The immunoregulation of autoimmune diabetes

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ABSTRACT OF THE DISSERTATION
The Immunoregulation of Autoimmune Diabetes

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How autoimmune diseases are regulated is a long-term research topic in the autoimmunity field. We use autoimmune diabetes as a model to study this. Autoimmune diabetes is a T cell-dependent autoimmune syndrome. The functions of T cells are regulated during their development and activation. Developmentally, T cells will undergo a stringent thymic selection: a process that self-reactive T cells are tolerized to become thymic derived Tregs or can be deleted by apoptosis based on binding affinity and avidity between the TCRs and self-peptide:MHC complexes. After T cells mature, they can also be tolerized in the periphery in many other ways, such as periphery induced regulatory T cell (pTreg) development, T cell anergy, and T cell exhaustion. pTregs generated extrathymically contribute to immune regulation of a given anatomical site. T cells anergy makes the T cells intrinsically functionally inactivated upon an antigen encounter, a process occurring during T cell priming due to the absence of costimulatory signals. T cells would remain alive but stay in a hyporesponsive state. Exhausted T cells can be functionally activated and differentiated into effector cells, but
due to the constant antigen exposure and TCR activation, these T cells gradually lose
the expression of the effector molecules at the same time express a series of inhibitory
molecules that maintain a hypofunctional status. In this thesis we mainly examined the
functional changes of the exhausted CD8 T cells in islets after immune checkpoint
blockade, a condition where facilitated diabetes progression occurs. This allows us to
understand how T cell exhaustion and subsequent immunoregulation are established in
autoimmune diabetes.

Briefly, we found blockade of PD-1/PD-L1 axis but not CTLA-4 promoted
diabetes progression in non-obese diabetic (NOD) mice. Both CD4 and CD8 T cells
responded to PD-1 blockade to proliferate. This was highly dependent on T cells as
antibody depletion of CD4 or CD8 T cells rescued anti-PD-1 diabetogenic effect.
Moreover, we noticed that PD-1/PD-L1 regulation was functionally established in islets
as islet T cells were sufficient to induce diabetes upon PD-1 blockade. Exhausted CD8
T cells expanded during diabetes progression. We identified two subsets of exhausted
CD8 T cells in islets: precursor exhausted T cells and terminally differentiated
exhausted T cells. In inflamed islets without anti-PD-1 treatment, most of the CD8 T
cells were exhausted T cells and more than 70% of the exhausted CD8 T cells were
precursor exhausted T cells; however, after PD-1 blockade, these precursor exhausted
T cells differentiated into terminally differentiated exhausted T cells that expressing a
large wave of effector molecules which contributed to the acute diabetes progression.

In addition to T cells, we found that macrophage was another set of cells that
underwent dramatic changes following PD-1 blockade. After anti-PD-1 treatment,
macrophages became highly activated, acquiring elevated signatures of NF-κB
signaling and IFNγ signaling which derived from their interaction with β cells and T cells, respectively. One subset of macrophages was derived from monocyte and these monocyte-derived macrophages displayed higher pro-inflammatory activity and can be recruited by activated T cells. The activated macrophages were cytocidal to β cells through nitric oxide (NO) production. Depleting monocytes with clodronate or inhibiting NO with aminoguanidine significantly reduced the incidence of PD-1 blockade induced diabetes. Therefore, we identified a novel killing component causing acute diabetes in context of PD-1 blockade. Our findings may help design treatment regimes in cancer immunotherapy to avoid autoimmune side effects.
Chapter 1: Introduction

1.1 Type 1 diabetes

1.1.1 Type 1 diabetes development in humans

Type 1 diabetes (T1D) is a deleterious autoimmune disease that develops early in life. Around 1.25 million Americans suffer from T1D, with about 40,000 new cases diagnosed each year (Dabelea et al., 2014). During the chronic course of T1D, the host immune system attacks and destroys β-cells of the pancreatic islets, resulting in permanent insulin loss and hyperglycemia. Over time, T1D complications can affect major organs in the body, including heart, blood vessels, nerves, eyes and kidneys, which could be life-threatening. There is no cure for T1D, and supplement of insulin is the only medication to control these complications.

Genome-wide association studies (GWAS) have identified that the primary T1D-susceptibility loci are the Class II human leukocyte antigen (HLA) genes in the major histocompatibility complex (MHC) (Todd et al., 2007; Burton et al., 2007). These T1D susceptible Class II MHC (MHC-II) molecules, such as HLA-DQ8 and HLA-DQ2, display a strong link to a high disease risk due to their biochemical structural feature lacking a canonical aspartic acid residue at position beta57 of beta chain (Lee et al., 2001; Todd et al., 1987). The non-aspartic acid residue at position 57 creates a P9 anchor pocket that was shallow, wide, and positively charged and the peptides selected by these unique HLA-II molecules contained acidic-rich residues at P9 position (Suri et al., 2005; Chang and Unanue, 2009; Godkin et al., 1997). A number of islet-derived antigens containing such binding motifs can be presented by the susceptible HLA-II, generating a
CD4 T cell repertoire skewing to diabetogenic reactivity. These antigens are involved in different stages of the disease, which includes insulin (Palmer et al., 1983), glutamic acid decarboxylase 65 (GAD65) (Petersen et al., 1994; Pardini et al., 1999), IA-2 (Pardini et al., 1999), gangliosde GM2-1 (Dotta et al., 1995), islet-specific Zn transporter ZnT8 (Slc30a8) (Nayak et al., 2014).

The activated islet-specific T cells help the autoreactive B cells, which generated islet cell autoantibodies before hyperglycemia. These autoantibodies have been used as reliable serological markers to predict the risk of T1D (Taplin and Barker, 2008). Moreover, antigen-specific T cells directly infiltrate into the islets and mediate the autoimmune attack (Willcox et al., 2009). In sum, the biochemical uniqueness of HLA-II provides an immunological foundation for the development of T1D.

1.1.2 A mouse model to study T1D

T1D is experimentally examined by the NOD mouse model which shares important features with the human disease. Importantly, the MHC class II molecule I-A\(^q\) expressed in the NOD mouse is structurally homologous to the human HLA-DQ8 and HLA-DQ2: it also contains a non-aspartic acid at the beta chain 57\(^{th}\) position, which makes it to select similar peptidomes shared with human HLA-DQ8 molecule (Suri et al., 2005, 2002). As a consequence, the NOD mice spontaneously develop diabetes due to the immunological attack of islet \(\beta\) cells. A single genetic correction of non-aspartic acid (serine) at beta57 to aspartic acid of I-A\(^q\) results in complete protection from autoimmune diabetes in NOD mice (Gioia et al., 2019).

Several key immunological features found in human T1D are recapitulated in the NOD mouse: 1) the early appearance of anti-islet antibodies in the serum with predictive
values of disease onset (Yu et al., 2000); 2) the local invasion of CD4 and CD8 T cells recognizing beta-cell antigens, forming a characteristic lesion in the islets, termed insulitis (Graham et al., 2012); 3) the chronic disease progression due to the effects of regulatory mechanisms to counter the pathogenic elements (Jeker et al., 2012); and 4) the persistence of autoreactive T cell responses post beta-cell destruction, leading to rapid rejection of islet transplants into already diabetic individuals (Burrack et al., 2017). All these valuable traits allow for examining immunological events taking place naturally for diabetes initiation and progression, which can be hardly achieved by studying human patients.

1.1.3 Insulin reactivity as the main driver of T1D

Similar with many other T cell-dependent autoimmune diseases, the autoimmune process in NOD mice is initiated by a single self-peptide followed intra- and intermolecular epitope spreading. A series of studies demonstrate insulin as the main antigen that drives the initial islet-specific immune responses.

First, insulin expression is β-cell specific and is more abundantly expressed compared with other islet antigens. Second, the levels of thymic insulin mRNA expression correlate with the risk of T1D, presumably due to impairment in negative selection of the insulin-specific T cells in the thymus (Snyder et al., 1997). Third, overexpression of proinsulin in the thymus and peripheral tissues under the promoter of MHC class II protects NOD mice from autoimmune diabetes (French et al., 1997). In agreement with this, proinsulin 2 (expressed in thymus) knockout mice develop accelerated insulitis and diabetes (Thébault-baumont et al., 2003). Fourth, anti-insulin T cells have been isolated from islets from humans with T1D and NOD mice and a major
portion of established T cell clones isolated from islets are insulin specific (Wegmann et al., 1994).

1.1.4 Insulin-specific T cells

The Wegmann group first described a series of islet-specific T cells lines from 7- or 12-week-old pre-diabetic NOD mice (Wegmann et al., 1994). 24 out of 54 T cell clones responded to native insulin protein. Almost all those insulin-specific T cell clones specifically recognized the 9-23 segment of the insulin B chain (Daniel et al., 1995). Substitution of the tyrosine at the 16th position with alanine (Y to A) abrogated the reactivity of these T cell clones in vitro (Abiru et al., 2000). A transgenic mice generated to express this Y16A mutant proinsulin gene only (native Ins1 and Ins2 gene were deleted, termed as NOD.B16A mouse) were free of insulitis and diabetes (Nakayama et al., 2005). These studies definitively showed insulin B:9-23 is the main epitope driving autoimmune diabetes development. Aside from B:9-23, Boitard group identified other insulin epitopes such as leader sequence, A chain and C-peptide of both preproinsulin I and preproinsulin II (Halbout et al., 2002).

Later studies including ours further explored the potential immunological registers within B:9-23 segments. We have identified two types of T cells targeting two overlapping peptides in B:9-23 segments: B:12-20 and B:13-21 (Mohan et al., 2010, 2011; Levisetti et al., 2007). The two registers are processed and presented by APC through different cellular pathways, which in turn activates the corresponding T cells. This issue will be discussed in detail in a following section. This observation reinforces the concept of the presentation to type A and type B T cells as we described previously (Peterson et al., 1999).
1.2 Type A and Type B T cells

1.2.1 Definition of type A and type B T cells

The conventional antigen presentation starts when the antigen presenting cells (APCs) take up and internalize protein antigens into the endocytic vesicles. Within the late endosomal or lysosomal compartment of the cell, the proteins are degraded and catabolized into peptides which are then loaded onto MHC class II molecules. The MHC class II molecules are synthetized in the endoplasmic reticulum (ER) which is full of unfolded or partially folded polypeptide chains. In ER, the MHC-II molecules are prevented from binding to peptides because the MHC class II-associated invariant chain (CD74) protein is folded on their combining site. The MHC class II molecules together with the invariant chain are transferred to a late lysosomal vesicle called the MHC class II compartment (MIIC). In MIIC the invariant chain dissociates from the MHC-II allowing other peptides to bind MHC class II. The chaperone protein H2-DM assists the peptide binding process in late endosomes or lysosomes while another protein H2-DO binds in the same location to H2-DM as MHC class II molecules bind, thereby preventing H2-DM from binding to MHC class II molecules and inhibiting the peptide exchange mediated by H2-DM. This finely tuned system allows stable peptide registers to be presented by MHC class II molecule.

In contrast to conventional peptide presentation, the denatured exogenous proteins or peptides are not taken into the deep vesicles where H2-DM and H2-DO are located. In recycling vesicles, the denatured proteins or peptides bind to MHC class II by peptide exchange. Thus, both high and low affinity registers can bind, albeit low
affinity ones turn over at a faster rate. A broader diversity of antigen presentation is achieved.

CD4+ T cells that recognize peptides processed through the conventional presentation pathway are defined as type A T cells whereas CD4+ T cells that recognize exogenous peptides presented in the unconventional way are defined as type B T cells.

1.2.2 Insulin-specific type A and type B T cells

Here I summarize our recent findings on insulin specific type A and type B presentation. Based on the structural features of I-A\textsuperscript{g7}, insulin B: 9-23 segment is predicted to contain two favorable binding core segments ending with either P20Gly (small amino acid) or P21Glu (acid-residue), which corresponds to 12-20: VEALYLVCG and 13-21: EALYLVCGE, respectively. These two registers independently bind to MHC class II molecules and shifting between the two can take place (Levisetti et al., 2007).

To further characterize the insulin-specific T cell response, we immunized NOD mice with the B:9-23 peptides emulsified in complete Freund’s adjuvant (CFA) and generated T cell hybridomas specific to this peptide (Mohan et al., 2010). Two types of T cells were identified: one set reacted with both insulin protein and the 9-23 peptide (type A), and the other only recognized the 9-23 peptide (type B) (Mohan et al., 2010). To further test the precise epitopes that can be recognized by the type A or type B T cells, DNA constructs containing the B:12-20 or B:13-21 segment covalently bound to I-A\textsuperscript{g7} beta chain were made. Register 2: 13-21 segment linked to I-A\textsuperscript{g7} was exclusively recognized by type A T cells, while type B T cells exclusively recognized the register 1: 12-20 segment bound with I-A\textsuperscript{g7} molecules (Mohan et al., 2011). This demonstrates that intact
insulin protein is internalized and processed to present B:13-21 by default whereas denatured insulin or free B chain peptides give rise to both B:13-21 and B:12-20 epitopes.

1.2.3 Pathogenicity of insulin-specific type A and type B T cells

During T1D progression in NOD mice, type B T cell response is dominant. Supporting this, immunization experiments showed that most of the T cell responses were directed to free insulin peptides, whereas conformational insulin induced little reactivity. Furthermore, insulin type B T cells were shown to be pathogenic and caused diabetes. Primary T cell clones induced insulitis and diabetes when transferred into the lymphopenic (NOD.SCID) mice whereas type A primary T cells did not (Mohan et al., 2010). Type B TCR transgenic mice displayed a high level of islet lymphocyte infiltration at an early age and developed rapid diabetes when they were crossed onto a RAG1-deficient background (Mohan et al., 2013). However, dispersed islets can present to either type A or type B insulin-specific T cell hybridomas because B:9-23 peptide containing secretory vesicles could be transferred to the intra-islet macrophages.

1.3 The regulation of autoimmune diabetes

1.3.1 The thymic regulation of insulin autoreactive T cells

To explain why type A and type B insulin specific T cells are different in their pathogenicity, the development and regulation of the two types of T cells should be considered. In NOD mice, the development of the type A insulin-specific T cell is regulated by the thymic epithelial cells (mTECs), a feature distinct from the type B T cells. These unique stromal cell population expresses genes encoding peripheral tissue proteins, a process under control of a transcriptional regulatory protein termed
autoimmune regulator (Aire). The tissue restricted antigens are therefore presented by mTECs to the thymocytes. Autoreactive T cells harboring high affinity TCRs against tissue restricted antigens can either be negatively selected or become tolerant, as a result, such T cells will not cause autoimmune. Insulin is expressed in mTECs under control of Aire. As mTECs process insulin protein, B:13-21 register should be presented to type A insulin-specific T cells; however, type B insulin-specific T cells can escape negative selection and peripheralize. When APCs encounter and present denatured insulin or free insulin peptides in the secretory granules in the islets, type B T cells can be activated, leading to the development of islet autoimmunity.

This hypothesis is supported by experiments using NOD.B16A mice (Mohan et al., 2011). The B16A insulin mutant does not activate B:9-23 specific T cells, both type A and type B presentation is abolished. In striking contrast to regular NOD mice, when NOD.B16A mice were immunized with the B:9-23 peptide, responses to13-21 can be found. These results indicate the exogenous peptide-specific T cell responses may play an unprecedented role in autoimmune type 1 diabetes. However, how insulin specific type A and type B cells are controlled during their development in thymus remains to be elusive.

1.3.2 The peripheral regulation of autoreactive T cells

Regulatory T cells

Regulatory T cells (Tregs) play a critical role in periphery immune tolerance. Treg-mediated suppression is a key regulatory mechanism of NOD mice and humans with T1D. It is well-documented that Treg depletion by anti-CD25 antibody accelerated the development of autoimmune diabetes (Mellanby et al., 2007; Marino et al., 2009).
NOD mice that lack Tregs due to Foxp3 knockout displayed rapid diabetes progression (Chen et al., 2005). Islet specific TCR transgenic mice (BDC2.5 and 8F10) developed diabetes only when they were crossed onto Rag1−/− background by which Treg development was abolished (Mohan et al., 2013). Although in NOD mice and T1D patients Treg cells are not deficient, the functionality of the Tregs is abnormal compared with healthy counterparts. In NOD mice, the islet Tregs express decreased levels of the IL-2 high affinity receptor CD25 and the survival factor Bcl2 (Tang et al., 2008). In T1D humans, the Treg functional marker GITR is expressed at a lower level (Xufré et al., 2013).

Treg-based therapeutic strategies have demonstrated notable efficacy in treating T1D. Treating NOD mice with low dose IL-2 increased Treg numbers and function, and therefore reversed the diabetes progression (Tang et al., 2008; Grinberg-Bleyer et al., 2010). Fc receptor-nonbinding anti-CD3 monoclonal antibody (teplizumab) has been tested in multiple clinical trials, and showed a role in stabilizing and preserving Treg functions (Skyler, 2013; Keymeulen et al., 2010; Herold et al., 2002, 2013; Penaranda et al., 2011). In a recent phase 2 trial, an early single course of teplizumab significantly slowed the disease progression in high-risk nondiabetic relatives of the patients with T1D (Herold et al., 2019). Aside from pharmacological-based therapies, cell-based therapies also suggested a significant role of Treg in controlling autoimmune diabetes. Repeated Treg adoptive transfer into neonatal NOD mice delayed the onset of diabetes (Wu et al., 2002; Tarbell et al., 2007). Similarly, clinical trials are testing in vitro expansion and adoptive transfer of Tregs to T1D patients (Marek-Trzonkowska et al.,
2012, 2014). In sum, a great body of studies have focused on examining peripheral
tolerance induction in autoimmune diabetes.

**Immune checkpoint regulation**

T cells are intrinsically regulated when activated. Upon TCR engagement by
peptide-MHC complexes, naïve T cells differentiate into effector cells capable of
secreting cytokines and cytotoxins; meanwhile, regulatory mechanisms are also set up
to fine-tune the effector functions of the T cells. One of such regulatory mechanisms are
mediated by checkpoint molecules. Two of the most studied checkpoint proteins are
cytotoxic T lymphocyte antigen 4 (CTLA-4) and Programmed Death 1 (PD-1). Drugs
that block these checkpoint proteins have been developed and can unleash the
regulatory control of the T cells allowing them to display their full functional potential.

CTLA4 down-modulates T cell priming by inhibiting co-stimulation signaling by
competing the CD28-B7 interactions. T cell express CD28, which engages the B7
molecules expressed by APCs, resulting in amplification of TCR signaling. This process
is required for the maturation of naïve T cells into effector T cells. However, CTLA-4 has
a higher affinity for B7 compared with CD28, and one molecule of CTLA-4 could bind
two molecules of B7 proteins (Linsley et al., 1994), which out-competes CD28 binding
with B7 and depletes B7 via “trans-endocytosis” (Qureshi et al., 2011). It has been
widely accepted that CTLA-4 plays an important role in T regulatory (Treg) cells to elicit
the suppressive functions (Wing et al., 2008); During initial T cell activation, CTLA-4 is
rapidly mobilized to the surface from intracellular protein stores. Thus, CTLA-4 controls
T cell activity primarily in secondary lymphoid tissues.
PD-1, similar with CTLA-4, is not expressed by naïve T cells, but is synthesized de novo after TCR engagement. PD-1 is mainly expressed by activated T cells. Upon binding with its ligand, PD-L1, PD-1 forms clusters with TCRs and transiently recruits the Src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2) (Yokosuka et al., 2012), possibly via its immunoreceptor tyrosine inhibitory motif (ITIM) and immunoreceptor tyrosine switch motif (ITSM) (Topalian et al., 2015; Okazaki et al., 2001). SHP-2 dephosphorylates key signal transducers of TCR signaling, thereby inhibiting the downstream effector gene expression. Whereas PD-1 expression is rapidly up-regulated during T cell activation, persistent exposure of the antigens to the T cells leads to sustained TCR stimulation, which results in substantially higher expression of PD-1 and the establishment of T cell exhaustion. Different from CTLA-4, PD-1 primarily exerts its function in already activated effector T cells, which normally reside in tissues, where its ligand, PD-L1, is widely expressed on various types of the cells, such as hematopoietic cells including T cells, B cells, macrophages, dendritic cells (DCs), and mast cells, and non-hematopoietic cells including vascular endothelial cells, keratinocytes, pancreatic islet cells and epithelial cells (Sun et al., 2018).

Immune checkpoint inhibitors targeting CTLA-4, PD-1, and PD-L1 have been widely studied and applied in treatment of a wide range of cancers. These treatments have achieved great successes clinically and several of them have been approved by FDA to treat cancer patients. While dramatically expanding the lifespan of the patients who are suffering from malignancies, these checkpoint inhibitors can cause immune-related adverse events (irAEs). Surprisingly, a lot of irAEs target endocrine tissues such as the pituitary gland, thyroid gland, adrenal glands, and pancreatic islets resulting in
endocrine autoimmune diseases (Sznol et al., 2017). A better understanding of how these life-saving drugs lead to autoimmune responses would be invaluable to avoid the adverse effects of anti-tumor immunotherapies.

There are increasing case reports from clinical trials using checkpoint blockade (anti-PD-1, anti-PD-L1, and anti-CTLA-4) showing that a handful, although less than 1% of cancer patients developed T1D after the treatment (Chae et al., 2017; Miyoshi et al., 2016; Godwin et al., 2017; Chokr et al., 2018; Way et al., 2017; Kapke et al., 2017; Hughes et al., 2015; Mellati et al., 2015; Zaied et al., 2018; Araújo et al., 2017). Usually, these patients had no history of T1D but developed the disease upon anti-PD-1 or anti-PD-L1 immunotherapy. In contrast to anti-PD-1, there are no cases of T1D in patients treated with anti-CTLA-4 monotherapy (Stamatouli et al., 2018). Very surprisingly, the patients developed fulminant onset of T1D shortly after the start of the immune checkpoint blockade, usually within 1 or 2 weeks to a few months. In many patients, islet-specific B cell responses did not develop, evidenced by the lack of autoantibodies against islet antigens (Zaied et al., 2018; Stamatouli et al., 2018).

Before the clinical studies of PD-1 inhibitors, PD-1 knockout mice were generated to study autoimmune diseases. C57BL/6 mice lacking the Pdcd1 gene spontaneously developed lupus-like proliferative arthritis and glomerulonephritis (Nishimura et al., 1999). PD-1 null BALB/c mice have dilated cardiomyopathy, which may cause fetal heart failure (Nishimura et al., 2001). The PD-1-PD-L1 axis is also reported to regulate the progression of autoimmune diabetes in NOD) mice. PD-1 knockout NOD mice or PD-1 blocking antibody treated NOD mice developed accelerated autoimmune diabetes (Wang et al., 2005; Pauken et al., 2013, 2015;
Okamura et al., 2019). Both autoreactive CD4 and CD8 T cells can respond to PD-1-PD-L1 blockade (Pauken et al., 2013, 2015; Okamura et al., 2019) and contribute to the acute diabetes development. The PD-L1 expressed by the islet parenchymal cells (Osum et al., 2018; Keir et al., 2006) is shown to limit the T cell function in the islets and mediate the peripheral T cell tolerance. A recent study by the Fiorina group developed a novel therapeutic approach by transplanting hematopoietic stem and progenitor cells (HSPC) with genetic overexpression or pharmacological restoration of PD-L1 (Nasr et al., 2017), and showed a role of hematopoietic cells in regulating autoimmune response via PD-1-PD-L1 axis.

These studies demonstrate that pathogenic T cells involved in T1D progression are tightly controlled by the PD-1/PD-L1 signaling in the periphery. The underlying mechanisms are not known and whether PD-1 plays an important role in regulating insulin specific T cells needs to be addressed. Also, urgent studies are required to identify promising biomarkers for the prediction of T1D progression in patients received cancer immunotherapies.
Chapter 2: Cytocidal macrophages in symbiosis with CD4 and CD8 T cells cause acute diabetes following checkpoint blockade of PD-1 in NOD mice

Chapter 2 is rewritten from the publication derived from this thesis. The paper has been published in Proc. Natl. Acad. Sci. USA on November 23, 2020 by Hao Hu, Pavel N. Zakharov, Orion J. Peterson, Emil R. Unanue.

2.1 Abstract

Autoimmune diabetes is one of the complications resulting from checkpoint blockade immunotherapy in cancer patients, yet the underlying mechanisms for such an adverse effect are not well understood. Leveraging the diabetes susceptible NOD mouse model, we identified a cascade of highly interdependent cellular interactions involving diabetogenic CD4 and CD8 T cells and macrophages. We demonstrated that exhausted CD8 T cells were the major cells that respond to PD-1 blockade and produce high levels of IFNγ. Most importantly, the activated T cells led to the recruitment of monocyte-derived macrophages that became highly activated in response to IFNγ. These monocyte-derived macrophages acquired cytocidal activity against β cells via nitric oxide and induced autoimmune diabetes. Collectively, this study reveals a critical role of macrophages in the PD-1 blockade induced diabetogenesis, providing new insights for the understanding of checkpoint blockade immunotherapy in cancer and infectious diseases.
2.2 Introduction

Immune checkpoint blockade (ICB) therapies are now approved for the treatment of various malignant cancers (Wei et al., 2018). Inhibitors to PD-1/PD-L1 and CTLA-4 are the most commonly used and studied ICB therapies, which results in T cell activation through removing the inhibitory signalings in cells. The current mechanistic model indicates that CTLA-4 dampens TCR signaling by competing with CD28 (a key co-stimulatory molecule) for the B7 ligands (CD80 or CD86) expressed on APCs in lymph nodes (Linsley et al., 1994). Thus, CTLA-4 blockade is thought to take effect during T cell priming stage. PD-1 is highly up-regulated induced by signal transduction via antigen receptors (TCR or BCRs) in T cells and B cells (Agata et al., 1996). In acute antigen exposure, PD-1 is transiently upregulated and then downregulated along with antigen clearance. However, in persistent antigen exposure such as in chronic viral infection and tumor settings, PD-1 is consistently highly expressed and results in T cell exhaustion. On the other side, the ligand of PD-1, PD-L1 (here we mainly discuss about PD-L1) is widely expressed in periphery tissues in response to pro-inflammatory cytokines such as IFNγ (Keir et al., 2006; Juneja et al., 2017). Thus, PD-1/PD-L1 regulation is generally thought to be established after T cells are primed and differentiated. However, the spatiotemporal regulation of PD-1/PD-L1 is less clear. In a Lymphocytic choriomeningitis virus (LCMV) infection model, upon systemic dissemination of the virus, PD-1 is transiently up-regulated in naïve T cells via recognition of cognate antigens during T cells priming in spleen, negatively modulating naïve-to-effector differentiation (Ahn et al., 2018).
Emerging evidence show that ICB treatment induces immune-related adverse events (irAEs) in cancer patients, resulting in autoimmune diseases such as autoimmune myocarditis, hepatitis, colitis, thyroiditis, and diabetes (Postow et al., 2018). These serious immune-related side effects hinder the application of ICB in clinic. Understanding the distinctive mechanisms of anti-tumor effects as well as autoimmune responses is critical for the development of new therapeutics. Intensive studies have been performed to delineate how anti-PD-1/PD-L1 or anti-CTLA-4 works in anti-tumor responses. Anti-PD-1 induces the expansion of exhausted CD8 T cells whereas anti-CTLA-4 promotes the expansion of ICOS+ Th1-like CD4 effector cells as well as exhausted CD8 T cells in tumor (Wei et al., 2017). The combination of the two has additive effects but still keeps its unique cellular responses (Wei et al., 2019). Another mechanism by which anti-CTLA-4 induces tumor rejection is via Treg depletion (Simpson et al., 2013; Selby et al., 2013). Evidence indicates that the effect of PD-1/PD-L1 blockade is positively correlated with tumor infiltrating CD8 T cells and PD-L1 expression by tumor and tumor-infiltrating immune cells (Tumeh et al., 2014; Herbst et al., 2014), suggesting that PD-1 regulation is established in situ. Therefore, methods to increase the T cell infiltration in tumor microenvironment augments anti-tumor responses of PD-1/PD-L1 blockade (Tang et al., 2016).

With regard to autoimmune disease, germline CTLA-4 deletion is lethal but deletion of CTLA-4 in mice during adulthood exacerbates collagen-induced arthritis but confers protection from experimental autoimmune encephalomyelitis (EAE) (Klocke et al., 2016; Paterson et al., 2015). This is due to that CTLA-4 deletion affects both conventional CD4 T cells and regulatory CD4 T cells. On the other hand, PD-1 signaling
deficiency or blockade in mice would result in several different autoimmune outcomes including lupus-like proliferative arthritis and glomerulonephritis in C57BL/6 mice (Nishimura et al., 1999), dilated cardiomyopathy in BALB/c mice (Nishimura et al., 2001) and autoimmune diabetes in Non-obese diabetic (NOD) mice (Wang et al., 2005; Guleria et al., 2007; Ansari et al., 2003; Hu et al., 2020).

A comprehensive transcriptional analysis such as single cell RNA sequencing (scRNA-seq) analysis will unbiasedly help us detect the populational and transcriptional changes under various conditions. In our recent paper, we utilized scRNA-seq to examine the changes in the islets of NOD mice at various stages during autoimmune diabetes development. We found that islets at the initial stage contained various effector and regulatory CD4 and CD8 T cells, which was followed by an apparent control phase, with the expansion of nonpathogenic and regulatory populations. In control phase (NOD mice at 8-12 weeks of age), a drastic increase of exhausted CD8 T cells can be observed, indicating the establishment of PD-1 regulation. Myeloid cells especially macrophages underwent a transition from homeostatic status to a pathogenic pro-inflammatory status after a putative two-step program of activation from 3 weeks of age to 15 weeks of age.

To better understand how PD-1 is regulating T1D, we will examine changes in various cellular components following PD-1 blockade by scRNA-seq. In summary, we identified a previously unexplored islet macrophage population derived from monocytes with high pro-inflammatory activity. In the islets of anti-PD-1 treated mice, the infiltration by monocyte-derived macrophage was under the influence of CD4 and particularly CD8 T cells. The CD8 T cells largely comprised the precursor exhausted T (T_{PEX}) cells that
were activated and differentiated to produce abundant IFNγ in response to PD-1 blockade, which in turn, activated the infiltrated monocyte-derived macrophages to promote diabetes progression. The anti-PD-1 induced development of acute diabetes was reduced by restricting the infiltration and function of such monocyte-derived macrophage in islets. Our study establishes that the myeloid cell compartment is an indispensable component of PD-1 regulation in autoimmune diabetes. Our study provides a novel cellular target- the monocyte-derived macrophage- that may minimize the adverse effects of checkpoint blockade immunotherapy.

2.3 Results

2.3.1 Rapid autoimmune diabetes develops after immune checkpoint blockade of the PD-L1/PD-1 pathway

To test whether NOD mice can phenocopy the fulminant diabetes observed in cancer patients received PD-1 checkpoint blockade immunotherapy. We evaluated the effects of anti-PD-1 treatment in NOD mice, especially during the early phase of the autoimmune process that starts at about the third week of age, the time that initial T cells are present in islets, mostly in contact with the resident macrophages (Carrero et al., 2017; Mohan et al., 2010). NOD mice of 6-8 weeks of age treated with anti-PD-1 developed diabetes within 2 weeks whereas control mice naturally became diabetic starting at around 20 weeks of age (Fig. 1A), consistent with previous reports (Nakayama et al., 2005; Ansari et al., 2003). By histological examination, the islets of the mice treated with anti-PD-1 showed marked leukocytes infiltration compared to the non-diabetic untreated mice (Fig. 1B). Younger mice of 3-5 weeks of age that have a limited number of infiltrating lymphocytes took longer to develop diabetes in contrast to
the 8-12-week-old mice (Fig. 2A), indicating a positive correlation between diabetogenicity of PD-1 blockade and the level of islet leukocyte infiltration.

Autoimmune diabetes in NOD shows gender bias, in our colony only 25% of males develop diabetes, as opposed to ~80% incidence among females. Anti-PD-1 treatment of 6-10-week-old mice abolished this gender difference with both sexes equally developing diabetes (Fig. 2B). Finally, to note is that around 20% of the NOD female mice in our colony do not develop diabetes when followed to 40 weeks of age. The 40-week-old non-diabetic NOD female mice that received anti-PD-1 acutely became diabetic even with only 1 injection of the antibody (Fig. 2C). Thus, PD-1 regulation explains part of the gender bias as well as the long-term resistance of many NOD mice to diabetes.

Islet resident macrophages, infiltrating dendritic cells, and CD45 negative cells including the endocrine cells (mostly β cells) and the endothelium, all expressed high levels of PD-L1 (Fig. 1C). Blockade of PD-L1 by anti-PD-L1 antibody also resulted in rapid diabetes development (Fig. 1D). However, CTLA-4 blockade failed to promote diabetes progression in adult mice (Fig. 1D, E), indicating that autoimmune diabetes was mainly controlled by PD-1/PD-L1 axis. In sum we confirmed that the PD-L1-PD-1 interactions had a profound controlling effect in autoimmune diabetes even from the early stages and extending to late stages.

2.3.2 Anti-PD-1 treatment enhances islet infiltration by CD4 and CD8 T cells- both are required for diabetes development

We evaluated the cellular response in islets from 6-week-old NODs following treatment with anti-PD-1 or isotype control antibody. Mice were euthanized one day
after the last treatment; islets were isolated, and their cellular infiltrates were analyzed by flow cytometry. Both CD4 T cells and CD8 T cells numbers increased in anti-PD-1 treated mice compared with control mice (Fig. 1F, Fig. 2D): the CD4 T cells increased about 4-fold but CD8 T cells had a more dramatic increase, about 40-fold, resulting in a sharp increase of CD8 T cell to CD4 T cell ratio (Fig. 1F, G, Fig. 2D). Of note, the level of CD8 T cells was always half of that of CD4 T cells in islets from mice with spontaneous diabetes even at later stage. The majority of both CD4 T cells and CD8 T cells entering the islets expressed high levels of PD-1 at steady state in contrast to the T cells in the pancreatic lymph nodes (pLN) and spleens (Fig. 1H, Fig. 2E). Intra-islet T cells displayed considerable cell division both in anti-PD-1 treated mice as well as in the controls assessed by BrdU incorporation (Fig. 1I, Fig. 2F). However, in anti-PD-1 treated mice, both CD4 T cells and CD8 T cells incorporated more BrdU during the 7-day treatment than in control mice, showing higher proliferative response (Fig. 1I, Fig. 2F).

CD4 T cells initiate the autoimmune responses in NOD diabetes followed by the involvement of CD8 T cells, both cells cooperate to promote the progression of diabetes (Miller et al., 1988; Bendelac et al., 1987). Both CD4 and CD8 T cells also were under PD-1 regulation: treatments that affected either CD4 T cells or CD8 T cells resulted in the loss of the diabetogenic effect of PD-1 blockade. For CD8 T cells, anti-CD8α (clone YTS169.4) reduced the CD8 T cell numbers in blood and pancreatic lymph nodes (Fig. 2G) and completely abolished the diabetogenic effect of PD-1 blockade (Fig. 1J). For CD4 T cells, treatment with a non-depleting clone YTS177 which inactivates CD4 T cell function, resulted in a complete protection from anti-PD-1 induced diabetes (Fig. 1K).
Depletion or inactivation of either CD8 T cells or CD4 T cells reduced the leukocytes infiltration of the islets (Fig. 2G). The ratio of intra-islet CD8 T cells versus CD4 T cells was also altered significantly (Fig. 2H). B cell response was not affected in anti-PD-1 induced acute diabetes. Anti-insulin autoantibody titers did not change neither was there an increase in T follicular helper cells in secondary lymphoid tissues (Fig. 2I, J). In sum, the numbers of both islet CD4 T cells and CD8 T cells were upregulated by anti-PD-1, re-emphasizing the importance of both MHC class II and class I presentation in NOD diabetes development. Lacking either arm of the T cell immunity affected the autoimmune responses against β cells.

2.3.3 Intraislet T cells are directly targeted by PD-1 blockade

Due to the fact that islet T cells expressed significant higher level of PD-1 compared with those from outside of the islets, we hypothesized that intraislet T cells were the main target of anti-PD-1. We tested this with two distinct ways.

First, we utilized FTY720, a small molecule agonist of sphigosine 1-phosphate (S1P) to block the trafficking of T cells from secondary lymphoid tissues to organs such as islets (Cartier and Hla, 2019). Fingolimod (FTY720) has been approved by the US Food and Drug Administration (FDA) for the treatment of multiple sclerosis (MS) (Cartier and Hla, 2019). It also shows promising therapeutic efficiency in preventing autoimmune diabetes in NOD mice (Maki et al., 2002; Penaranda et al., 2010). 6-week-old NOD mice were treated one day prior of first dose of anti-PD-1. Lymphocytes especially T cells in blood were significantly reduced with FTY720 treatment (Fig. 3A). We kept treating the mice with FTY720 every day and blood lymphocytes were measured every 3-5 days to ensure the efficacy of the FTY720 for 20 days while we monitored diabetes.
development (Fig. 3B). As a result of preventing entry of T cells into islets, anti-PD-1 failed to induce acute diabetes progression (Fig. 3C). We isolated the non-diabetic mice treated with FTY720 and found low level of leukocyte infiltration as expected (Fig. 3D, upper). However, we found the percentage of CD8 T cells was similar with that of CD4 T cells in islets, a phenomenon observed in mice treated with anti-PD-1 alone (Fig. 3D, lower), even though the mice were not diabetic. This indicated that the very few intraislet CD8 T cells directly responded to anti-PD-1 and proliferated to the level of CD4 T cells.

In the condition which we treated 12-week-old mice, an age when T cells are heavily infiltrated into islets, with FTY720 and anti-PD-1, even though FTY720 dramatically restricted the trafficking of the T cells into islets (Fig. 3E, F), mice were still progressing to become diabetic (Fig. 3G), with islets inflamed with leukocytes, no different from anti-PD-1 alone treated mice (Fig. 3H). These data showed intraislet T cells were sufficient to induce diabetes in response to PD-1 blockade.

Secondly, we depleted islet resident macrophages to further testify this hypothesis. Macrophages are the sole type of leukocytes residing islets, closely interacting with β cells and blood circulation (Calderon et al., 2015; Zinselmeyer et al., 2018). In NOD mice, macrophages were the beacons attracting β cell-specific T cells into the islets. High MHC class II molecules expressed by resident macrophages activated insulin reactive CD4 T cells and this initiated the whole process of autoimmunity in islets (Calderon et al., 2008; Unanue et al., 2016). We showed previously that depleting the islet resident macrophages at early age significantly reduced the entrance of the T cells into pancreatic islets and the incidence of diabetes
Thus, based on the observation we showed above, we speculated that depleting islet resident macrophages with anti-CSF1R (AFS98) would impair the diabetogenicity of PD-1 blockade. We tested this at early and late stage of the diabetes in NOD mice.

For early stage, 3-week-old NOD mice were treated with 2mg of AFS98 or control IgG2a for a week and then treated with 250μg for a second time. At 5-week-old, two groups of mice were treated with anti-PD-1 along with AFS98 or control antibody for three doses. Diabetes was followed and we found AFS98 treated mice were well protected from PD-1 blockade induced diabetes (Fig. 4A). Flow cytometry analysis was performed on isolated islets from the two groups of the mice. We found AFS98 treated mice showed significantly lower leukocyte infiltration compared with anti-PD-1 alone treated mice (Fig. 4B). Histology of the pancreas was performed and showed that AFS98 treated mice contained a large portion of intact healthy islets (Fig. 4C). Nearly a half of the islets in AFS98 plus anti-PD-1 group were healthy (score=0) whereas 40% of the islets in control IgG plus anti-PD-1 were massively infiltrated with leukocytes (score=4) (Fig. 4D). These data confirmed that targeting islet resident macrophages to reduce the entry of T cells can restrain the induction of the diabetes by PD-1 blockade in NOD mice.

We then tested this at late stage of the disease. 10-week-old NOD mice were treated with 2mg of AFS98 or IgG2a for a week and islets were isolated for examination. AFS98 depleted islet resident macrophages completely as shown by flow cytometry that F4/80+ Ly6C- islet resident macrophages were completely absent after AFS98 treatment (Fig. 4E, F). However, the newly recruited monocyte-derived macrophages
were not affected by AFS98 treatment (Fig. 4E, F). (We will show later in this chapter in detail that anti-PD-1 mobilized the monocytes entering islets and became highly pro-inflammatory, which played a key role in promoting diabetes progression in the context of anti-PD-1 treatment.) Similarly, both T cells (CD4 and CD8) and dendritic cells were not impaired by AFS98 treatment. As a result, AFS98 failed to protect diabetes from PD-1 blockade and mice acutely became diabetic within a week (Fig. 4G). This further indicated that T cells were required to enter the islets to be targeted by anti-PD-1 to induce diabetes.

2.3.4 ScRNA-seq analysis of islet infiltrating leukocytes in treated mice

As we showed above that anti-PD-1 mainly targeted lymphocytes in islets. Therefore, to better understand the cellular mechanisms of PD-1 immunoregulation in the islets, we performed scRNA-seq analysis of islet infiltrating leukocytes after anti-PD-1 treatment. 8-week-old NOD female mice were used for the experiments because we found that this is the age when extensive immunoregulations are set up in islets. Islet infiltrating leukocytes were isolated from the untreated NOD and anti-PD-1 treated NOD mice by flow cytometry sorting one day after the last injection of anti-PD-1 (see figure legend for the treatment protocol) (Fig. 5A). The cells were subjected to 10X Genomics single cell pipeline barcoding, library preparation and sequencing. A graph-based clustering was plotted to identify transcriptionally different leukocyte populations. In agreement with our recent study (Zakharov et al., 2020), clustering analysis revealed five major sets of leukocytes in both control and anti-PD-1 treated mice based on the expression of hallmark genes: T cells (both CD4 and CD8), B cells, conventional dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), macrophages. In each
subset a fraction of cells underwent proliferation and were grouped into a set of proliferating leukocytes (Fig. 5B, C). B cells, CD4 T cells and CD8 T cells were the predominant lymphoid cells but also included were a number of minor clusters of other lymphoid cells including ILC2, ILC3, NKT and NK cells described by our group (Zakharov et al., 2020). All clusters were identified in both control and anti-PD-1 treated conditions (Fig. 5D). Except for B cells, DCs, macrophages, and T cells especially CD8 T cells were highly represented in the islets of anti-PD-1 treated mice compared with control mice (Fig. 5D, E), a similar phenotype can be reproduced in the flow cytometry analysis (Fig. 5F).

Take a detailed look at the CD4 T cells, based on the differential gene expression, we identified five main clusters of CD4 T cells (Fig. 6A, Fig. 7A, B). These included naïve T cells with distinct expression of *Lef1* and *Tcf7* genes, effector T cells marked by high level of transcripts encoding cytokine *Ifng* and chemokine *Ccl5* expression, anergic T cells marked by the expression of inhibitory molecules *Cd200*, *Pdcd1*, *Lag3*, T regulatory cells (Treg) with high expression of *Foxp3* gene, and proliferating cells had upregulated expression of cell cycle genes (Fig. 7B). To our surprise, we did not observe a major populational shifts in CD4 T cells compared between anti-PD-1 treated sample and control sample indicated by the relative abundance of each subset (Fig. 6B, C). Especially, anti-PD-1 did not affect the frequency of Tregs in the islets, also validated by flow cytometry (Fig. 6C, D), The unchanged CD4 subpopulation highlighted by Treg subset after PD-1 blockade suggested that CD4 T cells may not be the critical component regulated by PD-1/PD-L1 signaling.
Then we focused our attention to CD8 T cells. Based on unique gene signatures including markers corresponding to various activation statuses (Fig. 7C, D), we identified five major subsets of CD8 T cells by scRNA-seq (Fig. 6E). We found naïve cells (Tcf7, Klf2), effector cells (Xcl1), proliferating cells (cell cycle genes), cytotoxic cells with high level expression of genes encoding granzymes and killer cell receptors (Gzma, Gzmk, Klrc1, Klrc1, etc), and exhausted T cells marked by the highest expression of Pdcd1, Tox, Ctla4, Lag3. More CD8 T cells were found upon PD-1 blockade (Fig. 5A), but the relative proportion of all five clusters of CD8 T cells showed no major changes comparing control and anti-PD-1 samples as well (Fig. 6F, G). But due to the fact that exhausted CD8 T cells largely responded to anti-PD-1 treatment, we would take a closer look at this population in the following section.

2.3.5 Exhausted CD8 T cells undergo a functional alteration in response to anti-PD-1 blockade

As I mentioned, the exhausted CD8 T cells (T_{EX}) with high level of PD-1 expression were shown to be the main target of PD-1 blockade in chronic lymphocytic choriomeningitis viral (LCMV) infection model. (Barber et al., 2006). TOX was recently identified as a reliable marker of exhausted CD8 T cells (Alfei et al., 2019; Khan et al., 2019; Scott et al., 2019) Along with TOX expression, two major subsets of exhausted T cells can be classified based on the expression level of TCF1 (encoded by Tcf7 gene): TOX+TCF1+, the stem-like precursor, (T_{PEX}), of the exhausted T cells and TOX+TCF1-, the terminally differentiated effector-like exhausted T cells (T_{TEX}) (Im et al., 2020, 2016; Utzschneider et al., 2016).
We next examined heterogeneity of the exhausted CD8 T cells by differential gene expression from scRNA-seq data and the distribution of each subset in the two conditions. Similar with the gene signatures reported in previous studies mentioned above, T\textsubscript{PEX} and T\textsubscript{TEX} were identified within the exhausted CD8 T cells in our dataset (Fig. 8A). Importantly, most of the exhausted CD8 T cells in the control sample were T\textsubscript{PEX} cells which accounted for over 75% of them. In contrast, 75% of the exhausted CD8 T cells in anti-PD-1 treated sample were T\textsubscript{TEX} cells (Fig. 8B).

Looking at the gene signatures, we found T\textsubscript{TEX} subset was characterized by high levels of pro-inflammatory genes (\textit{Nkg7}, \textit{Ccl3}, \textit{Ccl4}, \textit{Ccl5}, \textit{Gzmb}), which were related to the signatures related to effector cells (Fig. 8C). Strong effector signatures and their presence in anti-PD-1 treated sample indicated that this subset of exhausted T cells could be involved in the promoting diabetes development. In T\textsubscript{PEX} we observed upregulation of \textit{Tcf7} and genes related to protein translation (\textit{Rpl29}, \textit{Rpl27a}, \textit{Rpl35}, and \textit{Eilf4g2}), and cell motility (\textit{Tmsb10}) (Fig. 8C). The upregulation of protein translation genes might participate in self-renewal of such a subset, which had been described before that T\textsubscript{PEX} cells were stem-like cells with proliferative capacity. By gene set enrichment analysis (GSEA) (Subramanian et al., 2005), the transcriptional signature of T\textsubscript{TEX} cells resembled terminally differentiated exhausted (PD-1+ CD101+Tim3+) cells during chronic LCMV infection (Hudson et al., 2019) (Fig. 8D, E).

To further validate, we performed flow cytometry analysis on islets of 10-week-old NOD mice and used antibodies recognizing TOX, TCF1, and PD-1 to detect and quantify the two subsets of exhausted CD8 T cells identified by scRNA-seq. From the flow cytometry plot, we clearly identified T\textsubscript{PEX} cells expressing high levels of TOX and
TCF1 as well as T\textsubscript{TEX} expressing TOX but not TCF1 (Fig. 8F, Fig. 9A). Compared to TOX- CD8 T (non-T\textsubscript{TEX}) cells, both exhausted T cell subsets expressed high level of PD-1 and LAG3, consistent with scRNA-seq analysis (Fig. 8F, Fig. 9B).

Because the depth of the scRNA-seq was limited and we included all the leukocytes besides T cells which may further limit the capacity to detect specific genes, many well-known genes previously identified in exhausted T cells were not detected. Nevertheless, we checked the expression of some well-defined exhaustion markers such as TIM3, TIGIT, CXCR5, SLAMF6, and CD39 by flow cytometry. At protein level, both TIM3 and TIGIT showed an increased expression in exhausted CD8 T cells and T\textsubscript{TEX} revealed a higher level of TIM3 compared with T\textsubscript{PEX}, in line with other reports (Fig. 8F, Fig. 9B). As one of the first defined stem-like exhausted T cell markers in chronic LCMV infection model (Im et al., 2016), CXCR5 was also highly expressed in T\textsubscript{PEX} cells in islets in our autoimmune diabetes mouse model (Fig. 8F, Fig. 9B). With regard to SLAMF6 and CD39, which were identified as surface markers of precursor exhausted T cells and terminally differentiated exhausted T cells respectively (Chen et al., 2019; Gupta et al., 2015), PD-1\textsuperscript{+} CD8 T cells were divided into SLAMF6\textsuperscript{+}CD39\textsuperscript{-} and SLAMF6\textsuperscript{int/neg}CD39\textsuperscript{+} T cells (Fig. 8G, Fig. 9C). SLAMF6\textsuperscript{+} T cells expressed high level of TCF1, confirming they were T\textsubscript{PEX} cells. SLAMF6\textsuperscript{int/neg} T cells expressed low level of TCF1 (Fig. 8H). In sum, from many aspects, T\textsubscript{PEX} and T\textsubscript{TEX} cells were present naturally at a high level in inflamed islets during diabetes development. Due to the surface expression of SLAMF6 and CD39, we could further use these two as surrogate markers to represent T\textsubscript{PEX} and T\textsubscript{TEX} cells and perform more detailed analysis.
To functionally confirm we found exhausted CD8 T cells in islets, we examined the cytokine production in different subsets of T cells upon stimulation. Islets were isolated from 8-10-week-old NOD female mice and cultured in 96-well plates with anti-CD3 and anti-CD28 antibodies to stimulate activated T cells in vitro. IFNγ production from subsets of CD8 T cells was determined after a 4 hr culture by flow cytometry. Here we used PD-1, SLAMF6 and CD39 to differentiate non-T\textsubscript{EX}, T\textsubscript{PEX} and T\textsubscript{TEX} cells. While ~12% of the PD-1 negative non-T\textsubscript{EX} cells were IFNγ+, less than 5% of the exhausted T cells- T\textsubscript{PEX} or T\textsubscript{TEX} expressed IFNγ, confirming they were \textit{bona fide} exhausted T cells resistant to restimulation (Fig. 8H, Fig. 9D).

Our scRNA-seq data revealed that T\textsubscript{PEX} cells consisted of most of the exhausted T cells in the control sample and T\textsubscript{TEX} was the major subset in the anti-PD-1 treated sample (Fig. 4B), so we further validated this observation by flow cytometry analysis compared with mice treated with three injections of anti-PD-1 and untreated control mice at 8-week-old. Two staining methods including TOX/TCF-1 and SLAM6/CD39 were used to reliably determine the distribution of each subset in different conditions. In agreement with scRNA-seq data, TOX+TCF1+ T\textsubscript{PEX} cells predominated in control islets whereas TOX+TCF1- T\textsubscript{TEX} cells were highly represented in anti-PD-1 treated islets (Fig. 8J, upper panel). The same phenomenon was observed when SLAMF6 and CD39 were used to mark the two subsets of the exhausted T cells (Fig. 8J, lower panel).

Combining the results obtained from the two different staining protocols, 60% of the exhausted CD8 T cells were T\textsubscript{PEX}, whereas 40% of them were T\textsubscript{TEX} cells in control islets (Fig. 8K), reflecting the same subset distribution acquired from scRNA-seq data. On the other hand, the T\textsubscript{TEX} cells were significantly expanded in all mice treated with
anti-PD-1, accounting for 70% of the T\textsubscript{EX} cells (Fig. 8K). The phenotypic divergence on T\textsubscript{EX} cells induced by PD-1 blockade was better documented by examining the ratio between T\textsubscript{PEX} and T\textsubscript{TEX} cells with or without anti-PD-1 treatment (Fig. 8L). As in untreated control mice, the ratio scattered between 1 to 6, upon anti-PD-1 treatment, the ratio was mostly lower than 1 and very tightly distributed (Fig. 8L), suggesting a potent alteration induced by anti-PD-1 treatment. More importantly, the non-T\textsubscript{EX} was a minor component of the islet CD8 T cells in both conditions and the proportion of non-T\textsubscript{EX} following PD-1 blockade showed a trend of reduction, although it did not reach a significance (Fig. 8M). These data indicated that anti-PD-1 treatment promoted the differentiation of precursor cells to terminally differentiated cells, as shown in previous studies (Siddiqui et al., 2019; Miller et al., 2019; Chen et al., 2019; Odorizzi et al., 2015; Hudson et al., 2019).

To further examine these changes in the CD8 T cells response to anti-PD-1, we then tested for IFN\textgamma expression with the same protocol mentioned above. In this experimental setting, we included the anti-PD-1 treated mice. Islets from anti-PD-1 or control treated mice were isolated and IFN\textgamma expression was examined \textit{ex vivo} among the CD8 T cells after a 4 hr culture with anti-CD3 and anti-CD28 antibody. There was a definite increase of IFN\textgamma expression in all subsets of the CD8 T cells including non-T\textsubscript{EX} T cells, T\textsubscript{PEX} cells and T\textsubscript{TEX} cells (Fig. 8N, Fig. 9D, E). In brief the anti-PD-1 treatment had rescued the limited response of the exhausted T cells. Given that exhausted CD8 T cells represented the major subset after PD-1 blockade, we estimate that about 70% of the IFN\textgamma producing cells were derived from exhausted CD8 T cells in anti-PD-1 treated
mice (Fig. 8N, lower panel). Thus, anti-PD-1 cultivated an IFNγ copious microenvironment majorly derived from exhausted CD8 T cells.

2.3.6 Myeloid cells undergo remodeling in response to anti-PD-1 blockade

Another component with striking changes we found from our scRNA-seq analysis was the myeloid population. Dendritic cells (DCs) and macrophages both acquired marked remodeling with more striking differences in macrophages compared between control sample and anti-PD-1 treated sample (Fig. 10A). Both DCs and macrophages play critical roles in diabetes initiation and progression. We then further looked at each subset with detail by scRNA-seq.

For dendritic cells (DC), we specifically analyzed conventional DCs. In line with many previous studies, two conventional DCs were identified. They were conventional DC1 (cDC1) expressed genes corresponding to the Batf3-dependent CD103+ cDC1: Xcr1, Cd24a, Irf8, and Batf3. The conventional DC2 (cDC2) were heterogenous, comprising three subpopulations: cDC2(Cr7), cDC2(Mgl2), and cDC2(Ltb). High expression of Ccr7 in cDC2(Cr7) marked the CCR7+ cDC2 group with potential migratory activity from the sites of inflammation to the draining lymph node. The cDC2 (Mgl2) subset uniquely expressed Ccl17 and Mgl2 (encoding CD301b protein), which shares features with monocytes and macrophages. The third subset cDC2 (Ltb) expressed higher level of Havcr2 gene, encoding Tim-3, with immunosuppressive potential (Fig. 11A-C), in agreement with our recent study (Zakharov et al., 2020).

Both cDC1 and cDC2 were present in control and anti-PD-1 conditions (Fig. 11D). While cDC contained a superimposed inflammatory signal after PD-1 blockade,
manifested by IFNγ inducible genes such as Cxcl9 (Fig. 11A, Fig. 10A, B), the cellular composition in cDC cohort underwent only mild changes (Fig. 11C-E).

Four major clusters of islet macrophages can be identified by scRNA-seq analysis (Fig. 10A). Macrophages from control islets were mostly populated by cluster Mac-1 (Apoe) and Mac-2 (Atf3) (Fig. 10B, C). Specifically, the Mac-1 (Apoe) cluster was characterized by its expression of classical macrophage maturation genes such as Apoe, C1qa, Emr1 (encodes F4/80), and absence of a pro-inflammatory signature. The Mac-2 (Atf3) cluster was characterized by the expression of genes with NF-κB activation signatures such as Jun, Fos, Atf3, etc. (Fig. 10D). We previously showed that both clusters were also found in NOD.Rag1−/− mice (Zakharov et al., 2020), indicating their homeostatic role under physiological conditions. Mac-3 (Cxcl9) cluster was characterized by the high expression of IFNγ signature genes including Stat1, Cxcl9, Cxcl10, Isg15, and also displayed signatures of NF-κB activation (Fig. 10D). This subset increased during the progression of diabetes but was absent in NOD.Rag1−/− mice, indicating their association with an active autoimmune response. Similarly, the Mac-3 (Cxcl9) subset was also increased upon anti-PD-1 treatment. A minor cluster, Mac-4, was characterized by higher expression of Prdx-1, and potential anti-inflammatory activity (Fig. 10D); it was down-regulated during the development of the diabetes in NOD mice (Zakharov et al., 2020) but showed no major difference compared between control and anti-PD-1 treated mice.

We observed a marked change in macrophages with their subset distribution after anti-PD-1 treatment, especially in Mac-3 (Cxcl9) subpopulation. Therefore, we further analyzed the changes of the Mac-3 (Cxcl9). We found that these highly activated
macrophages as a whole outnumbered the other subsets following PD-1 blockade (Fig. 10B, C). In Mac-3 (Cxcl9) subpopulation, two more subsets can be further separated with distinct gene signatures: a resident subset, Mac-3-R and a monocyte derived subset, Mac-3-M (Fig. 10D). Compared with Mac-3-R, Mac-3-M expressed higher levels of Ly6c1, Ly6c2, Ccr2, Il1r1, and lower level of Emr1, Cx3xr1 (Fig. 10E), consistent with a monocyte derivation. In addition, many of the genes involved in IFNγ and NF-κB gene signatures exemplified by Cxcl9, Cxcl10, Stat1, Il1b etc., were even more elevated in Mac-3-M, showing a higher pro-inflammatory activity (Fig. 10E). The increased distribution of Mac-3 (Cxcl9) in anti-PD-1 treated mice was mainly from Mac-3-M, which represented half of the total islet macrophages in treated group while the frequency of the counterpart in control islets was extremely low (Fig. 10B, C).

To confirm the observation that we found from scRNA-seq, we performed flow cytometry analysis of islets after anti-PD-1 treatment to examine these two subsets of Mac-3 (Cxcl9). By scRNA-seq, Ly6c1, Ly6c2 genes were highly expressed and Emr1 gene was lower expressed in Mac-3-M compared with Mac-3-R. We thus utilized antibodies against Ly6C and F4/80 to differentiate these two subsets of macrophages by flow cytometry. Ly6C+ F4/80lo macrophages were monocyte-derived (MoMac) and Ly6C-F4/80+ macrophages were resident macrophages (ReMac) which might include several different subsets (Mac-1, -2, -4). From the flow cytometry data, we observed an increased infiltration of Ly6C+ F4/80lo MoMac following PD-1 blockade compared with control mice in which Ly6C-F4/80+ ReMac predominated (Fig. 10F, Fig. 11F). When checking other related markers identified from scRNA-seq data, we found MoMac expressed higher level of Ccr2 and MHC-II molecule I-Aγ7 and lower levels of Emr1 and
Cx3cr1 at protein level relative to ReMac (Fig. 10G). Taken together, in both transcriptional level and protein level, we detected increased infiltration of MoMac in the islets after anti-PD-1 treatment.

To understand how MoMac were recruited, we depleted T cells and examined their islet infiltration. When CD4 T cells or CD8 T cells were inactivated or depleted by antibody treatment, PD-1 blockade failed to recruit MoMac into the islets (Fig. 10F, H), and as was shown in Fig. 1, diabetes development was compromised (Fig. 1J, K). So T cells were responsible for the recruitment of MoMac cells. More importantly, during spontaneous diabetes, we could also find this subset of macrophages in the late stage of diabetes at 15-16-week-old right before mice started to show hypoglycemia; however, they are extremely rare at 6-weeks of age (Fig. 11G). This also indicated the presence of MoMac was positively correlated with T cell infiltration. As a control, we did not find any changes in the distribution of the spleen monocytes or red pulp macrophages compared between 16-week-old mice and 6-week-old mice, suggesting the changes of the monocytes were restricted to specific tissues such as islets in our case (Fig. 11H).

These data showed us two aspects of the T cells: firstly, the infiltrated T cells secreted chemokines to attract the monocytes from the blood to enter the islets; secondly, the activated T cells produced cytokines particularly IFNγ to stimulate the monocytes as well as resident macrophages to become pro-inflammatory. An IFNγ copious microenvironment triggered by anti-PD-1 treatment was crucial to drive the progression of autoimmune diabetes which we would discuss more in the later section.
2.3.7 Islet monocyte-derived macrophages are intrinsically more pro-inflammatory compared with islet resident macrophages

Several observations led us to think MoMac were more activated compared to activated ReMac and may participate more in promoting diabetes development after anti-PD-1 treatment. Firstly, scRNA-seq analysis revealed that Mac-3-M acquired a more profound pro-inflammatory activity (higher level of IFNγ signature) compared with Mac-3-R (Fig. 10E); secondly, PD-1 blockade treatment induced a biased differentiation to MoMac, which represented over half of the macrophages in the islets from the treated mice. To validate this hypothesis, we would compare the activation status of MoMac and ReMac with or without anti-PD-1 treatment in Figure 12. We isolated islets from older 10-12-week-old NOD mice, a time in which they were already heavily infiltrated with T cells and contained a level of MoMac cells (Fig. 13A, B). The islets were cultured with control antibody or with anti-PD-1 antibody for 48 hours. We examined the IFNγ production and the expression of the induced nitric oxide synthase (iNOS), encoded by Nos2 gene, as two parameters of T cell activation and macrophage activation. Following PD-1 blockade, we detected a higher level of IFNγ secreted from the islets (Fig. 12A), making the point that anti-PD-1 directly activated the intra-islet T cells to produce more IFNγ, as we showed in Fig. 3 and Fig. 4. Nitric oxide (NO) is one important bioactive molecule released by macrophages upon activated by IFNγ and is converted to be secreted by iNOS. Examining of iNOS expression is ideal because the expression of iNOS is controlled by NF-κB at steady state and can be augmented by IFNγ signaling (Gao et al., 1997), which implied the activation of both signalings. As we can see from
the flow cytometry analysis, following anti-PD-1, more of the islet macrophages but not the DC expressed iNOS (Fig. 12B, Fig. 13C, D).

To determine whether MoMac would be intrinsically more active than ReMac, we compared the expression of iNOS between the resident macrophages (ReMac) and the infiltrated MoMac in the isolated islets with or without anti-PD-1 treatment. Despite both subsets responded to anti-PD-1 antibody to express higher level of iNOS compared with no treatment control (Fig. 12C, D, Fig. 13E), more of the MoMac expressed iNOS in both untreated and treated conditions albeit a bit lower without anti-PD-1 treatment (Fig. 12E, F), suggesting that the MoMac cells were inherently more active than ReMac in islets.

To examine the role of IFNγ in the activation of macrophages, we performed the same experiments with IFNgR−/− mice and found the islet macrophages had no iNOS expression both at steady state and in response to anti-PD-1 (Fig. 12G, Fig. 13C). In sum, Islet macrophages especially infiltrated monocyte-derived macrophages became highly activated in response to IFNγ produced by T cells after PD-1 blockade.

2.3.8 Depletion of monocyte-derived macrophages inhibits the development of diabetes induced by PD-1 blockade

The next question we want to answer from the hypothesis was whether MoMac could contribute to diabetes development upon anti-PD-1 treatment. As monocytes were from blood, we treated mice with clodronate liposomes to transiently deplete monocytes and monitored the progression of diabetes after anti-PD-1 treatment. Clodronate liposome treatment completely depleted blood monocytes (Fig. 14A, B), but not the islet resident macrophages (Fig. 14C-E). When treated with anti-PD-1, mice
received clodronate liposome showed reduced monocyte infiltration into islets following PD-1 blockade, albeit not to the baseline level (Fig. 15A, B), indicating those MoMac were derived from blood. Similarly, mice with clodronate treatment also showed a reduction in total leukocytes infiltration (Fig. 15C). This result suggested that the activated macrophages could in turn affect the functionality and recruitment of the T cells. Based on our scRNA-seq data, activated MoMac expressed the highest level of Cxcl9 and Cxcl10 (Fig. 10E), chemoattractants that recruit T cells, and their reduction might be the cause for the drop in leukocyte infiltration. As a result, the clodronate liposome treated mice were largely protected from anti-PD-1 induced autoimmune diabetes- only 20% became diabetic- in contrast to almost all the control mice (Fig. 15D). Thus, blocking the infiltration of the monocyte into islets restricted the diabetogenicity induced by PD-1 blockade.

To better understand the pathways by which macrophages were involved in the induction of diabetes after anti-PD-1 antibody treatment we considered the role of interferons (particularly IFNγ) as well as the nitric oxide (NO) we examined above. To determine the role of IFNγ signaling, we generated three strains of mice: type 1 interferon deficient mice lacking Ifnar1 gene expression (NOD.IFNαR−/−), type 2 interferon deficient mice lacking Ifngr1 expression (NOD.IFNγR1−/−), and mice deficient in both type 1 and type 2 interferon signalings by crossing NOD.IFNαR−/− and (NOD.IFNγR1−/− mice to get NOD.DKO mice. We treated the three strains of mice and NOD wildtype mice (as control) encompassing a wide range of ages from 5 weeks to 16 weeks with anti-PD-1 and followed diabetes development. We found wildtype mice and NOD.IFNαR−/− mice developed diabetes rapidly. However, mice lacking IFNγ signaling
(NOD.IFNγR1−/− and NOD.DKO) remained diabetes free (Fig. 15E), pointing to the indispensable role of IFNγ signaling in anti-PD-1 diabetogenicity.

Although the germline knockout of IFNγ signaling prevented diabetes induced by anti-PD-1, there were concerns that T cells might be hypofunctional due to the knockout. We previously found splenocytes from NOD.DKO transferred diabetes to NOD NOD.Rag1−/− mice with similar kinetics compared with NOD wildtype splenocytes transfer, indicating the T cells from interferon deficient mice were normal regarding to diabetes transfer. To further exclude the role of IFNγ signaling in lymphocytes (especially T cells), we generated NOD. IFNγR1−/−Rag1−/− mice by crossing NOD.IFNγR1−/− with NOD.Rag1−/− mice in which T cells cannot develop. Using these mice, we can introduce wildtype splenocytes without IFNγ signaling deficiency. After reconstitution, mice would be treated with anti-PD-1 to follow diabetes induction. We would use NOD.Rag1−/− recipients as control which would develop diabetes rapidly when transferred with wildtype NOD splenocytes and treated with anti-PD-1. We found upon PD-1 blockade, NOD splenocytes induced diabetes in NOD.Rag1−/− recipients rapidly but not in NOD. IFNγR1−/−Rag1−/− recipients (Fig. 15F), indicating that IFNγ signaling in diabetogenesis was uncoupled from adaptive immunity and was dependent on innate immune system.

We then examined the role of nitric oxide in diabetogenesis mediated by anti-PD-1 treatment. As a relatively selective iNOS inhibitor, aminoguanidine (AG) was used in our experimental design to treat the mice. The mechanisms of how AG inhibited iNOS were probably by reducing tissue peroxyl and hydroxyl radicals using its free-radical scavenging activity. Due to its short half-life of AG in plasma, the mice needed to be
treated twice a day at 6mg/mouse with 12-hours interval daily to reach a better inhibition activity throughout the experiment. We found that mice treated with anti-PD-1 antibody together with AG were very well protected from acute diabetes induced by PD-1 blockade (Fig. 15G). However, the MoMac infiltration was not affected by AG treatment, shown by the same ratio between MoMac and ReMac between anti-PD-1 alone treatment and combination of anti-PD-1 and AG treatment (Fig. 15H, I), suggesting that the effect of NO in diabetes development was downstream of MoMac infiltration. Altogether, the findings indicated that the monocyte-derived macrophages actively participate in the pathogenesis of autoimmune diabetes.

2.3.9 Activated macrophages are cytotoxic to β cells

Next, we planned to test whether the activated macrophages can directly kill β cells. Here I only showed an *ex vivo* cytotoxicity assay. We also did an *in vivo* assay but would be discussed in later section. In the *ex vivo* assay, we cocultured a β cell insulinoma line Min6 with macrophages obtained from the peritoneal cavity. The macrophages were either untreated as a control or were stimulated in three conditions: LPS, IFNγ or a combination of LPS and IFNγ. To evaluate the activation status of the macrophages, the expression of iNOS was tested. We found that iNOS was mildly induced by LPS and increased to almost 100% positive by the combined treatment of LPS and IFNγ while IFNγ alone cannot induce its expression (Fig. 16A, Fig. 17A, B). When coculturing the macrophages with β cells for 16-18 hours, we detected the death of the β cells via flow cytometry by staining the cells with a dye that can differentiate dead cells from live cells. By doing so, we found macrophages stimulated by both LPS and IFNγ displayed a dose dependent cytocidal activity against β cells whereas
macrophages activated by either LPS or IFNγ alone did not (Fig. 16B). The death of the β cells was not through direct interaction with the LPS or IFNγ evidenced by the two facts: firstly, we rinsed the macrophages extensively to get rid of the LPS and IFNγ before coculture; secondly, the viability of β cells was not directly affected by culturing them with either of the reagents (Fig. 17C). However, it indeed required the interaction between the β cells and the macrophages to cause the death of the β cells as we did not see the demise of the β cells when they were separated by a transwell under any condition. (Fig. 16C).

To examine whether the killing was IFNγ dependent, we stimulated the macrophages with LPS and type I interferon IFNβ but found the β cells were not killed (Fig. 8D), suggesting the essential role of IFNγ. Moreover, when macrophages deficient in Ifngr1 were treated with LPS and IFNγ, iNOS cannot be induced to express and as a result and the β cells were not killed (Fig. 16E, F). Then we moved on to check the role of nitric oxide in macrophage killing β cells. Here we used a more potent and specific iNOS inhibitor 1400W in culture. While LPS and IFNγ stimulated macrophages killed β cells, iNOS inhibition completely abolished the cytocidal activity of these activated macrophages, suggesting the killing was via nitric oxide derived from the combined NF-κB and IFNγ signalings (Fig. 16G).

2.4 Discussion

We confirmed previous studies showing that PD-1 blockade but not CTLA-4 blockade induces acute diabetes development in adult mice (Ansari et al., 2003). Loss or blockade of PD-1 signaling compressed diabetogenicity into a few days so cellular interactions were more clearly defined (Wang et al., 2005; Ansari et al., 2003). It led to
the undisputed conclusion of a strong symbiosis between the intra-islet T cells- both CD4 and CD8 were required- and the innate cellular system. We documented the presence of an exhausted subset of CD8 T cells that, remarkably, appeared very early in the development of this autoimmunity, was functionally reversed by the anti-PD-1 treatment and was a major participant in the ensuing inflammatory reaction. Still, a notable observation was the documentation that the final effector reaction leading to the demise of β cells, derived from activated monocyte-derived macrophages: depleting them markedly reduced diabetogenesis.

Diabetes autoimmunity in both human and NOD mice was highly dependent for its initiation on recognition by CD4 T cells of β cell derived peptides presented on unique alleles of MHC-II proteins, I-A\(^{97}\) in the case of NOD mice (Wan et al., 2020). CD4 T cells set the autoimmune process in NOD by recognizing proinsulin derived peptides (Wan et al., 2018). Yet in NOD as in human T1D, CD8 T cells participate and play a significant role (Keir et al., 2007; Abdelsamed et al., 2020; Okamura et al., 2019). We saw this symbiosis in the present experiments where depletion of either T cell affected anti-PD-1-mediated diabetes development. We envision a multi-step reaction: an initial CD4 T cell in islets interacting with resident macrophages to be closely followed by entrance of DCs into islets, resulting in the recruitment and activation of CD8 T cells (Ferris et al., 2014). Such interactions are subjected to ensuing with complex modulatory changes that determines the final outcome, the demise of β cell mass.

We identified the heterogeneity of intra-islet CD8 T cells that included the exhausted subset and documented their functional changes in response to PD-1 blockade. These findings in an autoimmune situation are consistent with those in
chronic viral infection and cancer (Yao et al., 2019; Khan et al., 2019; Scott et al., 2019; Alfei et al., 2019). In our scRNA-seq data, a subset of CD8 T cells overexpressed Tox, an HMG-box transcription factor, the central regulator of the T “exhausted” cells. Another subset expressed additional high level of Tcf-7, identified as the precursor of the exhausted T cells. Those cells responded to anti-PD-1 treatment, leading to their high effector function, producing IFNγ, and creating the pro-inflammatory milieu that resulted in a diabetic endpoint. An issue to note is the presence of these exhausted sets of T cells very early in the development of the autoimmune process, also indicated in our recent scRNA-seq analysis, as early as 8 weeks of age (Zakharov et al., 2020). The obvious take home message is that the islet microenvironment modulates the effector function of the initial autoreactive T cells. How this mini organ which consists of highly secretory β cells, plus resident activated macrophages and activated endothelia regulates such T cell response is a major issue to investigate.

A major effector cell leading to diabetes after PD-1 blockade was an activated monocyte-derived macrophage, identified both by scRNA-seq analysis and flow cytometry. Their depletion reduced diabetes incidence. The monocyte-derived macrophages in response to IFNγ showed increased expression of MHC class II molecules and iNOS, that led to NO production and the killing of β cells. These findings underscore two contrasting functions of the islet resident macrophages: various subsets in an activated state having several functions such as homeostatic, local defense, and antigen presentation; and the monocyte derived, entering islets upon cues derived from an inflammatory state, and having a cytocidal role.
The findings on the cytocidal role of macrophages and their development requiring IFNγ raise several important issues. First, what is their role and that of IFNγ in regular diabetes? Early studies had pointed to the effector role of macrophages in NOD diabetes based on experiments depleting monocytes by clodronate liposomes administration or other treatments (Jun et al., 1999a; b; Lee et al., 1988; Calderon et al., 2006). (As shown here, clodronate liposome selectively depleted blood monocytes and did not affect the islets resident macrophages.) In the present analysis on islets from non-manipulated NODs just before dysglycemia, as well as in our recent scRNA-seq examination, the activated macrophages were strongly represented. Such subset was not found in the absence of IFNγ receptor (Zakharov et al., 2020) but is highly represented in the present experiments with PD-1 blockade. This study shows that NOD.IFNGR1−/− and NOD.DKO female mice failed to respond to anti-PD-1 to induce acute diabetes, in striking contrast, spontaneous diabetes still developed in these mice albeit with decreased incidence (Carrero et al., 2018; Serreze et al., 2000; Hultgren et al., 1996). This indicates alternative effector mechanisms during conventional diabetes, most likely pointing to CD8 cytolytic T cells.

Much discussion has taken place on the role of CD8 T cells and their association with β cell killing. Diverse experimental manipulations have been tested, from gene knockouts of MHC-I on β cells or APC, to gene knockouts of the cytolytic pathways in CD8 T cells, examining the roles of Fas-Fas-L and perforin-granzymes (Hamilton-Williams et al., 2003; Dudek et al., 2006; Varanasi et al., 2012; Mollah et al., 2012). While presentation by MHC-I peptide complex is absolutely required for diabetes progression, the association with direct cytolytic function may vary depending on the
stage of the process. In our experiments we cannot exclude a role for CD8 T cells in
direct killing of β cells, a level of diabetes was still apparent after monocyte depletion. In
such a complex and chronic autoimmune process, it is likely that several effector
reactions may develop with time and contribute to the demise of β cells.

Finally, our study opens the question whether activated macrophages should be
considered as the effector downstream of T cells in situations of checkpoint blockade in
cancer and infectious diseases. Blockade of PD-1 signaling in context of cancer and
chronic infection has mostly focused on CD8 T cells. However more recent studies are
pointing to diverse roles of macrophages that supersedes their suppressive function. As
for cancer, in a T3 MCA sarcoma mouse model, a subset of macrophages with both
pro-inflammatory and monocyte signatures increased dramatically upon immune
checkpoint blockade (Gubin et al., 2018). Genetically depletion of PD-1/PD-L1 signaling
between T cells and macrophages favored a M1-associated macrophage signaling
including IL-6, TNFR, iNOS and chemokine production (Diskin et al., 2020).
Blockade/deletion of PD-1 expression on tumor associated macrophage (TAM) restored
phagocytosis, induced metabolic reprogramming of the macrophages, increased T cell
effector function, promoted antigen presentation, and increased the survival of mice
bearing tumors (Gordon et al., 2017; Strauss et al., 2020). Tumor infiltrating
macrophages expressed high level of Trem2 which when inhibited modulated the tumor
microenvironment, improved intratumoral T cell responses and further augmented anti-
PD-1 checkpoint immunotherapy (Molgora et al., 2020; Katzenelenbogen et al., 2020).
To the extent that activated macrophages will be the final effector may much depend on
the features of the particular cancer and its susceptibility to macrophage effector molecules.

With regard to the resident macrophages at steady state, we showed in our study that islet resident macrophages were activated even without T cell infiltration and they were essential for the initiation of diabetes development. This was also the case concerning to PD-1 blockade induced diabetes. Different from islet resident macrophages, at steady state, tumor associated macrophages were generally considered as immunosuppressor. Thus, targeting the resident macrophages pharmaceutically might be beneficial to prevent diabetes while keep anti-tumoral responses of anti-PD-1. Although in this study, we did not examine whether tumor bearing mice would have a better control of tumor progression when depleting resident macrophages via anti-CSF1R plus anti-PD-1, accumulative studies showed that CSF1R blockade enhanced T cell migration, reinvigorated T cell responses in tumor and rendered tumors more susceptible to T cell dependent immunotherapy including anti-PD-1 treatment (DeNardo et al., 2011; Mitchem et al., 2013; Mok et al., 2014; Zhu et al., 2014; Peranzoni et al., 2018; Hoves et al., 2018). This dramatic difference between tumor associated macrophages and islet resident macrophages provides the rationale for CSF1R inhibition to uncouple the toxic effect to β cells and anti-tumor response induced by PD-1 blockade.

In addition to cancer, PD-1 blockade had been largely studied in mouse models of LCMV infection where the role of cytolytic CD8 T cells is major component. Clinically, anti-PD-1 therapy has been tested to treat different chronic infectious diseases resulting in enhanced immune responses against viruses and in some cases, viral
clearance (Day et al., 2006; West et al., 2013; Gardiner et al., 2013). Thus, it will be an issue to consider whether besides T cells, activated macrophages also play a role in infectious diseases modulated by the PD-1 pathway. Paradoxically, PD-1 blockade induced macrophage activation leads to reactivation of *Mycobacterium tuberculosis* infection. The reactivation is dependent on augmented TNF-α secretion by macrophages (Tezera et al., 2020). This would add another layer of complexity of targeting macrophages in context of PD-1 blockade. Therefore, the biological effects of macrophage/monocyte activation downstream of PD-1 blockade is very much context dependent. Hence, more efforts should be made to understand the biology of macrophages downstream of PD-1 blockade to uncouple their efficacy to complement therapeutics and toxicity to avoid adverse effects.

### 2.5 Materials and Methods

#### 2.5.1 Mice

NOD/ShiLtJ (NOD), NOD.129S7(B6)-Rag1tm1Mom/J (NOD.*Rag1*−/−), and B6.NOD-(D17Mit21-D17Mit10)/LtJ (B6g7) were originally obtained from the Jackson Laboratory, NOD.*Ifnar1*−/−, NOD.*Ifngr1*−/−, NOD.DKO (double knockout) (Carrero et al., 2018), and NOD. *Ifngr1*−/−. *Rag1*−/− mice were generated in this study. All mice were bred and maintained and experimented in our pathogen-free animal facility in accordance with the Division of Comparative Medicine of Washington University School of Medicine (Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accreditation no. A3381-01).
2.5.2 Cell line and isolation of peritoneal macrophages

Insulinoma cell line, Min6 was obtained from ATCC and maintained in our laboratory in DMEM media supplemented with 15% FBS (v/v). Cell were propagated in vitro when the density of the cells reached 70% confluence. Peritoneal exudate cells were collected by flushing the peritoneum with 5 ml PBS with 2.5 mM EDTA and 0.2% BSA and cultured overnight after which any lymphocytes were removed by extensive washing.

2.5.3 Treatment and diabetes monitoring

Mice were treated intraperitoneally with 250μg anti-PD-1 (RMP1-14), anti-PD-L1 (10F.9G2) or anti-CTLA-4 (9D9) on days 0, 3, and 6. For controls, mice were injected with 200μg of rat IgG2a isotype control antibody or PBS. Antibodies were diluted in PBS prior to injection for a total volume of 0.2ml per mouse.

For CD4 T and CD8 T cell depletion, 250μg of anti-CD4 (YTS177.1) and anti-CD8 (YTS169.4) were injected intraperitoneally three days before the first injection of anti-PD-1 and during the 3 doses of anti-PD-1 injection.

For NOD splenocytes transfer, splenocytes from NOD (10-12 weeks old) were harvested and red blood cells were lysed with ACK (Ammonium-Chloride-Potassium) Lysing Buffer. Cells were washed with DMEM plus 10% FBS once and then with PBS for twice. Single cell suspension was made in PBS buffer and 10×10^6 cells were transferred in 100μl into 8-10 weeks old NOD. Rag1^-/- recipients or NOD. Ifngr1^-/-. Rag1^-/- recipients.
For monocyte depletion, 100μl/mouse clodronate liposome or PBS liposome were injected on days -3, 0, 3, and 6 intravenously and anti-PD-1 were given on days 0, 3, and 6.

For iNOS inhibition, aminoguanidine (6mg per mouse) diluted in PBS were injected twice a day intraperitoneally (8:00 in the morning and 20:00 in the evening) intraperitoneally starting from day 0 to day 8, then once a day from day 9 to 20 (8:00 in the morning). Anti-PD-1 were injected on days 0, 3, and 6.

Blood glucose was monitored every day after the third dose of anti-PD-1 until day 20 to 40 or weekly after splenocytes adoptive transfer. After two consecutive readings of >250 mg/dL (Chemstrip 2GP; Roche Diagnostics, Indianapolis, IN), mice were considered diabetic.

2.5.4 Fluorescence-activated cell sorting and conventional flow cytometry

Pancreatic islets were isolated as described (Calderon et al., 2015) and dispersed into single-cell suspension using non-enzymatic Cell Dissociation Solution (Sigma-Aldrich) for 3 min at 37°C. To block Fc-receptors engaging, the cell suspensions were incubated at 4°C for 15 min in PBS (pH 7.4) supplemented with 2% FBS and 50% of FC-block (made in-house). For surface staining, cells were incubated with fluorescently labeled antibodies (1:200 v/v) at 4°C for 20 minutes. Cells were then washed and analyzed by flow cytometry or subjected to FACS sorting.

For staining of intracellular transcription factors, islet cells were stained with surface antibodies for 20 minutes, fixed, and permeabilized using Foxp3/Transcription factor staining buffer set (Thermo Fisher Scientific) per manufacturer's instructions. Cells were reacted with FOXP3, TCF1, TOX antibodies at room temperature for 30 min.
For intracellular cytokine and protein staining, isolated islets (~100 islets per well in 96-well plate) were placed at 37°C in DMEM, 10% FBS (GE Healthcare), anti-CD3ε (10μg/ml), anti-CD28 (5μg/ml) and 10μg/ml Brefeldin A for 4 h for IFNγ detection. For iNOS staining, no stimulation was needed. Islets were then dispersed and washed with PBS, 1% BSA, and cell surface stained with fluorescent antibodies for 30 min at 4°C. Then the cells were fixed and permeabilized using BD Fixation/Permeabilization Solution Kit (Thermo Fisher Scientific) according to the instruction. The cells then were stained with fluorescent antibodies recognizing intracellular proteins IFNγ and iNOS for 30 min at 4°C.

For BrdU incorporation assay, mice were treated intraperitoneally with 1mg per mouse BrdU in PBS starting from day 0 to day 6. The mice were separated into anti-PD-1 treated group (three injections of anti-PD-1 were given on days 0, 3, and 6) and untreated group. At day 7, islets were isolated from the two groups and BrdU incorporation were assessed according to the manufacturer's instruction (BD Pharmingen).

All conventional flow cytometry was done on BD FACSCanto II (BD Biosciences, San Jose, CA, USA) and all the FACS sorting was done on BD FACSARia II sorter (BD Biosciences) and analyzed using FlowJo 10.0 software (Tree Star).

2.5.5 Histology

Pancreata were isolated, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Microscopy was performed, and images were collected on the NanoZoomer (Hamamatsu Photonics K.K.).
2.5.6 Single-cell RNA sequencing, library preparation, alignment, and analysis

The scRNA-seq analysis was performed on live islet CD45+ leukocytes from 8-week-old NOD female mice either untreated or treated with anti-PD-1, 4-8 mice per one sample. After sorting, cells were loaded onto the Chromium Controller (10X Genomics) (Zheng et al., 2017). The resulting samples were processed using the Chromium Single Cell 3′ Library & Gel Bead Kit (10x Genomix, v2) following the manufacturer’s protocol. The libraries were sequenced on Illumina NovaSeq6000. The 10X Genomics Cell Ranger (v2.1.1) was used for raw reads mapping (GRCm38) and counting unique molecular identifiers (UMI). The library preparation and sequencing was done at the Genome Technology Access Center core facility (GTAC, Washington University in St. Louis, https://gtac.wustl.edu/). The 8-week-old control sample was the same used in the time course data (Zakharov et al., 2020). The Seurat package (v2.3.4) (Satija et al., 2015) was used for heterogeneity analysis and differential expression analysis. The Wilcoxon rank sum test was used to identify differentially expressed genes. A canonical correlation analysis was run to align datasets using the RunCCA function. For dendritic cells analysis, the inflammatory-driven signal affecting separation by cell types was reduced before the clustering.

2.5.7 Cytotoxicity assay

Cytotoxicity killing assay was performed using Min6 β cell line as target cells and peritoneal exudate macrophages cells. The day before coculture, peritoneal cells were plated and treated with different stimulations: untreated, 100ng/ml LPS, 100ng/ml IFNγ, 100ng/ml IFNβ, LPS+IFNγ or LPS+IFNβ. β cell line Min6 were plated at 50,000 cells per well and rested overnight. The next day, peritoneal cells were washed, and
macrophages were collected and added to β cells at various ratios starting from 10:1 (macrophage to β cells). 24 hours later, the cells were stained with indicated antibodies at 37°C for 20min. To detect cell death, cell viability dye 780 (ebioscience) was used along with other markers including CD45, CD11b, I-A^q, and iNOS to identify macrophages. Then the cells were washed at least 3 times with PBS and trypsinized and collected for cell death detection by flow cytometry.

2.5.8 Statistical analysis

Paired or unpaired two-tailed Student's t tests were performed when two groups of samples were compared. Mantel-Cox log-rank test was performed to compare survival curves in mice from different treatment. All the p values were calculated using GraphPad PRISM 7 with the following significance: n.s. p>0.05; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Statistical details for each experiment can be found in the figures and legends.

2.5.9 Data availability

The scRNA-seq datasets generated during this study was deposited in National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE150178.
Figure 1 PD-1 blockade promotes rapid autoimmune diabetes in NOD mice. (A) Incidence of diabetes in female NOD mice treated with anti-PD-1 at 6-8 weeks of age (n=20, red) or without treatment (n=24, blue). Mice were given three injections of anti-PD1 every three days and diabetes were followed starting at 6-weeks of age. Results are pooled from 3 independent experiments. (B) Representative H&E histology of pancreas from 6-8-week-old NOD mice treated with or without anti-PD-1. Samples were prepared one day after the last injection. Scale Bar, 100μm. Data are representative of n=5 mice. (C) Flow cytometry analysis of PD-L1 expression in indicated cells isolated from islets of 8-week-old NOD female mice. Data are representative of 3 independent experiments. (D) Incidence of diabetes following three injections of anti-PD-L1 (n=4, red) or anti-CTLA-4 (n=6, blue) in 8-week-old NOD female mice. Experiment was performed one time. (E) Representative H&E histology of pancreas from 6-8-week-old NOD mice treated with or without anti-CTLA-4 from (D). Scale Bar, 100μm. Similar results were found in three other mice examined. (F) Percentage of CD4 and CD8 T cells in islets from untreated or anti-PD-1 treated mice. ****p<0.0001. Experiments were performed three times with at least 3 mice in each group per experiment. (G) Ratio between CD8 T cells and CD4 T cells in islets. ****p<0.0001. Data are pooled from 5 independent experiments. (H) Expression of PD-1 in CD4 T cells and CD8 T cells in islets, pancreatic lymph nodes (pLN), and spleen from untreated 8-week-old NOD female mice. Results are representative from n=5 mice from 3 independent experiments. (I) Proliferative capacity of CD4 T cells (**p=0.0087) and CD8 T cells (**)p=0.0043) in islets measured by BrdU incorporation. Results are from n=6 mice from 2 independent experiments. (J) Incidence of diabetes in 8-week-old female NOD mice
treated with anti-PD-1 plus isotype control antibody (n=11, blue) or anti-PD1 plus anti-CD8α (n=8, red). ***p=0.0009. Results are pooled from 2 independent experiments. (K) Incidence of diabetes in 8-week-old female NOD mice treated with anti-PD-1 plus isotype control antibody (n=8, blue) or anti-PD1 plus anti-CD4 (n=8, purple).

****p<0.0001. Results are pooled from 2 independent experiments. All the comparison of survival curves was performed using the Mantel-Cox log-rank test and all the P values in dot plots were calculated using an unpaired two-tailed Student’s t-test. Each data point in the dot plot indicates an individual mouse.
Figure 1

A) Diabetes free (%) over time for No treatment and α-PD-1.

B) Images showing NOD_Ctrl and NOD_α-PD-1.

C) Flow cytometry analysis of MACs, DCs, and CD45− PD-L1.

D) Diabetes free (%) over time for α-PD-L1 and α-CTLA-4.

E) Images showing NOD_Ctrl and NOD_α-CTLA-4.

F) Bar graph showing % of total islet cells for CD4 T, CD8 T.

G) Scatter plot comparing CD8 T/CD4 T in Ctrl α-PD-1.

H) Diagram showing PD-1 expression in CD4 T and CD8 T.

I) Bar graph showing BrdU+ (%) for CD4 T and CD8 T.

J) Diabetes free (%) over time for various treatments: Ctrl, α-PD-1, α-CD8α+α-PD-1.

K) Diabetes free (%) over time for various treatments: Ctrl, Isotype ctrl+α-PD-1, α-CD4+α-PD-1.
Figure 2 Checkpoint blockade and diabetes development. (A) Incidence of diabetes induced by anti-PD-1 in 3-5-week-old NOD female mice (n=35, blue) and 8-12-week-old NOD female mice (n=17, red). ****p<0.0001. (B) Incidence of diabetes induced by anti-PD-1 in NOD male mice (n=18, blue) and NOD female mice (n=21, red). (C) Incidence of diabetes induced by anti-PD-1 in >40-week-old NOD female mice. Mice were given one injection of anti-PD-1 (n=4, red) or isotype control antibody (n=4, blue). (D) Flow cytometry analysis of islet CD45+ leukocytes from NOD female mice treated with anti-PD-1 or isotype control antibody. (E) Mean Fluorescence Intensity of PD-1 expression in CD4 T cells and CD8 T cells from islets, pancreatic lymph nodes (pLN), and spleen of 8-week-old NOD female mice (n=5). (F) BrdU incorporation in CD4 T cells and CD8 T cells from islets of 8-week-old NOD female mice treated with or without anti-PD-1. (G) Flow cytometry analysis of CD4 T cells and CD8 T cells in pLN and islets after antibody treatment. (H) Ratio between CD8 T cells and CD4 T cells in islets in the indicated conditions from (G). (I) Level of insulin autoantibody, IgG1 and IgG2b, in the serum of 8-week-old NOD female mice treated with (n=6) or without (n=5) anti-PD-1 measured by ELISA. To determine the specificity one aliquot of sera was incubated with a 10-fold excess of insulin before measurement (Right). (J) Flow cytometry analysis of T follicular helper cells in spleen and pLN from 8-week-old NOD female mice treated with three injections of isotype control antibody or anti-PD-1 antibody.
Figure 2
Figure 3 Intraislet T cells are required for anti-PD-1 to drive diabetes development. (A) representative flow cytometry analysis of blood of 6-week-old NOD mice treated with saline or FTY720. (B) Blood T cells during the diabetes monitoring under the conditions of anti-PD-1 plus FTY720 or anti-PD-1 plus saline. (C) Incidence of diabetes in 6-week-old mice treated with FTY720 plus anti-PD-1 (n=7) or saline plus anti-PD-1 (n=7). Experiment is performed once with n=7 mice in each group. **P=0.0042. (D) Upper: representative flow analysis of islet cells from the 6 remaining non-diabetic mice from FTY720 plus anti-PD-1 treated mice. Lower: Ratio between CD8 and CD4 T cells after treatment. The data from the 6 remaining non-diabetic mice were compared with the record data generated before. ****p<0.0001. (E) representative flow cytometry analysis of blood of 12-week-old NOD mice treated with saline or FTY720. (F) Blood T cells during the diabetes monitoring under the conditions of anti-PD-1 plus FTY720 or anti-PD-1 plus saline. (G) Incidence of diabetes in 6-week-old mice treated with FTY720 plus anti-PD-1 (n=9) or saline plus anti-PD-1 (n=8). Experiment is performed once with multiple mice in each group. (H) H&E histology of pancreatic islets in the two conditions. Results are from non-diabetic mice from each group.
Figure 3
Figure 4 Restriction of T cells infiltration by CSF1R blockade serves as a prophylactic strategy to prevent PD-1 blockade induced diabetes. (A) Incidence of diabetes in NOD mice treated with AFS98 plus anti-PD-1 or IgG2a control antibody plus anti-PD-1 at 3-week-old. Data are pooled from n=13 mice from each group from three independent experiments. ***P=0.0003. (B) Representative flow cytometry of the infiltrated CD45 leukocytes in islets from AFS98 plus anti-PD-1 treated mice or anti-PD-1 alone treated mice. (C, D) Representative H&E histology (C) and insulitis scores (D) of pancreatic islets from the two conditions. (E) Representative flow analysis of islet cells after AFS98 treatment in 10-week-old mice. (F) Quantification of each subpopulation of APCs from the two conditions from (E). Data are pooled from n=4 mice in each condition. **P=0.0068; *P=0.0150. (G) Incidence of diabetes in NOD mice treated with AFS98 plus anti-PD-1 (n=5) or IgG2a control antibody plus anti-PD-1 (n=4) at 13-week-old.
Figure 4
Figure 5 Single-cell RNA sequencing analysis identifies anti-PD-1 responsive islet infiltrating leukocytes. (A) Flow cytometry sorting of CD45+ leukocytes from either 8-week-old untreated mice or anti-PD-1 treated female mice. (B) t-SNE plot of intra islet leukocytes combining the control and anti-PD-1 treated NOD mice. (C) Expression of canonical immune cell markers in clusters of islet infiltrating leukocytes from (B). (D) t-SNE plot from (B) split by the two conditions. (E) Fraction of immune cell populations relative to the total islet cells number in the two conditions from scRNA-seq data. (F) Fraction of immune cell populations relative to islet cells calculated based on flow cytometry analysis. Bars indicate SD.
Figure 5
Figure 6 Islet T cell heterogeneity after anti-PD-1 treatment. (A-B) t-SNE plot of intra-islet CD4 T cells from the 8-week-old untreated mice and anti-PD-1 treated mice described in Fig. 5; both are conditions merged (A) or shown separately (B). (C) fraction of each cluster in CD4 T cells relative to total islet cells. (D) Flow cytometry analysis of Foxp3+ Treg cells in islets treated with or without anti-PD-1. Islets were examined on Day 7 after the third injection of anti-PD-1. Result is representative of 2 independent experiments with n=2-3 mice in each group. (E-G) Examination of CD8 T cells done as in (A-C).
Figure 6

A. CD4 T cells

B. Ctrl vs. α-PD-1

C. % of islet cells

D. Islet CD4 T cells

E. CD8 T cells

F. Ctrl vs. α-PD-1

G. CD8 T cells
Figure 7 Single-cell RNA-seq analysis of islet infiltrating leukocytes. (A) Expression of marker genes in CD4 T cell clusters (see Fig. 6A). (B) Heatmap showing differentially expressed genes that characterize clusters of CD4 T cells. (C) Expression of marker genes in CD8 T cell clusters (see Fig. 6E). (D) Heatmap showing differentially expressed genes that characterize the clusters of CD8 T cells. For (B, D), differentially expressed genes with P adj. < 0.05 (Wilcoxon rank sum test) are shown. The data showed the combined cells from both anti-PD-1 and control mice.
Figure 7
Figure 8 PD-1 blockade drives the functional alteration of exhausted CD8 T cells. (A) Left: t-SNE plot of CD8 T cells merged as in Fig. 6E. Right: t-SNE plot of exhausted CD8 T cells extracted from the CD8 T cell population. (B) Population of exhausted cells from (A) split by the two condition: TTEX predominates after anti-PD-1 treatment. Right: Quantification of the frequencies of each subpopulation. (C) Heat map illustrates the differences between two exhausted CD8 T cells subsets (P adj < 0.05, Wilcoxon rank sum test). (D) GSEA plot comparing the TTEX gene signature from (C) against published transcriptional dataset of exhausted CD8 T cells (BioProject: PRJNA497086) (nominal P-value < 1e-5). (E) Heat map showing expression of enriched genes from (D) in the reference dataset used above. (F) Flow cytometry analysis of exhausted T cell in islets from 10-week-old untreated NOD mice. Histogram shows the expression of key molecules associated with exhausted T cells including PD-1 TIM3, TIGIT, LAG3, and CXCR5 (numbers in the box indicated MFI: mean fluorescent intensity). Flow analysis is representative from n=12 mice from 4 independent experiments. The staining of each inhibitory molecule was repeated 2-3 times. (G) Flow cytometry analysis of TPEX and TTEX cells examining for SLAMF6 and CD39. Results are representative from n=3 mice. (H) Expression of TCF1 in SLAMF6+ TPEX and SLAMF6- TTEX and quantification of TCF1 expression in each subset. Data are representative from n=3 mice. (I) IFNγ expression in TPEX, TTEX, and non-TEX. ***p (TPEX vs non-TEX)=0.0002; ***p (TTEX vs non-TEX )=0.0009. Results are pooled from n=4 mice from 2 independent experiments. (J) Flow cytometry analysis using TOX and TCF1 (upper) and SLAMF6 and CD39 (lower) to examine the distribution of TPEX cells and TTEX cells in control and anti-PD-1 treated 8-week-old NOD mice. Data are representative from 2 independent experiments in each
staining protocol. (K) Percentage of T\textsubscript{PEX} cells and T\textsubscript{TEX} cells by each condition, pooled from the results of the two staining protocols in J. ****p<0.0001. Each dot represents one experiment. (L) Ratio of T\textsubscript{PEX} cells and T\textsubscript{TEX} cells based on the results of (K). **p=0.0024. (M) Percentage of non-T\textsubscript{EX} cells in control and anti-PD-1 treated mice, taken from the results in (J). (N) Upper: IFN\textgamma expression in T\textsubscript{PEX}, T\textsubscript{TEX}, and non-T\textsubscript{EX} under the indicated treatment. **p=0.0097 (non-T\textsubscript{EX}); **p=0.0025 (T\textsubscript{PEX}); **p=0.0037 (T\textsubscript{TEX}). Lower: pie chart indicating the percentage of IFN\textgamma+ cells from non-exhausted T cells and exhausted T cells in each condition. Results are pooled from n=4 mice from 2 independent experiments.
Figure 8

(A) CD8 T cells

(B) Exhausted T cells

(C) TEX

(D) Nominal p-value < 1e-5

(E) Enrichment score

(F) CD8 T cells

(G) CD8+PD1+

(H) CD8+PD1+

(I) IFNγ+CD8 T (%)

(J) TOX

(K) SLAMF6

(L) CD39

(M) % of CD8 T

(N) IFNγ+CD8 T (%)
Figure 9 Identification of two subsets of exhausted CD8 T cells in islets. (A) Representative flow cytometry gating strategy to identify the two subsets of exhausted CD8 T cells based on TOX and TCF1 expression. (B) Inhibitory molecules expressed by non-TEX, TPEX, and TTEX cells. (C) Representative flow cytometry gating strategy for the identification of exhausted CD8 T cells based on SLAMF6 and CD39 expression. (D, E) Gating strategy and expression of IFNγ in TPEX cells, TTEX cells and non-TEX cells in islets in control (D) and anti-PD-1 treated mice (E).
Figure 9
Figure 10 PD-1 blockade drives the infiltration of monocyte-derived macrophages into islets. 
(A) Combined t-SNE plot of myeloid cells obtained from untreated and anti-PD-1 treated NOD female mice. 
(B) t-SNE plot from (A) split by the two conditions. 
(C) Fraction of each cluster of macrophages relative to all islet cells in the two conditions. 
(D) Heat map showing the differential expression gene analysis among subsets of macrophages (P adj. < 0.05, Wilcoxon rank sum test). 
(E) Volcano plot shows the differential gene expression between Mac-3-R and Mac-3-M (P adj. < 0.05, Wilcoxon rank sum test). 
(F) Flow cytometry validating the infiltration of monocyte-derived macrophages (MoMac) under the indicated treatment. 
(G) Expression of several protein marker comparing MoMac and ReMac. *p (F4/80)=0.0123; **p (CX3CR1)=0.0053; *p (CCR2)=0.0445; *p (I-A^*7)=0.0119. P value is calculated using a paired two-tailed Student’s t-test. 
(H) Ratio between MoMac and ReMac in islets. **p=0.0079. Results are pooled from 2 independent experiments.
Figure 10
Figure 11 Identification of monocyte-derived macrophages in islets following anti-PD-1 treatment and in islets from pre-diabetic NOD mice. (A) t-SNE plots showing the expression of marker genes in myeloid cell clusters from Fig. 10A. (B) Heatmap showing differentially expressed genes that characterized clusters of dendritic cells (P adj. < 0.05, Wilcoxon rank sum test). (C) Combined t-SNE plot showing DCs obtained from untreated and anti-PD-1 treated NOD female mice. (D) t-SNE plot from panel (C) split by the two conditions. (E) Fraction of each cluster of dendritic cells relative to islet cells in the two conditions. (F) Flow cytometry gating strategy used in Fig. 10F. (G) Monocyte-derived macrophages in islets from 6 and 16-week-old NOD female mice. (H) Identification of different subsets of macrophages in spleen from 6 and 16-week-old NOD female mice.
Figure 11
Figure 12 Monocyte-derived macrophages are more pro-inflammatory compared with islet resident macrophages. (A) Quantification of IFNγ production in supernatants from 100 cultured islets isolated from 10-12-week-old NOD mice from 12 independent experiments (each data point). **p=0.0018, P value was calculated using a paired two-tailed Student’s t-test. (B) Expression of iNOS in macrophages from islets cultured with or without anti-PD-1 for 48h as in (A). **p=0.0022. (C-F) Expression of iNOS in monocyte-derived macrophages or resident macrophages with or without anti-PD-1 treatment. *p (C)=0.0438; **p (D)=0.0055; *p (E)=0.0411; *p (F)=0.0427. P values of (E, F) were calculated using a paired two-tailed Student’s t-test. (G) Expression of iNOS in macrophages from 100 islets isolated from 10-12-week-old NOD wildtype mice or NOD.IFNγR−/− mice.
Figure 12

A

B

C

D

E

F

G

IFNγ (pg/ml)

iNOS+ (% of Mac)

Ctrl

α-PD-1

iNOS+ (% of Mac)

Ctrl

α-PD-1

iNOS+ (% of Mac)

Ctrl

α-PD-1

Ctrl MoMac

α-PD-1 MoMac

Ctrl ReMac

α-PD-1 ReMac

Ctrl

α-PD-1

NOD

NOD.IFNγR1

n.s.

**

*
Figure 13 Monocyte-derived macrophages are more pro-inflammatory compared with islet resident macrophages. (A) Representative flow analysis showing the infiltration of the isolated islets from 10-12-week-old NOD female mice treated with or without anti-PD-1 (50μg/ml) in culture for 48h before analysis. Result is representative of 9 independent experiments. (B) Frequency of CD45+ leukocytes and CD3+ T cells in untreated or anti-PD-1 treated islets from (A). (C) Representative flow cytometry showing iNOS in islet macrophages from 10-12-week-old NOD or NOD.IFNγR−/− mice. (D) iNOS expression in DC from the untreated or anti-PD-1 treated islets. (E) Representative flow cytometry for Fig. 12 C-F.
Figure 13
Figure 14 Clodronate liposome depletes monocytes in the circulation but not the islet resident macrophages. (A) Levels of monocytes in blood of PBS liposome or clodronate liposome treated mice by flow cytometry. (B) Percentage of blood monocytes as in (A). *p=0.0286. (C-E) Representative flow cytometry analysis showing the percentage of monocytes in blood and resident macrophages in 6-8-week-old NOD mice (C) or the non-diabetic strain B6.g7 mice (D) treated with PBS liposomes or clodronate liposomes. The results showing the percentage of resident macrophage in islets was pooled from NOD and B6.g7 mice (E).
Figure 14
Figure 15 Decreasing the infiltration of monocyte-derived macrophage into islets prevents diabetes following PD-1 blockade. (A) Flow cytometry analysis showing the effects of clodronate liposome treatment on islet myeloid cells. (B) Ratio between MoMac and ReMac in islets from the data of (A). Untreated age matched NOD mice (n=5) were examined as control. ***p=0.0007, *p=0.011. (C) Percentage of infiltrating CD45 leukocytes from the data of (A, B). ***p=0.001, *p=0.0496. (D) Diabetes incidence of NOD mice treated with anti-PD-1 plus PBS liposomes (n=13, red) or anti-PD-1 plus clodronate liposomes (n=12, blue) at 6-8-weeks of age. ****p<0.0001. (E) Diabetes incidence of NOD (n=35, blue), NOD.IFNAR−/− (n=10, red), NOD.IFNGR−/− (n=17, green), and NOD.DKO (n=13, purple) treated with anti-PD-1. ****p<0.0001. (F) Diabetes incidence of NOD. Rag1−/− recipients (n=14, red) or NOD. IFNγR1−/− Rag1−/− recipients (n=17, blue) transferred with NOD splenocytes (10⁷ per mouse). Four weeks later the mice were treated with three injections of anti-PD-1 antibodies and monitored diabetes for 4 weeks. ****p<0.0003. (G) Diabetes incidence of NOD mice treated with anti-PD-1 plus aminoguanidine (AG, 6 mg/mouse, n=13, blue) every 12h for 20 days or anti-PD-1 alone (n=9, red) starting at 5-7-weeks of age and followed for 4 weeks. **p=0.004. Results are pooled from 2 independent experiments. (H) Flow cytometry analysis showing the effects of aminoguanidine treatment: 5-7-week-old NOD mice were treated as in (G). 4 weeks later, islets from the two groups of mice were isolated and examined by flow analysis. Data are representative from n=4 mice from 2 independent experiments. Two mice from each group are combined for analysis in second experiment. (I) Ratio between MoMac and ReMac in islets from the data of (H). **p (CTRL vs anti-PD-1) =0.0026; **p (CTRL vs anti-PD-1+AG)=0.0016.
Figure 15
Figure 16 Activated macrophages acquire cytocidal activity against β cells. (A) Expression of iNOS in peritoneal macrophages treated with LPS (100ng/ml) or IFNγ (100ng/ml) or a combination of both. (B) Cytotoxicity analysis of macrophages treated with different stimuli to β cells (Min6) at various effector to target cell (E:T) ratios. (C) Cytotoxicity analysis when separating macrophages from β cells by a transwell. Results are pooled from 2 independent experiments. (D) Macrophages treated with LPS plus IFNγ or LPS plus IFNβ (100ng/ml) for 18h for cytotoxicity assay. (E) Expression of iNOS in peritoneal macrophages from NOD.IFNγR−/− mice treated with various stimuli. (F) Cytotoxicity analysis comparing NOD wildtype macrophages and IFNγR−/− macrophages. (G) Cytotoxicity analysis of NOD wildtype macrophages treated with nitric oxide inhibitor 1400W (100μM).
Figure 16
Figure 17 Activated macrophages acquire cytocidal activity to β cells. (A) Flow cytometry analysis of peritoneal macrophages from NOD mice. (B) Expression of iNOS (%) in macrophages treated with indicated stimuli for 18h ex vivo. (C) Viability of β cell line (Min6) treated with indicated stimuli for 24h. β cell death was quantified by flow cytometry using cell viability dye.
Figure 17

A

CD45+Live cells

CD11b

Ly6C

F4/80

Peritoneal Macrophages

INDO (%) vs.

Cell viability (%)

Ctrl

LPS (100ng/mL)

IFNγ (100ng/mL)

LPS+IFNγ

β cells (Min6)
Chapter 3: Conclusions and future directions

In the study of autoimmunity, great efforts have been made to tolerize the responses of self-reactive T cells in order to control the disease. The NOD mouse model allows us to study the general regulations in T1D. Insulin has been identified as the prime antigen that drives the development of the disease. The autoimmune diabetes in mice phenocopies the T1D in human. The disease susceptible gene MHC class II molecules are structurally similar in NOD mice and T1D patients. The disease in both mice and human develop spontaneously. And more recently, it has been found that cancer immunotherapy causes fulminant diabetes in patients while the same observations in mice can be seen when NOD mice are treated with anti-PD-1 blocking antibody. These findings indicate an essential role of PD-1 in the regulation of T1D.

In this thesis project, we studied the cellular and molecular mechanisms of PD-1 regulation in autoimmune diabetes. We found PD-1 regulation was established early after T cells were primed. We demonstrated that reducing the islet infiltration of T cells prevented the diabetes development induced by PD-1 blockade. Importantly, we found islet resident macrophages could be targeted. Depletion of islets resident macrophages by anti-CSF1R antibody at early stage dramatically reduced diabetes incidence.

One important information from this project is that we identified an alternative killing mechanism besides that of T cells in diabetes development, and this is especially essential in anti-PD-1 induced diabetes. After unleashing the PD-1 regulation, the exhausted CD8 T cells were highly activated to produce pro-inflammatory cytokines such as IFNγ. The abundant cytokine and chemokine production in the compact
microenvironment in islets led two events: 1) attracted more leukocytes to islets among which monocyte-derived macrophages accounted for a large portion of the infiltrate; 2) activated the myeloid cells such as macrophages and dendritic cells. Dendritic cells can acquire and present antigens to further activate both CD4 and CD8 T cells. Macrophages in response to these pro-inflammatory cues became effector cells and caused the demise of the β cells. In our study, we proved the cytocidal role of the macrophages with high NF-κB and IFNγ signatures. Ex vivo experiments directly demonstrated macrophages activated by LPS and IFNγ killed β cells.

In conclusion, we identified that the macrophage is a major player in contributing diabetes development. On one hand, the islet resident macrophages serve as a beacon for the T cells to enter the islets and become activated. In such a scenario, macrophages could acquire the antigens from the β cells in multiple ways including phagocytosis, micropinocytosis, and granule uptake and then present the insulin peptide to CD4 T cells. It has been shown these CD4 T cells are the first infiltrate in islets. On the other hand, at the later stage of the disease when T cells are activated and islets are largely inflamed, monocyte can be recruited into the islets. These macrophages encounter the pro-inflammatory cues and become cytocidal, cooperating with CD8 T cells to kill the β cells via nitric oxide. Thus, macrophages can be targeted to slow down the progression of autoimmune diabetes. During spontaneous diabetes development, we also observed the infiltration of monocyte-derived macrophages in islets at later stage in NOD mice at 15-16 weeks of age.

There are many unresolved questions regarding to this project and we would like to further pursue the following in the future.
1) How do macrophages cause β cell death in vivo? In chapter 2, we described the cytocidal role of macrophages to β cells ex vivo. Questions will be raised whether the same mechanisms happen in vivo. We performed a pilot experiment using NOD.Rag1−/− mice in order to exclude the effects of T cells. We injected LPS to provide the NF-κB signaling. After 18h, mice were sacrificed. The islets were isolated and IFNγ was added. β cells death was tested by immunofluorescent imaging via propidium iodide staining in situ. There were few or no dead cells in the control, LPS alone treated or IFNγ alone treated islets. But when both signals were activated, the death of β cells took place. This experiment indicates that both NF-κB signaling and IFNγ signaling bring together the death program in vivo.

Another way to interrogate this question is to use genetically modified mouse strains. Because IFNγ signaling is playing a significant role in killing, we will generate conditional Ifngr1 knockout mice by introducing loxP sites flanking the Ifngr1 gene and cross this strain with NOD strains expressing lineage specific Cre, such as Lym-Cre (IFNγR conditionally deleted in macrophages), Xcr-1-Cre (IFNγR conditionally deleted in DC1). The two Cre mice are already generated in our lab. After we generate the strains of interest, we will treat the mice with anti-PD-1 to see whether macrophage specific IFNγR knockout results in diabetes development. In this way, we will have a solid conclusion that macrophages activated by IFNγ are detrimental to β cells.

Why monocyte-derived macrophages are intrinsically more active compared with resident islet macrophages. In order to understand the detailed molecular mechanisms, I will examine the macrophages by bulk RNA sequencing under different conditions: macrophages from non-diabetic NOD background (NOD.H2b mice which express a
different non-diabetic susceptible MHC class II) treated or untreated with anti-PD-1; resident macrophages from untreated NOD mice; resident and monocyte-derived macrophages from anti-PD-1 treated NOD mice. By doing this, we could acquire a differential transcriptional landscape among normal islet resident macrophages, islet resident macrophages at the initial stage of the diabetes and resident macrophages as well as monocyte-derived macrophages at the late stage of the diabetes development. This will help us focus on genes that may contribute to the killing of the β cells.

2) How are the antigen specific T cells regulated? It would be interesting to understand how the autoreactive T cells are regulated during diabetes progression. As we found the exhausted CD8 T cells can be further categorized into precursor exhausted CD8 T cells and terminal exhausted CD8 T cells. Which category will those autoreactive CD8 T cells fall into? How about the CD4 T cells?

To answer this question, we plan to use tetramer-based technology to check the functionality of antigen specific T cells. We have some pilot experiments performed on the insulin specific CD4 T cells. From chapter 1, we hypothesized that 13-21 specific CD4 T cells are deleted by negative selection in thymus induced by mTECs. But when mice were treated with anti-TGFβ or anti-CTLA-4 prior immunization, 13-21 responses can be detected (unpublished data), indicating that 13-21 specific T cells are not deleted in the thymus but regulated in the periphery. We would like to take insulin specific T cells as an example to study the regulation of antigen specific T cells.

In a pilot experiment, we used tetramers to detect the 13-21 and 12-20 specific T cells, respectively. We identified 13-21 specific T cells in the periphery lymphoid tissues, indicating that these cells escape thymic negative selection. Compared with 12-20
specific T cells, most of 13-21 specific T cells are Tregs, expressing Foxp3 and CD25. The Treg differentiation is dependent on insulin expression in thymus since in NOD.\textit{Ins2}$^{-/-}$ mice, 13-21 specific T cells are not Tregs. This adds another layer of T cell regulation besides PD-1/PD-L1 axis discussed in the thesis. I plan to further examine this in the future.

To interrogate whether thymic insulin expression is important for the Treg differentiation of 13-21 specific T cells. We will first test the presentation of insulin peptide in thymus. Three strains of NOD mice can be tested: NOD.wt, NOD.\textit{Ins2}$^{-/-}$, and NOD.B16A (see chapter 1). We can test the presentation of insulin by mTEC, B cells, pDCs, and conventional DCs using hybridoma lines IIT3 (13-21 specific) and 9B9 (12-20 specific). CD69 and IL-2 production will be evaluated by flow cytometry and CTLL reporter cell line to determine the presentation of the insulin peptide.

3) How does the islet microenvironment foster an immunosuppressive cue to induce T cell exhaustion? It is surprising to find that in a very early stage, majority of T cells are polarized to an exhausted phenotype. At later stage, more than 70% of the CD8 T cells are exhausted CD8 T cells based on transcriptional analysis and the expression of the protein markers of exhaustion. Since islets contain the endocrine cells, endothelial cells, and resident macrophages, we could examine these three components to answer this question. First, endocrine cells including the main cell type $\beta$ cells. As with increased T cell infiltration, $\beta$ cells undergo dramatic changes metabolically and immunologically. The altered secretion from the stressed $\beta$ cells or the dead $\beta$ cells may induce an immunosuppressive cue to cause T cell exhaustion. It is not known the influence of metabolites to the exhausted T cell differentiation. This key
question needs further investigation. But recently, vascular endothelial growth factor-A (VEGF-A) has been shown to induce the expression of TOX and T cell exhaustion and VEGF-A is highly expressed by the stressed β cells (Kim et al., 2019). Second, the activated endothelial cells. When becoming activated, the endothelial cells express high level of MHC II and MHC I, which may serve as a platform to cause T cell exhaustion. In addition to this, the endothelial cells may interact with T cells via inhibitory molecules such as PD-L1 and galectin to suppress the T cell function (Nambiar et al., 2019). Third, the macrophages. Recently the Colonna group demonstrated the Trem2+ macrophages in tumor are immunosuppressive. When blocking Trem2, the suppressive macrophages can be shifted into pro-inflammatory macrophages and restored the T cell function and cause tumor rejection (Molgora et al., 2020). Islet macrophages express high levels of Trem2 during the control phase. It would be interesting to block Trem2 using the antibodies provided by the Colonna group to examine if this would accelerate autoimmune diabetes development.
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