenesis. J Immunol 2013;191:6147–6155
41. Gomez-Pinilla PJ, Farro G, Di Giovangiulio M, et al. Mast cells play no role in the pathogenesis of postoperative ileus induced by intestinal manipulation. PLoS ONE 2014;9:e85304
42. Duttlinger R, Manova K, Berrozpe G, et al. The Wsh and Ph mutations affect the c-kit expression profile: c-kit misexpression in embryogenesis impairs melanogenesis in Wsh and Ph mutant mice. Proc Natl Acad Sci USA 1995;92:3754–3758
43. Nigrovic PA, Gray DH, Jones T, et al. Genetic inversion in mast cell-deficient (Wsh) mice interrupts corin and manifests as hematopoietic and cardiac aberrancy. Am J Pathol 2008;173:1693–1701
44. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 2010;464:1293–1300
45. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745–763
46. Brown MA, Sayed BA, Christy A. Mast cells and the adaptive immune response. J Clin Immunol 2008;28:671–676
47. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nat Rev Immunol 2008;8:478–486
48. Oka T, Kalesnikoff J, Starkl P, Tsai M, Galli SJ. Evidence questioning cromolyn's effectiveness and selectivity as a 'mast cell stabilizer' in mice. Lab Invest 2012;92:1472–1482
49. Feng ZC, Li J, Turco BA, Riopel M, Yee SP, Wang R. Critical role of c-Kit in beta cell function: increased insulin secretion and protection against diabetes in a mouse model. Diabetologia 2012;55:2214–2225
50. Dudeck A, Dudeck J, Scholten J, et al. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. Immunity 2011;34:973–984
51. Lilla JN, Chen CC, Mukai K, et al. Reduced mast cell and basophil numbers and function in Cpa3-Cre; Mcl-1fl/fl mice. Blood 2011;118:6930–6938
52. Hatfield J, Quirion M, Rao K, Brown M. NODckit$^{W/Wv}$ mast cell deficient mice fail to develop type 1 diabetes (Abstract). J Immunol 2010;184:135.39
53. Katz HR, Austen KF. Mast cell deficiency, a game of kit and mouse. Immunity 2011;35:668–670
54. Hübner MP, Shi Y, Torrero MN, et al. Helminth protection against autoimmune diabetes in nonobese diabetic mice is independent of a type 2 immune shift and requires TGF-β. J Immunol 2012;188:559–568
55. Metz M, Piliponsky AM, Chen CC, et al. Mast cells can enhance resistance to snake and honeybee venoms. Science 2006;313:526–530
56. Schneider LA, Schlenner SM, Feyerabend TB, Wunderlin M, Rodewald HR. Molecular mechanism of mast cell mediated innate defense against endothelin and snake venom sarafotoxin. J Exp Med 2007;204:2629–2639
57. Marichal T, Starkl P, Reber LL, et al. A beneficial role for immunoglobulin E in host defense against honeybee venom. Immunity 2013;39:963–975
58. Palm NW, Rosenstein RK, Yu S, Schenten DD, Florsheim E, Medzhitov R. Bee venom phospholipase A2 induces a primary type 2 response that is dependent on the receptor ST2 and confers protective immunity. Immunity 2013;39:976–985