Inflammatory status of the pancreas in NOD mice that do not develop overt diabetes

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Abstract
Type 1 diabetes (T1D) is an autoimmune disease in which immune cells target the pancreatic islets and destroy the β-cells, resulting in hyperglycemia and decreased plasma insulin levels. The non-obese diabetic (NOD) mouse is the most used animal model for studying diabetes because it spontaneously develops T1D and shares similarities with the human disease. A hallmark feature of this model is the appearance of insulitis, defined as an inflammatory cell infiltration of the pancreatic islets. However, a small percentage of NOD mice do not develop overt diabetes even after 28–35 weeks of age. Thus, we questioned the status of the pancreatic islets in these non-diabetic NOD mice, with particular focus on islet inflammation and plasmatic insulin levels, in comparison to pre-diabetic (11 weeks old) and new-onset diabetic mice. Diabetes progression was evaluated by assessing blood glucose and pancreas histology. The inflammatory score was determined on Hematoxylin–Eosin (HE)-stained sections of pancreas. Plasma insulin was detected by enzyme-linked immunosorbent assay (ELISA). The results showed that inflammation increased in an age-dependent manner in all mice, irrespective of their diabetic status. Mostly affected within the analyzed groups were the 28 weeks old non-diabetic NOD mice, in which insulin production was reduced and inversely correlated with the inflammatory status. We conclude that in NOD mice, pancreatic inflammation progresses independently of diabetes onset and clinical signs of disease. Most likely, the NOD females that do not develop overt diabetes preserve a small mass of functional β-cells, which is able to provide the physiological insulin levels and avoid diabetes onset.

Keywords: type 1 diabetes, NOD mice, histological inflammatory score, insulin, CD45+ inflammatory cells.

Introduction
Type 1 diabetes (T1D) is a complex, organ-specific autoimmune disorder characterized by pancreatic β-cells destruction induced by autoreactive T-cells, resulting in a decrease of plasma insulin levels and hyperglycemia. The histopathological hallmark of T1D is defined by insulitis, a characteristic inflammatory lesion consisting of lymphocyte infiltrates around and within the islet. The onset of diabetes correlates with the progression of inflammation of the pancreatic islets, as well as the amount of β-cells mass which becomes non-functional over time [1, 2].

Islet inflammation associated with diabetes was first reported in 1902, in the pancreas of a 10-year-old child, as peripheral focal points of small cell infiltration [1]. Later on, the lesion was called insulitis by the Swiss pathologist Hanns von Meyenburg [1]. A consensus guideline established that more than 15 cluster of differentiation (CD)45+ cells per islet encountered in minimum three different islets represent a criterion to define insulitis. According to this guideline, the pathology report is done based on the following parameters: the entire number of islets analyzed, the percentage of islets with insulitis, the percentage of pseudoatrophic islets, and the spatial relationship of the infiltrate to the insulin-positive islet cells [3].

Currently, several animal models are extensively used to bring new insights into the immune, genetic, and environmental mediated mechanisms involved in T1D pathology in humans. Two types of murine models of diabetes are most commonly used, i.e., chemically-induced diabetic mice (for instance, by injection of Streptozotocin), and non-obese diabetic (NOD) mice, which spontaneously develop diabetes [4].

NOD mice are an appropriate model of spontaneously developed autoimmune diabetes because this strain shares many genetic and cellular features with the human disease. Furthermore, insulin is likely an early autoantigen in the NOD mouse as in humans [5]. A higher incidence of diabetes in NOD females compared with males was reported. However, the development of diabetes in NOD mice is apparently affected by environmental factors, including housing conditions, health status, and diet. These evidences could explain the different levels of diabetes incidence registered in NOD mice colonies from different laboratories [6–8].

The inflammatory infiltrate involves lymphoid, as well as myeloid, cell types. The most abundant cell type in the inflammatory infiltrate is represented by T-cells, particularly CD3+CD8+ lymphocytes, CD3+CD4+ lymphocytes, CD20+ B-lymphocytes and macrophages are also present [9–12].

Histological analysis in NOD mice showed that the immune infiltrate begins to surround the pancreatic islets at about 4–5 weeks of age (peri-insulitis), and after about 10 weeks, the immune cells can be already found within the pancreatic islets (insulitis). The inflammatory infiltrate consists initially of dendritic cells and macrophages, which infiltrate the islets before other CD45+ cells. In time (at 12–14 weeks of age), lymphocytes become the predominant...
cellular type, while CD11b+ F4/80+ macrophages are present in lower numbers [13–15].

**Aim**

In this study, we evaluated a group of NOD-resistant mice, with particular focus on islet inflammatory status and plasmatic insulin levels, in comparison to pre-diabetic (11 weeks old) and new-onset diabetic mice. Our results showed that in NOD mice, the pancreatic inflammation progressed independently of the diabetes onset and the clinical signs of disease. Importantly, despite their severe insulitis, the diabetic-resistant NOD females preserve physiological insulin levels due to a minimum mass of functional β-cells, which avoids the overt diabetes onset.

**Materials and Methods**

**Mice**

NOD/ShiLtJ mice were purchased from Jackson Laboratory (Stock No. 001976) and maintained in our Animal Facility under specific pathogen-free conditions. The animals were housed in 12 hours light and 12 hours dark cycle.

All experimental procedures involving animals were conducted in accordance with the European Union (EU) Directive 2010/63/EU and approved by the national competent authority (Authorization No. 390/10/07/2018).

For this study, NOD female mice were divided into four groups: pre-diabetic (11 weeks old) (n=6), new-onset diabetic (n=6), non-diabetic at 28 weeks (n=5) and non-diabetic at 35 weeks (n=4).

**Diabetes monitoring**

Blood glucose levels were measured weekly in all female mice starting 10 weeks of age, by puncture of the lateral tail vein and using a standard glucometer. Mice were considered diabetic after two consecutive blood glucose measurements exceeding 200 mg/dL. The new-onset diabetic NOD females were included in the study in the first week after diabetes confirmation.

**Tissue preparation**

Mice were anesthetized with Ketamine/Xylazine (100/20 mg/kg) by intraperitoneal injection. Pancreatic tissue samples were harvested and fixed in ice-cold phosphate-buffered saline (PBS) with 1.5% paraformaldehyde and 0.1% glutaraldehyde overnight, at 4°C, and then they were immersed in 30% sucrose (Sigma-Aldrich). The pancreatic tissue was embedded in optimal cutting temperature (OCT) compound (Tissue Freezing Medium, Leica), frozen in isopentane suspended in liquid nitrogen and sectioned (5 μm) using a Leica CM1850 cryostat (Leica Biosystems).

**Histological evaluation of inflammatory cells infiltrated in the pancreatic islets of NOD mice**

For each mouse, serial cryosections were cut, with three sections per slide, five slides per level and between 15–20 levels per mouse. The histological evaluation was performed on sections cut 150 μm apart to prevent double evaluation of the same islet. These sections were stained with Hematoxylin–Eosin (HE Fast Staining Kit, Carl Roth). Before HE staining, slides were air-dried and rehydrated. After dehydration by a graded series of ethanol solutions and clarification with xylene, all slides were mounted with coverslips using CV Mount (Leica). After staining, all sections were analyzed using a Nikon microscope.

**Determination of inflammatory score/insulitis**

The degree of inflammation (insulitis) was assessed by histological score. At every 150 μm interval, the serial cryosections with 5 μm thickness (corresponding to the first slide from each level) were stained with HE. A number of 42–60 pancreatic islets from each pancreas were examined to assess the degree of infiltration. Insulitis scoring was performed according to the following criteria: 0 – no insulitis, absence of cell infiltration; 1 – peri-insulitis, infiltration restricted to the periphery of the islet; 2 – less than 50% of the islet area is infiltrated; 3 – severe insulitis, 50% or more of the islet area is infiltrated and the islet structure disrupted.

**Immunofluorescence for insulin and CD45+ cells detection**

Sections rehydrated in PBS were incubated in 5% mouse serum in PBS for 30 minutes at room temperature to block non-specific binding sites. Then, the cryosections were immunostained with monoclonal mouse anti-insulin Alexa Fluor 594 (2D11-H5) (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, sc-8033AF594), Phycocyanin (PE) anti-mouse CD45 (1:50, BioLegend, Catalogue #103106), and PE rat immunoglobulin G2b (IgG2b), k isotype control (1:150, BioLegend, Catalogue #400607) diluted in PBS with 1% bovine serum albumin (BSA). Nuclei were labeled with Hoechst-33342 (1:1000). The slides were mounted with antifade mounting medium (ProLong™ Gold Antifade Mountant, Thermo Fisher Scientific) and visualized under a fluorescence microscope. Pictures were taken using a fluorescence microscope (Leica).

**Blood collection/Quantification of plasma insulin by enzyme-linked immunosorbent assay (ELISA)**

Blood samples were collected by cardiac puncture and transferred into vacutainers containing ethylenediaminetetraacetic acid (EDTA), and then centrifuged at 3000×g for 10 minutes, at 4°C. Plasma samples were assessed in anti-mouse insulin precoated 96-well strip plate using mouse insulin ELISA kit (Thermo Fisher Scientific). Absorbance was measured on an ELISA plate reader set at 450 nm and 550 nm, using insulin standards for calibration. The 550 nm values were subtracted from the 450 nm values to correct for optical imperfections in the microplate.

**Statistical analysis**

The results were analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc.) by one-way analysis of variance (ANOVA) test and Tukey’s multiple comparison test post-hoc analysis. P-value, defined as <0.05, was considered as statistically significant.
Results

Insulitis scoring based on the infiltration of inflammatory cells into the pancreatic islets

Blood glucose levels were monitored weekly in non-fasted female mice from the NOD colony, starting 10 weeks of age. Animals aged 11 weeks were considered pre-diabetic and all had glycemia levels less than 200 mg/dL. Two consecutive measurements of glycemia higher than 200 mg/dL were considered as a clinical indication of diabetes onset. The mice within the first week after diabetes confirmation were considered new-onset diabetics. Mice with no diabetes onset until 28 weeks old were named non-diabetic. A few non-diabetic mice were continuously monitored up to 35 weeks of age.

Islet inflammation was established on HE-stained slides by assigning each islet a score between 0 and 3, according to the protocol explained above. Representative images of islets with corresponding score are illustrated in Figure 1. Islets with score 0 had normal architecture, with no inflammatory cells and were clearly delimited from the surrounding parenchyma. Islets with score 1 still had a clear delimitation from the adjacent parenchyma; however, small nuclei, characteristic of inflammatory infiltrate, became apparent at one pole of the islet periphery. The islets with score higher than 1 were usually completely surrounded by inflammatory infiltrate, but the overall infiltration was less than 50% (for score 2 islets) or higher than 50% (score 3 islets). There were cases in which the morphology of the islet was completely lost, yet its presence was recognizable, in which case the attributed score was also 3.

Figure 1 – Illustrative histological images of pancreatic islets with different levels of inflammation. Insulitis scoring was performed on HE-stained sections and evaluated according to the following criteria: (A) Score 0 – no insulitis, absence of cell infiltration into the islet; (B) Score 1 – peri-insulitis, infiltration restricted to the periphery of the islet; (C) Score 2 – less than 50% of the islet area is infiltrated; (D) Score 3 – severe insulitis, 50% or more of the islet area is infiltrated and islet structure is disrupted. Scale bars, 100 μm. HE: Hematoxylin–Eosin.

Islet inflammatory score increases with age, irrespective of the diabetic status

Comparative analysis of the insulitis score in the three experimental groups, i.e., pre-diabetic (n=6), diabetic new-onset (n=6), and non-diabetic at 28 weeks (n=5) groups, showed an overall decrease in the percentage of score 0 islets, concomitant with an overall increase in the score 3 islets with progressive ageing of mice (Figure 2A). Thus, around 24% (±7%), 21% (±15%) and 17% (±4%) of islets were scored 0 in pre-diabetic, diabetic new-onset, and non-diabetic mice, respectively (Figure 2A). On the contrary, around 18% (±9%), 22% (±10%) and 36% (±4%) of islets were scored 3 in pre-diabetic, diabetic new-onset, and non-diabetic mice, respectively. Furthermore, the percentage of score 3 islets...
was even more increased in animals that were euglycemic at the age of 35 weeks \((n=4)\) (Figure 2B). These data suggest that the inflammatory score in NOD female mice increases with age, irrespective of the diabetic status. No significant differences in islets scored 0, 1 and 2 were found between the experimental NOD mice groups.

**Detection of insulin level in non-diabetic NOD female mice**

Having the continuous deterioration of the pancreatic islets in NOD mice with age, we asked whether the insulin level is maintained at physiological range in non-diabetic females. To answer this question, blood was collected from mice and plasma insulin level was determined using an ELISA kit. Despite the high variation noted in the plasma insulin level in the pre-diabetic group, an apparent decrease in the insulin level in diabetic new-onset and non-diabetic groups was observed (Figure 3A). Although this decrease did not meet the statistical significance (which is likely a consequence of the highly spread values in pre-diabetic group), the data suggested a direct correlation between the insulitis score and insulin level. This was further confirmed by immunofluorescent examination of the pancreatic islets for the CD45 pan-hematopoietic immunomarker, which stained both T- and B-cells infiltrating the pancreatic islets, and insulin. The qualitative analysis of inflammatory cells and insulin in serial pancreatic sections from non-diabetic mice showed that the presence of insulin was inversely correlated to the presence of CD45+ inflammatory cells (Figure 3B). The highly infiltrated islets, confirmed with either HE or CD45 immunostaining, revealed an apparently weaker capacity to produce insulin, in comparison to a score 1 islet.

**Highly infiltrated islets do not completely lose the capacity to produce insulin at a low level in non-diabetic mice**

To explain the resistance to diabetes of a few female mice with high level of insulitis, islets with various degree of infiltration have been selected from non-diabetic females and assessed for the presence of insulin by immunofluorescence. The results showed that many pancreatic islets with severe insulitis still had functional \(\beta\)-cells that produced insulin (Figure 4), thus supporting the idea that these non-diabetic NOD mice are still able to maintain the insulin at physiological levels and thus avoid diabetes onset.

![Figure 2](image_url) **Figure 2** – Inflammatory score in NOD mice: (A) Comparative analysis of islet inflammatory score in three NOD experimental groups – pre-diabetic \((n=6)\), diabetic new onset \((n=6)\), non-diabetic at 28 weeks \((n=5)\), showing that inflammation increases with age independently of diabetic status. Insulitis was scored by examining a minimum of 42 islets per mouse \((n=58, \pm 6)\). (B) Diagram showing the increase in the percentage of score 3 islets in NOD females with age, irrespective of the diabetes onset. The statistical analysis was performed by one-way ANOVA with Tukey’s multiple comparison test post-hoc analysis, \(p<0.05\) (*) and \(p<0.01\) (**). ANOVA: Analysis of variance; NOD: Non-obese diabetic.
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Figure 3 – (A) Plasma insulin in NOD mice as detected by ELISA assay. Note that the insulin level decreases with age (from 11 to 28 weeks) and is inversely correlated to the inflammatory status. (B) Insulin (left image) and CD45+ inflammatory cells (middle image) staining of cryosections from non-diabetic NOD pancreatic tissue that were counterstained with DAPI for nuclei labeling (blue fluorescence). On the right image, a successive HE-stained section is shown. Note that positive staining for insulin is inversely correlated with the presence of CD45+ inflammatory cells, mostly evident in pancreatic islets severely infiltrated. Scale bars, 100 μm. CD45: Cluster of differentiation 45; DAPI: 4’,6-Diamidino-2-phenylindole; ELISA: Enzyme-linked immunosorbent assay; HE: Hematoxylin–Eosin; NOD: Non-obese diabetic.

Figure 4 – (A–F) Insulin detection by immunofluorescence in pancreatic islets with different degrees of infiltration from non-diabetic NOD females (representative pictures). Immunofluorescence staining was performed with monoclonal mouse anti-insulin Alexa Fluor 594 (2D11-H5) antibody (1:200). Note that positive staining for insulin negatively correlated with the degree of inflammation. In contrast with islets scored 0 or 1, insulin was detected less in pancreatic islets with severe insulitis (score 3). Scale bars, 100 μm. NOD: Non-obese diabetic.
Discussions

T1D occurs as a direct result from an autoimmune attack directed towards the pancreatic insulin-secreting $\beta$-cells. The first consequence of this attack is the inflammation of the pancreatic islets, the so-called insulitis. In time, the exacerbation of this process leads to the gradual death of the $\beta$-cells which translates at clinical level in the development of chronic hyperglycemia.

There are significant differences between the insulitic lesions in humans as compared to NOD mice [16]. In humans, insulitis is found mainly in young individuals with recent onset of the disease, although it can be clearly detected in only a small fraction of islets. In chronic patients, insulitis is less frequent, even rare, possibly indicating a different kinetic of the $\beta$-cell loss. Irrespective of the disease duration, most T1D patients still have residual insulin positive islets. In NOD mice however, most of the islets are affected by a robust lymphocytic infiltration. Whereas in humans the level of infiltration is mild, in NOD mice there is a massive accumulation of inflammatory cells, initially displayed at the periphery of the islets [3, 14].

As main characteristic of T1D, insulitis is defined as the multifocal infiltration of the pancreas with inflammatory cells resulting in severe loss of $\beta$-cells and impaired insulin production [17]. Considering the role of insulitis as a determining factor of T1D pathogenesis, different experimental approaches have been used to elucidate the onset of autoimmune diabetes [18, 19]. Studies performed on animal models have clearly identified the CD4$^+$ and CD8$^+$ lymphocytes as the predominant cellular type that infiltrate the pancreatic islets and described molecular aspects associated with insulitis in NOD mice [7–9, 20]. On the contrary, in the pancreas of non-diabetic mice, such as C57BL/6, immune cells are rarely found [21].

In the present study, the inflammatory score and islet histopathology were evaluated in different groups of NOD female mice (pre-diabetic, diabetic new-onset and non-diabetic) corresponding to various stages of disease progression. The damage of each islet was scored into three stages, based on the infiltration level, as previously described by others [22]. Several differences were noted, i.e., the increase in the percentage of the severely infiltrated islets (score 3) with animal age, rather than the diabetic status and the capacity of infiltrated islets to keep secreting insulin at low extent in mice that remained euglycemic for 35 weeks. Thus, islets appeared to progressively change their morphology with animal age, which was also correlated to the disease stage: insulin-containing islets with no inflammation were gradually replaced by islets with a normal mass of $\beta$-cells and a small number of inflammatory cells at the periphery. With ongoing progression of disease, a significant number of islets with severe infiltration and reduced numbers of $\beta$-cells appeared, which in some cases progressed to islets totally infiltrated with inflammatory cells and completely devoid of $\beta$-cells.

This paper shows that diabetes resistant NOD female mice have inflammatory scores in the pancreas even higher that new-onset diabetic mice, and therefore their resistance to diabetes may seem peculiar. Our results showed that the percentage of islets with severe insulitis increased with age, independent of the diabetic status. Furthermore, the decreased expression of insulin in affected islets was inversely correlated with the degree of inflammatory infiltration. However, a small supply of functional $\beta$-cells was still preserved, which was enough to maintain normal glycemia.

Our results contribute to a better understanding of the dynamics of diabetes onset in NOD mice and may provide an explanation for diabetes resistance in this model. However, the relation between insulitis, dynamics of $\beta$-cell loss and coexisting mechanisms of dysfunction, still remains a major goal to clarify; an improved understanding of these processes may be the key towards designing new therapeutic strategies that target multiple mechanisms in chronic autoimmune diseases.

Conclusions

There was no correlation between diabetes resistance associated with absence of clinical signs of diabetes in non-diabetic NOD mice at 28 and 35 weeks and a low status of islet inflammation. On the contrary, data obtained from inflammatory scoring analysis in NOD mice suggested an increase in inflammatory status with age, independent of the onset of diabetes. Moreover, plasma insulin levels negatively correlated with the degree of inflammation. Most likely, in non-diabetic NOD mice there is still a reserve of functional $\beta$-cells sufficient to prevent the onset of diabetes.

Conflict of interests

The authors declare that they have no conflict of interests.

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