



The Immunoreactive Platform of the Pancreatic Islets Influences the Development of Autoreactivity

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Diabetes 2019;68:1544–1551 | <https://doi.org/10.2337/dbi18-0048>

Tissue homeostasis is maintained through a finely tuned balance between the immune system and the organ-resident cells. Disruption of this process not only results in organ dysfunction but also may trigger detrimental autoimmune responses. The islet of Langerhans consists of the insulin-producing β -cells essential for proper control of body metabolism, but less appreciated is that these cells naturally interact with the immune system, forming a platform by which the β -cell products are sensed, processed, and responded to by the local immune cells, particularly the islet-resident macrophages. Although its physiological outcomes are not completely understood, this immunoreactive platform is crucial for precipitating islet autoreactivity in individuals carrying genetic risks, leading to the development of type 1 diabetes. In this Perspective, we summarize recent studies that examine the cross talk between the β -cells and various immune components, with a primary focus on discussing how antigenic information generated during normal β -cell catabolism can be delivered to the resident macrophage and further recognized by the adaptive CD4 T-cell system, a critical step to initiate autoimmune diabetes. The core nature of the islet immune platform can be extrapolated to other endocrine tissues and may represent a common mechanism underlying the development of autoimmune syndromes influencing multiple endocrine organs.

Each organ has unique anatomical and physiological features that determine how its immunological reactions are programmed. Included among the many critical components of each immunological platform are the biology of the cells that compose the organ, their contents of resident or migratory innate immune cells, and the vascularization and blood flow of the organ. All of these components determine how the organ reacts and protects itself from

infection and how it responds in autoimmune situations. In the specific context of autoimmunity, two distinct issues are important: first, how the tissue conveys its immunologically relevant information to the immune system and, second, how it serves as the target of the immune effector reactions. How the immunologically relevant information is conveyed becomes critical and will depend on 1) how a tissue cell processes its “antigens”; 2) how it transfers such antigens either locally, to the phagocytes, the antigen-presenting cells (APCs) that inhabit the organs, or systemically, to the peripheral lymphoid tissues; and 3) whether the cells in the organ express the genes encoding the MHC. It is through the MHC with its function of binding peptides that a cell can interact with CD4 and CD8 T cells. Finally, the nature of the effector reactions is conditioned by factors associated with the tissue environment. Two major issues are the degree of vascularization and the permeability of the vasculature system. These will influence the entry of lymphocytes and blood-derived proteins, including antibodies and complements.

The pancreatic islet is a well-vascularized mini-organ consisting of various sets of endocrine cells. The major set is the β -cells (~1,000), which respond to increases in blood glucose levels. The islets are the target of autoimmune reactivity that results in the specific demise of β -cells, leading to type 1 diabetes (T1D), a disease extensively analyzed clinically and experimentally. The autoimmune process has a strong genetic component in which the MHC genes play a major role. Most prominently, allelic variants of class II MHC (MHC-II) are highly important, an issue first made evident in a major report by Todd et al. (1) that examined T1D patients. Indeed, CD4 T cells reactive to β -cell-derived peptides are critical for disease initiation, later followed by the involvement of CD8 T cells. These fundamental observations in the disease, i.e., the dependency

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Received 5 April 2019 and accepted 27 May 2019

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on particular MHC-II alleles, are echoed in the NOD mouse, a unique strain that spontaneously develops autoimmune diabetes. Of particular interest, the MHC-II allele of the NOD mouse, I-A^{g7}, has structural features akin to HLA-DQ8, that is, an absence of aspartic acid, an acidic residue, at position 57 of the MHC-II β -chain (2,3). This results in an unpaired arginine at the α -76 position that favors binding to peptides with acidic residues at their carboxy end (4–7). In the NOD mouse, transgenic expression of MHC with a correction of serine to aspartic acid at β -57 results in complete absence of autoimmunity, an indication of how a single amino acid residue determines the outcome of immunological reactions (8,9).

This Perspective summarizes our attempts to understand the immune platform of an islet and how it conditions an autoimmune program. By the logistics of this Perspective, we cannot cover the extensive literatures on this complex autoimmune process. Rather, we summarize our biased views on how the process is initiated based on our experiences examining the NOD mouse (10–13) (Fig. 1). We agree that insulin is the major autoantigen driving forward the autoimmune process. In particular, our evidence points to degradative products of insulin as giving rise to the set of CD4 T cells that initiate this process and that bypass autoregulatory control mechanisms. Because of the nature of presentation of denatured insulin or insulin peptides, the CD4 T cells driving the process are

subject to less regulation. The insulin peptides are generated in the β -cell itself from degradation of excess insulin dense core granules (DCGs) in crinophagic bodies (crinosomes). Presentation of insulin peptides takes place both in peripheral lymphoid organs as well as in the islets by resident macrophages. In peripheral lymphoid organs, their presentation takes place continuously as the contents of the crinosomes are released into the blood, obeying responses to glucose stimulation. Such peripheral presentation is important in the response of the T cells. In islets, presentation of insulin epitopes takes place by islet-resident macrophages first and later by dendritic cells that enter the islets. This local presentation sets the stage for the amplification of the response, including the participation of T cells to other epitopes, including CD8 T cells. Below, we briefly describe our experiences examining the nature of the CD4 T cells to insulin and the biology of presentation in islets and in peripheral lymphoid organs.

THE NATURE OF THE INSULIN-SPECIFIC T CELLS

The initiation of islet autoimmunity has been well recorded in the NOD mouse since islets and pancreatic lymph nodes can be examined for autoreactive T cells weeks before there is any evidence of glycemic changes. The first autoreactive T cells are found surprisingly early in the life of the mouse, by 3 weeks of age (14). The first seminal reports that examined the nature of the CD4

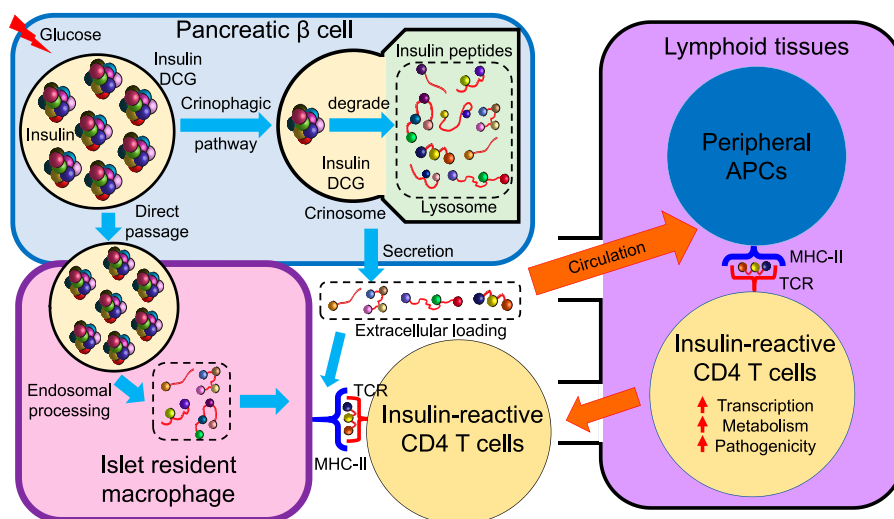


Figure 1—Overview of the islet immune platform and its communication with the lymphoid tissues. The pancreatic β -cells contain two sets of vesicles that provide antigenic materials. The regular insulin DCG mainly contains abundant insulin molecules that are secreted to regulate glucose metabolism. Via the crinophagic pathway, the insulin DCG is fused with lysosomes to degrade the excessive amounts of insulin production, resulting in the generation of a set of denser granules, the crinophagic bodies or crinosomes. This is a physiological process required for maintaining cellular homeostasis but simultaneously generates catabolized insulin fragments that may give rise to the majority of the T-cell responses. The β -cell delivers its immunological information both locally and systemically. First, the β -cell-derived granules are taken up by the islet-resident macrophages that naturally express high levels of MHC-II, resulting in a robust presentation of β -cell antigens. In NOD mice, this process is crucial for proper amplification of the islet-infiltrating T cells. As the autoimmune process moves forward, other APCs, including dendritic cells, may also play a role in presenting islet antigens, an issue not depicted in the figure. Second, the peptide fragments, mostly in the crinosomes, are secreted from the β -cells after glucose challenge. These materials reach various secondary lymphoid tissues and can be recognized by the corresponding T cells. Although divergent T-cell responses could be generated during such peripheral recognition, a representative insulin-reactive T cell acquires an activation phenotype that favors the invasion into the islets and the ability to cause diabetes. TCR, T-cell receptor.

T cells showed them reacting with insulin epitopes; such CD4 T cells recognized peptides derived from the B chain of insulin and caused diabetes when transferred to non-diabetic mice (15,16). These fundamental studies were confirmed and extended by different experiments, all pointing out that autoreactivity to insulin is a major component in the initiation of the process. Several reviews have examined these issues in detail (11,17,18). At the same time, clinical studies in patients with T1D reported that an initial feature of T1D was the presence of insulin autoantibodies (19,20). The presence of such autoantibodies has predictive values. It is important to emphasize that the presence of such autoantibodies indicates the presence of “helper” CD4 T cells, precisely the sets of T cells that initiate and drive the autoimmune process (21).

Other T cells have been shown to react with peptides derived from different β -cell proteins; these have been considered as an example of epitope spreading and/or as minor epitopes (11,22). These are operational terms, but detailed examination of such T cells and their interactions with peptide–MHC-II complexes is wanting. Included are more recent and provocative reports on the isolation of T cells directed to hybrid insulin peptides, i.e., made by the fusion of two peptides in highly catabolic compartments (23–27). In brief, although there is broad T-cell autoreactivity in T1D, the evidence for insulin autoreactivity as the major driver of the process is strong and convincing.

More Than One CD4 T Cell Recognizes Epitopes in the B:9–23 Peptide

Our early studies confirmed the finding of CD4 T cells to insulin epitopes centered on the insulin B-chain segment 9–23 as well as on segments of the C-peptide. A new perspective became apparent when Levisetti et al. (28) and Mohan et al. (29–31) examined in detail such CD4 T cells through very extensive studies. A novel approach that was followed combined several ex vivo and in vivo approaches: 1) isolating and cloning of the T cells, 2) examining their fine specificity using variant peptides with different mutations of key residues, 3) examining their binding to purified I-A^{g7} molecules, 4) testing their presentation when APCs were offered insulin, and finally, 5) examining their biological reactivity in vivo.

Such a combination of cellular and biochemical analyses allowed the identification of two subsets of CD4 T cells based on their recognition of different peptide segments in the B:9–23 peptide (Table 1). One set recognized the 12–20 segment and the other recognized the 13–21 segment, a one-amino shift. This shift of one single residue was decisive. The 12–20 segment with a terminal glycine binds weakly to I-A^{g7}, whereas in contrast the 13–21 segment, having a terminal glutamic acid, binds relatively stronger. Two important issues then became apparent. When insulin was offered to APCs, only the 13–21 peptide was presented. The presentation and processing of insulin that takes place in lysosomal-like vesicles of the APC had eliminated the weak binding segment B:12–20. Previous

studies using protein antigens had shown that intracellular processing of a protein restricts the peptide repertoire that is presented, favoring better binding peptides. In contrast, the 12–20 segment was recognized only when peptides or denatured insulin were offered to the APCs; that is, such T cells recognized already processed products of insulin. But the important correlate is that the T cells recognizing the weak-binding B:12–20 peptide, which is not processed from insulin, predominated during the early diabetic stage; we have termed such T cells as type B. In contrast, the conventional T cells, those that recognized insulin and the B:13–21, termed as type A, were few (29–31).

These findings correlated with functional analysis of the T-cell responses in young NOD mice immunized with insulin or peptides. Immunization with insulin or with the B:13–21 peptide did not result in any T-cell response (30). At face value, the NOD mouse is unresponsive to native insulin and to its main processing product, the B:13–21 segment. In striking contrast, immunization with the B:9–23 peptide or to B:12–20 resulted in a strong response; the NOD mouse responds to insulin peptides.

Insulin Presentation in the Thymus

The issue is whether the expression of insulin in the thymus by the medullary thymic epithelial cells (mTECs) (32) represents a control mechanism for the insulin-reactive T cells. Expression of the insulin gene in the thymus is under genetic control with allelic variants that influence the extent of expression and the incidence of T1D (33–36). Specifically, a T1D susceptibility locus maps to the polymorphisms in the variable number of tandem repeat (VNTR) of the human insulin gene (33). Of note, the class III VNTRs associated with T1D resistance correlate with higher insulin expression in the human thymus, suggesting a role of thymic insulin expression in promoting tolerance (34). In the mouse, two genes encode for insulin and only one, *Ins-2*, is expressed in the thymus; its selective depletion results in accelerated diabetes (37). Following such results, we speculate that the expression of insulin by mTECs results in the presentation of the B:13–21 peptide segment, imposing a certain level of negative selection and/or regulation of these T cells. This will not happen with the T cell reactive with the B:12–20 peptide, which is not presented from insulin processing.

Examination of the B16A mouse lends credence to such an interpretation. This mouse strain was developed by Maki Nakayama in the Eisenbarth laboratory (38,39); it lacks both the *Ins-1* and *Ins-2* genes but contains a transgene under the rat insulin promoter expressing a modified *Ins-2* gene (a change of tyrosine to alanine at the 16th residue of the insulin B chain). Very telling, this mouse does not develop diabetes. The T cells to B:12–20 and to 13–21 react poorly to such a change. In contrast to the regular NOD mouse, the B16A mouse when immunized exhibits a T-cell response to insulin and to B:13–21 (30). Thus, for the B16A mouse, regular insulin is foreign, and the T cells react to it.

Table 1—Summary of the two sets of insulin-reactive CD4 T cells

Register	B:12–20	B:13–21
Sequence	SHLVEALYLVCGERG	SHLVEALYLVCGERG
Affinity	Low	High
Dissociation rate	Fast	Slow
Native insulin	No	Yes
Insulin peptides	Yes	Yes
Internal processing	Eliminated	Selected
Sites of presentation	Islet/periphery	Islets/periphery
T-cell reactivity	Strong	Minimal
Diabetogenicity	Strong	Undetermined

CD4 T cells have been identified in patients with T1D by a few laboratories; these T cells were isolated from blood and from islets (27,40–44). In T1D, the T cells were isolated at a time when the autoimmune process was in progress and likely represent not only those that initiate the process but also the sets that expand and diversify. A number of the T cells recognized the insulin B-chain segment and C-peptides, very similar to the NOD mouse, including a number that only recognize insulin peptides.

Do our findings on type B T cells have a wider application (10)? Repeated examples have been found in the literature of immunoreactivity to denatured self-proteins or peptides (45). The self/nonself discrimination paradigm applies to most proteins, but such fundamental discrimination may not hold when applied to a denatured protein. Note recent reports on the T cells in patients with narcolepsy (46) and juvenile idiopathic arthritis (47); both studies identified self-reactive T cells only recognizing peptides but not the protein of a target autoantigen. Exocytosis of peptides from intracellular catabolism of a self-protein may be a common feature in autoimmunities and particularly in endocrine autoimmunity where crinosomes contain catabolic products that, as in the β -cells, can be exocytosed.

MHC-II Epitopes Found in Crinosomes

The findings of type B T cells that recognize denatured proteins or peptides, moieties that avoid protein processing, raises two important issues. What is the source of the peptides? Where are such peptides/denatured proteins in the β -cells? And how are they presented? Many of the denatured proteins/peptides reside in the crinophagic bodies of the β -cells (48). These peptides are exocytosed, captured, and presented by local APCs in islets and peripheral lymphoid tissues since they are also released into the circulation (48). Crinophagic bodies or crinosomes were first described by Smith and Farquhar (49) in the pituitary gland, resulting from the fusion of the hormone-containing vesicle to lysosomes. Farquhar envisioned that these organelles exist to control the excessive production of hormones and thus maintain cellular homeostasis. Crinosomes have been described in all endocrine organs

and particularly in the pancreatic β -cells. In β -cells they serve to regulate the excessive production of DCGs (50).

Using monoclonal antibodies specific to the B:9–23 insulin peptide and unreactive with native insulin, we identified Lamp-1–positive vesicles that did not coreact with insulin and were compatible with the crinosomes (29). Such vesicles were separated from the DCGs by differential centrifugation, and by mass spectrometry analysis contained a number of insulin peptides (48), some of which stimulated the T cells specific to B:12–20 (51). Remarkably, many of the peptides were those that had been predicted based on immunological analysis of the T cells, both in NOD mice and in patients with T1D, including the insulin B:9–23 peptide. As analyzed below, these peptides were released after a glucose challenge and then disseminated out of the islets.

THE ISLET IMMUNE PLATFORM: CENTRAL PRESENTATION BY THE RESIDENT MACROPHAGES

We have paid considerable attention to the islet-resident macrophages in studies that were started by Boris Calderon and later expanded by others in the laboratory, particularly by Stephen Ferris and Pavel Zakharov (52–55). Islets in nondiabetic strains of mice harbor 2–10 typical macrophages per islet. Islet-resident macrophages are found from birth; lineage tracing experiments indicate that they derive from definitive hematopoiesis and self-replicate (52). Islet macrophages are normally in a high state of activation, independent of the diabetic status of the mice. They express high levels of MHC-II molecules as well as costimulatory molecules and Toll-like receptors (53). Their gene transcriptional program is similar to macrophages in the lung mucosa as well as those in the intestine, the “barrier” macrophages. We found expression of TNF as well as IL-1 β and α . Islet macrophages are strong APCs. Compared with the islets, the pancreatic stroma harbors a much higher number of macrophages, 20–30 times more (52). Most of these derive from yolk sac hematopoiesis and some from circulating monocytes. Stromal macrophages express low levels of MHC-II and are CD206 positive, a marker used to define M2-like

macrophages; they express a number of genes involved in tissue repair and homeostasis (52). Our data strongly support the findings of others showing that the tissue conditions the program of differentiation of the resident macrophages.

Islet macrophages play an important role in the early development of islets. This issue is evident in mice having a spontaneous mutation in the colony-stimulating factor 1 (CSF-1) protein. Such mice lack macrophages since birth and have a marked reduction in islet mass; the mice respond poorly to glucose challenge and develop hypoinulinemia (52,56).

In live imaging studies, the macrophages are found anchored to blood vessels and constantly throwing long filopodia that sample large areas of the islets (Fig. 2A). Importantly, occasional filopodia enter the blood lumen. Bernd Zinselmeyer, who led these studies in the laboratory, documented that the islet macrophages capture small particles from blood (55). Ultrastructural studies documented that the islet macrophages are in very close contact with the β -cells (Fig. 2B and C) and, importantly, take up the insulin-containing DCGs as well as vesicles having different luminal content, most likely from crinosomes (48,51). The granules are taken up intact, with identifiable membrane of the vesicles inside the phagocytic vesicle. This mode of uptake is far different from the canonical degranulation after a glucose challenge in which the membrane of the DCG is incorporated into the plasma membrane for releasing insulin. It resembles the process of transferring melanophores from melanocytes into keratinocytes. The electron microscopic images suggest a pinching process where two thin macrophage extensions pinch out the area containing the DCG.

These series of findings raise two important questions: 1) what causes the macrophage activation, and 2) what is

its physiological role? The macrophage activation is likely due to a variety of interactions most prominent with the insulin DCG that contains a number of bioactive molecules, such as insulin, ATP, and granins, as well as with circulating blood components. The macrophages need to integrate a number of signaling events, as reflected in their complex transcriptome (53). The role of the activated macrophage is not known, but a protective role is a logical speculation, perhaps against circulating viruses or other pathogenic moieties, such as endotoxins.

Under conditions that favor diabetic autoimmunity, i.e., genetic and environmental triggers, the islet-resident macrophage plays a major role in the initiation of the autoimmune process (54). Autoimmunity in the NOD mouse is already evident by the time of weaning, at the 3rd week of age. Our own studies indicated that at this time a percentage of the resident macrophages had an added program of activation over that of macrophages of nondiabetic strains, and coincident with the first appearance in islets of CD4 T cells to insulin. Most of the T cells entering islets were in close contact with the resident macrophages. To examine whether the macrophages were instrumental in the entrance of the T cells, we eliminated the islet-resident macrophages by injecting the mice with monoclonal antibodies to the CSF-1 receptor (CSF-1R). This led to a loss of T-cell entry and a marked reduction in diabetes incidence (54). In sum, all the evidence points to the macrophage as opening the islets to the circulating diabetogenic T cells.

THE ISLET IMMUNE PLATFORM: PRESENTATION BY PERIPHERAL LYMPHOID ORGANS

Peripheral lymph nodes have a powerful influence on the development of autoimmune diabetes. Surgical removal of pancreatic lymph nodes resulted in a marked reduction in diabetes incidence (57). The pancreatic lymph nodes

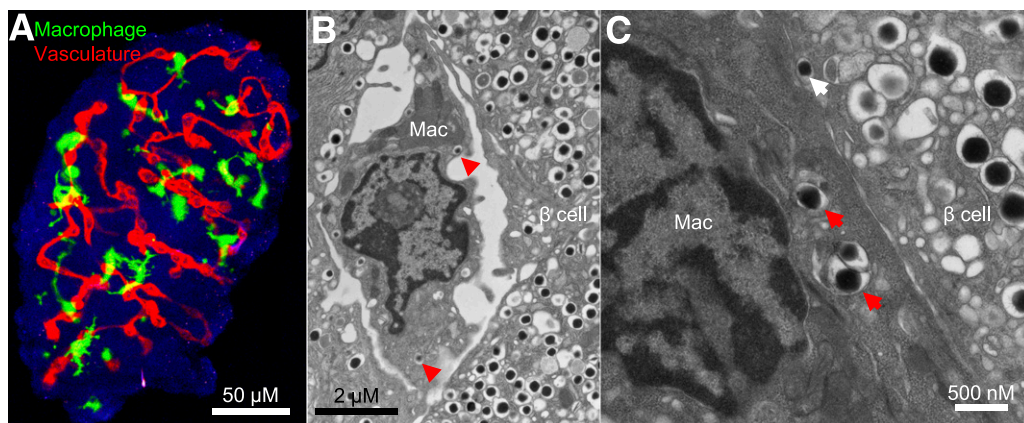


Figure 2—Microscopic studies that reveal the close interactions among the resident macrophages, the vasculatures, and the β -cells in the islets. A: An immunofluorescent image showing the positioning of the islet macrophages (green, labeled by CX₃CR1-GFP) adjacent to the intra-islet vascular structures (red). Note that some macrophages throw their filopodia into the lumen of the blood vessels. Electronic microscopy showing the intimate interactions between the islet macrophages and the β -cells in 4-week-old B6 (B) and NOD (C) mice. The red arrows indicate the insulin DCG taken up as intact by the islet macrophages; the white arrow in C may denote a granule during the passage from the β -cell to the macrophage. Mac, macrophage.

harbor antigenic material that can be detected by the proliferation of injected T cells to various CD4 and CD8 diabetogenic epitopes. In our studies, we ablated all lymphoid tissues by injecting pregnant NOD mice with lymphotoxin β -Fc fusion protein (58). Interaction of lymphotoxin- β with its receptor is required for the development of lymph nodes during embryonal life. Such a treatment resulted in total inhibition of the autoimmune process, with a complete lack of T-cell activation. Thus, lymph nodes are essential for diabetes to develop.

These two findings need to be placed in the perspective of our recent investigations in which we documented the release of a number of peptides from islet crinosomes into the circulation after a glucose challenge (48). Our studies included islets from nondiabetic mice as well as from human subjects without diabetes. This periodic seeding of peptides resulted in their presentation to insulin-reactive T cells in all peripheral lymph nodes tested. We made an estimate of the amounts of peptides in each node based on the concentration of insulin in blood before and after a glucose challenge and the relative amounts of insulin peptides released compared with insulin. Our estimate was of 2×10^{10} molecules of peptides throughout the lymphoid tissues, an amount that would lead to single digits of peptide-MHC complexes from a given APC. To be able to document the very low levels of presentation of insulin peptides in lymph nodes, it was necessary to change the approach for examining antigen presentation to a highly sensitive one; labeled T cells that recognized insulin peptides were transferred into NOD mice, and their trajectory through lymph nodes was recorded by live imaging (48). A high level of presentation results in the tight adherence of the T cells to the APCs. In contrast, a low level of presentation results in a brief contact interaction and release; it can be documented by scoring the mobility of the T cells for a period of time. This approach documented that the various lymph nodes contained insulin peptides; such an effect was not found in the B16A mouse that did not contain immunogenic insulin, a highly relevant control. The presentation of the B:12-20 epitope was not affected by administration of S961, a blocker of the insulin receptor, proving that it had to derive from free insulin peptides. Such presentation was not found in lymph nodes from C57Bl/6 mice that express a different allele of MHC-II that does not bind the insulin peptides.

The conclusion is that peripheral nodes are constantly being seeded by insulin peptides derived from the degranulation of β -cells. This results in an intermittent and low level of presentation that has biological effects on T cells that peripheralize from the thymus. We went on to examine the possible effects of such peripheral presentation. T cells to the B:12-20 peptide upon transfer into NODs circulated through the nodes interacting with the MHC-bound peptides, resulting in a gene signature of partial activation, and importantly, these T cells acquired enhanced diabetogenicity (48). This was an unexpected result that challenges some of the paradigms relating to

T-cell activation in peripheral nodes, i.e., that contact of a T cell with an APC presenting self-antigens does not result in activation unless the APC has been stimulated before. We suggest that such an interpretation needs reevaluation and that it may depend very much on the nature of the T cells, the density of the peptide-MHC complex, and the prior history of the T cells. Future studies analyzing different autoreactive T cells should provide us with a critical comparison.

SUMMARY

Islets contain all the elements required for an effective immune response to develop. First, islets normally harbor an activated resident myeloid cell (a macrophage) having all its molecular armamentarium capable of activating CD4 T cells. Moreover, such macrophages critically take up insulin DCG and crinosomes from β -cells that provide potential autoantigenic peptides. Second, β -cells are constantly exocytosing peptides derived mainly from crinosomes that disseminate throughout the lymphoid tissues. Thus, β -cells are constantly challenging the immune system by seeding the peripheral lymphoid organs. The main autoantigenic challenge is insulin simply from being the major protein of the β -cell. The finding that autoreactive peptides are being presented in lymphoid tissues imposes a number of responses of autoreactive T cells generated after thymic development. Some of these responses will result in their activation whereas others may be modulatory. The balance of these two outcomes may determine the development and progression of diabetic autoimmunity. The go/no-go decision on whether autoimmunity develops or remains dormant is the challenge for us to understand. It will obviously depend on genetic components, such as the critical MHC molecules that constitute the main propensity factor, plus local and environmental conditions, which include infections and components of the microbiomes.

Acknowledgments. The authors thank the members of our laboratory for their contributions to the work mentioned in the text.

Funding. The research work was supported by research grants from the National Institutes of Health (AI-114551, DK-058177, and DK-120340) and JDRF. The laboratory receives general support from the Kilo Diabetes & Vascular Research Foundation.

Duality of Interest. This work was supported by Janssen Pharmaceuticals. No other potential conflicts of interest relevant to this article were reported.

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