Development of Type 1 Diabetes

Monocytes and dendritic cells in the pancreas

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Development of Type 1 Diabetes

Monocytes and dendritic cells in the pancreas

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Monocyten en dendritische cellen in de pancreas

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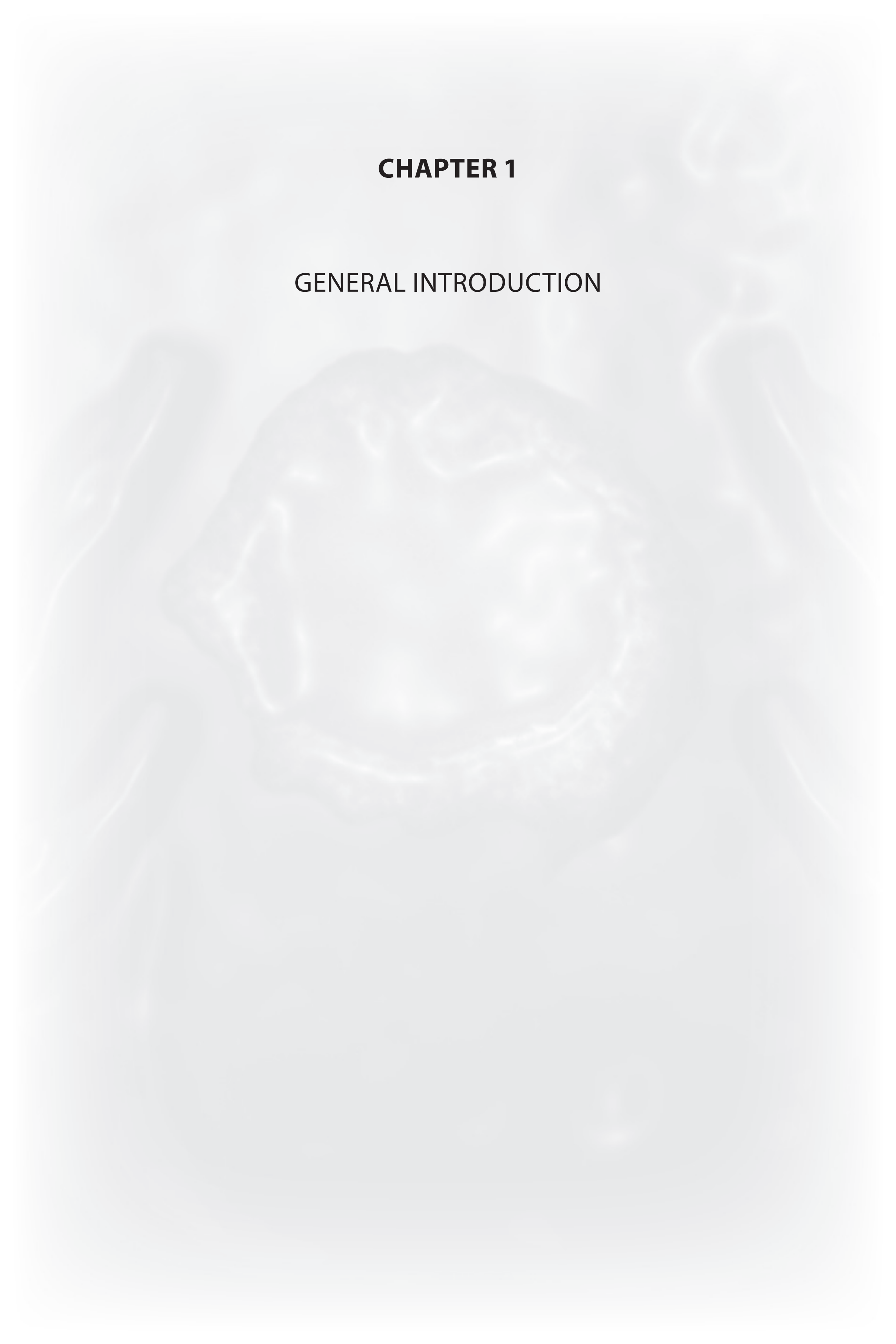

CHAPTER 1

GENERAL INTRODUCTION

General introduction

This thesis focuses on the presence of precursors for dendritic cells and the characterization of dendritic cell subsets in the normal pancreas in mice and humans as well as in the pancreas of the NOD mouse, a type 1 diabetes mouse model. Therefore, we give a short introduction to dendritic cells, the NOD mouse and type 1 diabetes.

Innate and adaptive immunity

The immune system is divided in two arms, the innate and adaptive immune system [1]. These two arms of the immune system have unique properties to protect the host from pathogenic organisms, and to create an effective immune response. The innate immune system is the first line of defense and contains cells and soluble mediators that defend the host from infection by pathogens in a non-specific manner. Besides anatomical barriers (epithelial surfaces) and protein families (complement) the innate immune system contains different cell types, such as granulocytes and macrophages (MΦs) [2].
Adaptive immunity has evolved to provide a broad and finely tuned repertoire of recognition for both self- and nonself-antigens [3]. Adaptive immunity involves a regulated interaction between antigen-presenting cells (APCs) and T and B lymphocytes, which facilitate pathogen-specific immunologic effector pathways, generation of immune memory and regulation of host immune homeostasis [4, 5].

Dendritic cells

Dendritic cells (DCs) are APCs which interface on both innate and adaptive immunity. They are crucial for the innate and adaptive immune response to infections and for maintaining immune tolerance to self-tissues and commensal microorganisms [6]. DCs can be divided into two main subtypes: conventional DCs (cDCs) and plasmacytoid DCs (pDCs).
cDCs are $CD11c^+$ and are divided into two main classes based on their localization in tissues and migratory properties: migratory cDCs and lymphoid tissue-resident cDCs. Migratory cDCs are immature and sample antigens in peripheral tissues and subsequently migrate as veiled cells to local lymph nodes (LNs) via the afferent lymphatics and develop into mature or semi-mature cDCs [7, 8]. Semi-mature cDCs are thought to induce tolerance and mature cDCs primarily induce immunity and have a high expression of co-stimulatory molecules and MHC class II [9]. Lymphoid tissue-resident cDCs are found in lymphoid organs, such as LNs, which play a major role in priming $CD4^+$ and $CD8^+$ T cells.
pDCs are found in the bone marrow (BM), blood, secondary lymphoid organs and re-enter the blood before homing to other lymphoid organs in both steady-state and inflammatory conditions [10]. Unlike migratory cDCs, which enter LNs via the afferent lymphatics, pDCs enter LNs through high endothelial venules (HEV) [11-13]. Yet, similar to cDCs, pDCs are in

the steady state immature. pDCs need to be activated to start releasing massive amounts of IFN type I and/or take up and present antigens [14].
The different DC subsets will be described more in detail in the following paragraphs, but first the origin of DCs will be discussed.

Origin of DCs

The development of DCs from myeloid precursors in the BM has been studied extensively. Such DCs (myeloid DCs) arise in the BM from a common myeloid progenitor (CMP), which develops into the so called MΦ and DC precursor (MDP) (Fig.1). The MDP gives rise to monocytes or via the common DC precursor (CDP) to cDCs or pDCs [15, 16]. The CDP is restricted to produce cDCs (via the precursor DCs) and pDCs, but not monocytes [17, 18]. Although most of the cDCs are from myeloid origin, some DCs have a lymphoid origin (Fig.1) and arise from a common lymphoid progenitor (CLP), which develops into cDCs or pDCs [19, 20].

Monocyte-derived DCs

Besides the generation of DCs via the CDP, monocytes can also give rise to cDCs, the so-called monocyte-derived DCs. Mouse blood monocytes can be divided into two major subsets: immature $Ly6C^{hi}CD43^{+}$ ($CD14^{hi}CD16^{-}$ in humans) and mature $Ly6C^{low}CD43^{hi}$ monocytes ($CD14^{+}CD16^{hi}$ in humans) [21, 22]. Recently, immature and mature monocytes were redefined as classical and nonclassical monocytes, respectively [23]. Mouse monocytes express the macrophage colony-stimulating factor receptor (M-CSFR), which is essential for their development, and the CX3C-chemokine receptor 1 (CX_3CR1) [21, 24]. It has been shown that classical monocytes are $CCR2^{hi}CX_3CR1^{low}$, whereas nonclassical monocytes are $CCR2^{low}CX_3CR1^{hi}$ [22, 25].
Classical $Ly6C^{hi}$ monocytes are thought to diapedese to inflamed tissues and differentiate into the so called inflammatory TNF-α/iNOS producing DCs (TipDCs) [22, 24-26]. Classical monocytes can also contribute to the renewal of several resident subsets of MΦs and DCs, such as mucosal DCs and lung DCs, or develop into nonclassical $Ly6C^{low}$ monocytes [16, 24, 27].
The nonclassical $Ly6C^{low}$ monocytes patrol blood vessels in the steady state and are also capable of extravasating during inflammation [28]. It has in addition been described that $Ly6C^{low}$ monocytes differentiate into tolerogenic DCs and induce T cell tolerance through programmed death ligand 1 (PD-L1) [29].

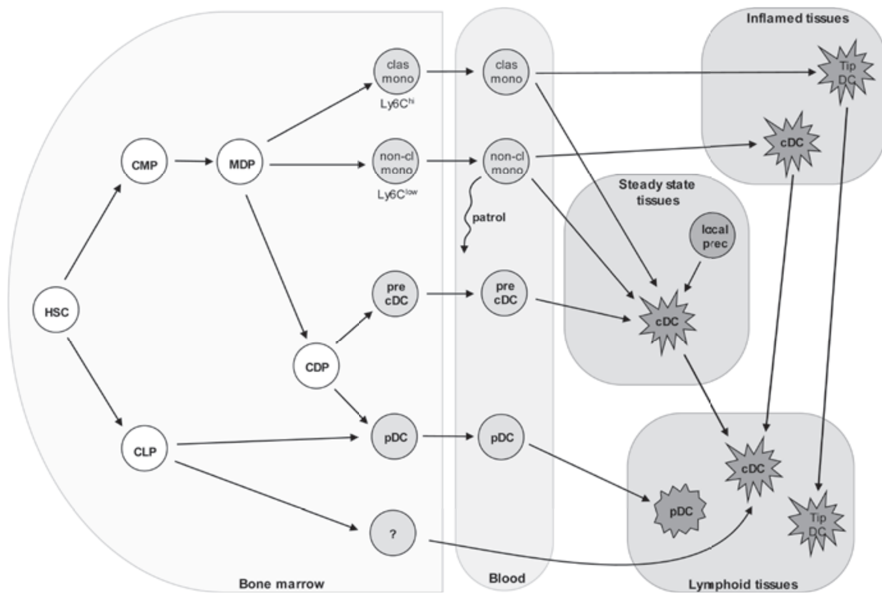

Figure 1. Hematopoietic scheme for DC development.

Hematopoiesis is initiated in the bone marrow (BM) by hematopoietic stem cells (HSCs). HSCs differentiate into common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). CMPs then further differentiate into macrophage DC progenitors (MDPs), which differentiate into classical monocytes (clas mono), nonclassical monocytes (non-cl mono) or common DC progenitors (CDPs). Classical monocytes (Ly6C^{hi}) migrate to inflamed tissues or steady state non-lymphoid tissues and differentiate into inflammatory DCs (Tip-DCs) or conventional DCs (cDCs) respectively. Nonclassical monocytes (Ly6C^{low}) patrol blood vessels in the steady state and migrate to inflamed tissues or steady state non-lymphoid tissues lymphoid tissues and differentiate into cDCs. CDPs differentiate into pre-cDCs or plasmacytoid DCs (pDCs). Pre-cDCs migrate to non-lymphoid tissues and differentiate into cDCs. pDCs migrate to lymphoid tissues. In non-lymphoid tissues local precursors (local prec) can also differentiate into cDCs. Finally, after antigen uptake both Tip-DCs and cDCs in steady state tissues and/or in inflamed tissues migrate to lymphoid tissues to present antigens to naïve T cells.

Local precursors for DCs in tissues

Apart from the development of DCs via CDPs in the BM or from monocytes, DCs also arise from precursors in non-lymphoid peripheral tissues. In organs, such as the skin and brain, local precursors for MΦs and Langerhans cells have been detected [30-32]. We earlier described the presence of local precursors for MΦs in the fetal pancreas of C57BL/6 mice [33]. A study of local pancreatic precursors for DCs will be an important part of the thesis.

The described origin of DCs reflects the current state of the art research (depicted in Figure 1) and is under ongoing debate and investigation. It has to be noted that DCs with a comparable phenotype can have different origins.

Conventional DCs

cDCs in immunity

As mentioned previously, cDCs can be divided into two main classes: migratory cDCs and lymphoid tissue-resident cDCs. Migratory cDCs, which are immature, can be identified as $CD11b^+$ DCs and $CD11b^-$ DCs, which express CD103 (integrin αE) [34, 35]. Migratory cDCs are highly phagocytic and take up foreign- or self-antigens. This antigen is processed and coupled to MHC molecules on the cell surface, which is required for the presentation of antigen to either $CD4^+$ or $CD8^+$ T cells [36]. cDCs use several receptors or mechanisms to acquire and present antigen, such as the mannose- or Fc-receptors and Toll-like receptors (TLRs), which indirectly or directly recognize microbial products [37-39]. cDCs can also be activated by pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) [37]. Once activated, cDCs upregulate co-stimulatory molecules (CD80 and CD86) for productive T cell stimulation, downregulate the expression of the chemokine receptors CCR1, -2 and -5, and upregulate CCR7, which promotes migration to draining LNs [40-42]. After activation cDCs are capable of producing cytokines, such as IL-1β, IL-6, IL-12 and TNF-α [43], in considerable amounts.
Lymphoid tissue-resident cDCs can be further classified by the expression of the surface markers CD4 and CD8α into $CD4^+$ cDCs, $CD8\alpha^+$ cDCs and $CD4^-CD8\alpha^-$ cDCs [44, 45]. $CD8\alpha^+$ cDCs have a high capacity to cross-present antigens on MHC class I and play a major role in priming cytotoxic $CD8^+$ T cell responses [46-48]. This cDC subset is also thought to be involved in tolerance induction during steady state [49-51] (See paragraph cDCs in tolerance). $CD4^+$ cDCs and $CD4^-CD8\alpha^-$ cDCs are more efficient in presenting antigens to $CD4^+$ T cells by MHC class II [52-54].

cDCs in tolerance

cDCs are not only immunogenic but can also induce antigen specific unresponsiveness or tolerance. The tolerogenic function of cDCs involves various mechanisms, including induction of T cell anergy, immune deviation, regulatory T (Treg) cell activation and promotion of apoptosis of activated T cells. Recently, cDC populations involved in tolerance induction have been identified and aberrations in these tolerogenic cDCs may contribute to the development of autoimmunity [55]. In the mouse the $CD8\alpha^+$ cDCs are thought to be involved in tolerance induction during steady state [49, 51]. It has been described that $CD8\alpha^+$ cDCs are mainly generated by fms-like tyrosine kinase 3 ligand (Flt3L) stimulation [51]. Interestingly, Flt3L injection protected nonobese diabetic (NOD) mice from type 1 diabetes (T1D) by enhancing Treg cells frequency in the pancreas draining LNs (pLNs) and by modulating the balance of cDC subsets towards $CD8^+$ cDCs [50, 56].
Other surface molecules expressed by cDC populations that are suggested to be involved with tolerogenic functions are CD103, Langerin and CCR5. $CD103^+$ cDCs and their function

are well characterized in mucosal murine tissues and secondary lymphoid organs [57-60]. $CD103^+$ cDCs isolated from the gut and mesenteric LNs are capable of driving the differentiation of Foxp3-expressing Treg cells via a TGF-β dependent mechanism [61]. Furthermore, $CD8\alpha^+CD103^+Langerin^+$ cDCs isolated from the spleen were predominantly involved in phagocytosis of apoptotic cells and presented cell-associated antigens to T cells, in order to induce self-tolerance [62].
With regard to CCR5, in a mouse model for experimental autoimmune encephalitis (EAE), oral tolerance could be induced in wild-type, but not in $CCR5^{-/-}$ mice [63]. In addition, $CCR5^{-/-}$ mice show increased levels of IL-12 in the gut associated lymphoid tissue. Recently, it has been described that the development of diabetes is accelerated in $CCR5^{-/-}$ NOD mice [64]. Furthermore, cDCs isolated from NOD salivary glands lack CCR5, in contrast to their counterparts in control mice [65]. Collectively these studies point to a pivotal role for $CCR5^+$ cDCs in tolerance induction.
Regarding the molecular pathways which are involved in DC tolerance several molecular interactions have been unraveled. Fas-FasL interactions induce apoptosis [66], PD-1:PD-L interactions deliver inhibitory signals to T cells [67, 68] and depletion of tryptophan promotes activation-induced cell death by the enzyme indoleamine 2,3 dioxygenase (IDO) [69]. In autoimmunity, apoptosis of potentially autoreactive lymphocytes by IDO-expressing cDCs could maintain peripheral tolerance [70]. Recent evidence suggests that Treg cells can induce tolerogenic cDCs to promote autocrine activation of IDO [71]. Also cytokines, such as IL-10, IFN-γ and TGF-β, can actively induce tolerance by inducing $Foxp3^+$ Treg cells or by depleting reactive T cells [72-75]. Finally, interaction between CD80/CD86 on cDCs and CTLA-4 on Treg cells is important for proper function of the Treg cells [76, 77].

Plasmacytoid DCs

pDCs in immunity

pDCs are a recently included member of the DC family [78-80]. pDCs have been known for several decades as "plasmacytoid T cells" or "natural interferon-producing cells" [81-84]. pDC activation is mediated through TLR7 and TLR9 stimulation by CpG nucleotides, which results in IFN type I production and/or antigen uptake followed by processing and presentation to T cells [85, 86]. Their function under various pathological conditions is still ill-defined, but the ability to secrete large amounts of type I interferons (IFN) in response to encountered viruses has defined them as initiators of the adaptive immune response in viral infections [87-90]. Although pDCs are capable of producing high amounts of IFN type I, they play an important role as APCs as well. When given peptide Ag in a mixed leukocyte reaction, pDCs potently stimulate proliferation of allo-specific T cells [91-93]. In addition, the presence of Ag-specific immunoglobulins stimulates the uptake of Ag by human pDCs and promotes

the presentation to $CD4^+$ T cells or $CD8^+$ T cells [94, 95]. According to some studies, pDCs only present endogenous antigen and fail to cross-present exogenous antigens to $CD8^+$ T cells [96, 97]. A recent study shows that highly purified BM-derived mouse pDCs also take up, process and present soluble antigen to antigen-specific T cells in the presence of serum components, albeit at lower levels than cDCs [98].
In the mouse pDCs are characterized as $CD11b^-CD11c^{low}B220^+Ly6C^+$ [99]. A more precise definition of mouse pDCs has been obtained using the pDC-specific molecules plasmacytoid dendritic cell antigen-1 (PDCA-1) and Siglec-H [100, 101].

pDCs in tolerance

It is known that human pDCs induce development of regulatory T cells in vitro [102]. A tolerogenic function of mouse pDCs has also been found in several experimental models. In allergic-asthma mouse models and after allogeneic hematopoietic stem cell or heart transplantation tolerance is maintained by pDCs [103-105]. A tolerogenic role of pDCs has been suggested in models for autoimmune diseases as well. In the NOD pancreas pDCs could be depleted and this led to an acceleration of insulitis and a loss of local IDO [106] (see also paragraph The NOD mouse model, Involvement of pDCs in NOD insulitis). A recent study also showed the protective role of pDCs against the development of diabetes after infection with lymphocytic choriomeningitis virus (LCMV) [107]. They showed that pDCs produced TGF-β and thereby inducing the conversion of naive anti-islet T cells into Treg cells. A mouse model for systemic lupus erythematosus (SLE) showed that a low-dose peptide tolerance therapy stimulates pDCs to produce TGF-β and induces autoantigen-specific Treg and suppression of inflammatory Th17 cells in the kidneys [108]. Furthermore, a recent study of rheumatoid arthritis in mice also shows that depletion of pDCs in vivo enhanced the severity of arthritis and enhanced T and B cell autoimmune responses against type II collagen [109].
pDCs are also capable of inducing tolerance by the suppression of other DCs and T cells. In a model of allergic inflammation pDCs suppress the generation of effector T cells induced by cDCs [105]. pDCs could also stimulate the formation of Treg cells in an ICOS-L-dependent way [110].

Type 1 diabetes

Clinical characterization and incidence

Diabetes is a metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion and/or insulin action. Two main types of diabetes have been characterized. Type 2 diabetes (T2D) results from insulin resistance, a condition in which cells fail to use insulin properly. T2D is the most common form of diabetes and is primarily due to lifestyle factors and genetics. T1D is characterized by autoimmune-mediated

destruction/silencing of pancreatic beta cells resulting in insulin deficiency. The onset of T1D is associated with infiltration of the islets of Langerhans by mononuclear leukocytes (insulitis). The autoimmune pathogenesis is further illustrated by several auto-antibodies specifically associated with the disease, such as islet cell antibodies (ICA), glutamic acid decarboxylase (GAD)-65 antibodies and insulin auto-antibodies (IAA) [111]. Both T1D and T2D result from a complex interaction of genetic and environmental factors (diet and viral infections).
The classical symptoms of diabetes are polyuria (frequent urination), excessive thirst, hunger and fatigue. Symptoms may develop rapidly (weeks or months) in T1D, while they usually develop much more slowly and may be subtle or absent in T2D. In all forms of diabetes there is an increased risk of long-term complications. The major long-term complications are related to damage of blood vessels, like cardiovascular disease, retinopathy, neuropathy and nephropathy.
Globally, as of 2010, an estimated 285 million people have diabetes, especially T2D (90%). The incidence is increasing rapidly, and by 2030, this number is estimated to almost double. T1D has a large regional variation in incidence, varying from 10.3 cases per 100,000 per year in Lithuania to 52.6 cases per 100,000 per year in Finland [112]. In the Netherlands the incidence of T1D is 18.6 per 100,000 per year [113]. In Europe, between 1989 and 2003, the overall increase in incidence was 3.9%. In a study of T1D incidence, a doubling of new cases of T1D in European children younger than 5 years has been predicted from 2005 to 2020, and the prevalence of cases younger than 15 years will rise by 70% [112]. T1D is treated with insulin replacement therapy, either via subcutaneous injection or insulin pump, along with attention to diet, and careful monitoring of blood glucose levels using glucose meters. Untreated T1D commonly leads to coma, often from diabetic ketoacidosis, which is fatal if untreated.

Symptoms of T1D often occur when the insulitis and beta cell destruction/silencing are more or less at the end stage, which makes it very difficult to study the pre-diabetic phase in humans. Therefore, animal models, such as the NOD mouse, are often used to study the development of autoimmune diabetes. Although the autoimmune diabetes development in animal models is not completely comparable with the development in T1D patients, this is still the most suitable approach. The NOD mouse model will be discussed in more detail in the next paragraph.

The NOD mouse model

History

The nonobese diabetic (NOD) mice is an inbred mouse strain developed in a breeding program to establish a cataract-prone subline (CTS) from non-inbred ICR (CD-1) mice [114]. The NOD mouse strain is not cataract-prone, but the first case of insulin-dependent diabetes was observed in a NOD female at the 20th generation of inbreeding. After a further six generations of inbreeding, a cumulative diabetes incidence by 30 weeks of age of 60-80% in females versus only about 20% in males was observed [114].

Clinical features of diabetes in NOD mice are quite similar to human T1D and include abrupt onset between 12 and 17 weeks (equivalent to early adolescence period in humans). However, discrepancies between mice and humans have been described as well, such as the absence in the mouse of auto-antibodies against islet-antigens other than insulin [115, 116]. Also the insulitis in humans is less invasive as compared to the insulitis in the NOD model [117]. Furthermore, the male/female ratio in humans is approximately 50/50, while in mice this ratio is around 10/90 [114, 118].

Besides the development of autoimmune diabetes in NOD mice these mice develop several other autoimmune diseases. NOD mice develop lymphocytic infiltrations in the submandibular gland [119], a declined salivary flow [120] and increased number of anti-nuclear antibodies and anti-SS-A antibodies [121], similar to those seen in Sjögren's syndrome patients. Other autoimmune inflammations are observed in the colon and thyroid gland [122, 123]. Furthermore, immune-mediated hearing loss is observed [124]. It is known that the NOD mouse is also susceptible to EAE [125] and shows exacerbation of asthma in an OVA-induced model [126].

NOD-related strains

Related mouse strains that are often used as control strains for the NOD mouse are NOD/scid, NON/Lt and NOR/Lt mice.

NOD/scid mice were generated by backcrossing the scid (severe combined immune deficiency) mutation with NOD mice [127]. These mice lack functional lymphocytes and do not develop diabetes [127]. NOD/scid mice are widely used for adoptive transfer of diabetes via lymphocyte transplantation [128].

NON/Lt (nonobese non-diabetic) mice are closely related to NOD mice as they have been developed from the ICR strain [129]. However, these mice do not develop diabetes, although small leukocyte infiltrations can be present in the pancreas [129].

The NOR/Lt (nonobese diabetic resistant) strain is a diabetes-free strain produced from an outcross between NOD and C57BL/KsJ followed by a backcross to the NOD background [130]. These diabetes-resistant mice share 88% of the NOD genotype, including the

diabetogenic MHC H-2g7 haplotype [130]. NOR mice exhibit the same peri-insulitis, with DC and lymphocyte accumulation, characteristic of NOD mice, but do not develop diabetes. In addition to their diabetes resistance, NOR mice are distinguished from NOD mice by exhibiting a more robust suppressor T cell function in an allogeneic mixed leukocyte reaction [130].

Insulitis

NOD mice develop spontaneous autoimmune diabetes (around 15-20 weeks of age) that is preceded by peri- and intra-insulitis (from 4-5 weeks of age onwards). Before the insulitis (at 3 weeks of age) an increase in perivascular and periductular extracellular matrix (ECM) is observed. At this age cDCs and MΦs are the first mononuclear leukocytes present in the perivascular para-islet connective tissue but not in the islets. From 4-5 weeks of age onwards cDCs and MΦs start to accumulate at the islet edges (Fig.2). From 10 weeks onwards large numbers of T and B cells surround the islets and finally cDCs, MΦs and lymphocytes infiltrate the islets. At that time the beta cells are vanished, which lead to massive loss of beta cells and overt diabetes [131-136].

Several studies described the diabetogenic role for cDCs in the initiation of T1D [137]. Depletion of phagocytic cells (including cDCs) in the NOD pancreas using clodronate-loaded liposomes results in the resolution of inflammatory infiltrates and a delayed diabetes development [138]. In addition, depletion of cDCs in the early phase of NOD insulitis, using a diphtheria toxin model, shows that DCs are crucial to the development of diabetes in the NOD mouse [106]. Furthermore, several reports have suggested that cDCs from NOD mice have increased ability to activate T cells through higher IL-12 production and co-stimulatory molecule expression [139, 140].

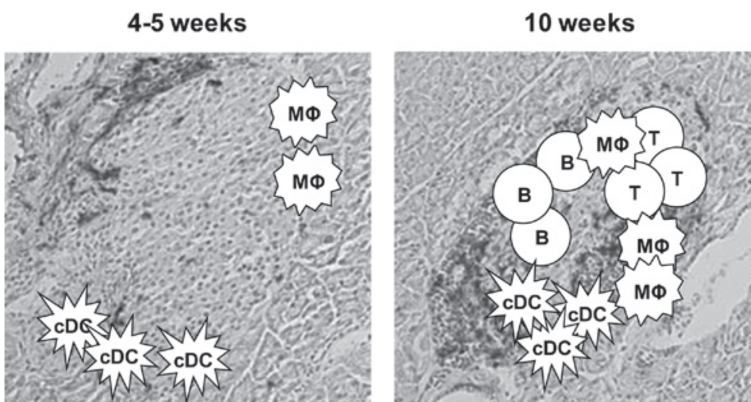

Figure 2. Insulitis development in the pancreas of NOD mice.

Early abnormalities in the islet milieu of the NOD mouse

Besides the influx of immune cells in the pancreas of NOD mice there is evidence that abnormalities in islet function and islet micro-environment are present at the start of autoimmune insulitis. Intriguingly, the first islet anomalies that were described were already present at birth. These anomalies are: higher numbers of paradoxically hyperactive beta cells with pre-proinsulin II expression, high percentages of immature islets, elevated levels of APCs and $FasL^+$ cells, and abnormalities of ECM protein expression [33, 141-145]. These data show that from birth onwards both islet-endocrine and islet-immune abnormalities are present in the NOD mouse. These data suggest that tissue-specific autoimmune reactions could arise from developmental phenomena taking place during fetal life in which ECM-immune cell interaction may play a key role. This subject is part of my thesis questions.

Early abnormalities in the differentiation of monocytes and DCs in NOD mice

Monocyte abnormalities

In the NOD mouse several monocyte abnormalities have been described. NOD mice, as well as NOR mice, have an abnormally high number of nonclassical (mature) $Ly6C^{low}$ monocytes in the circulation [146]. In addition, although monocytes from C57BL/6 mice downregulated their capability to adhere to fibronectin and intercellular adhesion molecule-1 (ICAM-1) upon maturation, the nonclassical (mature) NOD monocytes retained the high adhesive capacity that is characteristic of immature cells [147]. Both monocyte subsets of NOD mice show enhanced differentiation into MΦ-like cells in vitro [146]. Furthermore, NOD mouse monocytes show a decreased migration in vivo towards the pro-inflammatory chemokines CCL2 and -3, but an increased migration towards the lymphoid tissue-related CCL19 [148].

DC abnormalities

DC precursors in BM of NOD mice show proliferation/differentiation abnormalities and from these precursors abnormal DCs arise with a spontaneous high pro-inflammatory set point [149]. These abnormal DCs have a high level of NFkB, a high acid phosphatase content, a high IL-12 and low IL-10 expression [139, 150, 151]. These DCs are incapable of sufficiently sustaining the proliferation of Treg cell populations in the NOD mouse [152]. It has been shown that correction of these DC abnormalities prevents the development of autoimmune diabetes [153, 154]. In addition, DCs of NOD mice show an increased fibronectin adhesion in vitro [155].

The involvement of pDCs in NOD insulitis

A recent study used the BDC2.5/CD11c-DTR/NOD transgenic model to investigate the effect of pDC ablation on the insulitis development. At the time when these mice have ongoing

peri-insulitis pDC depletion increases the rate and severity of insulitis and injection of pDCs resulted in an inhibition of the progression of insulitis [106]. In this study, a role for IDO in the insulitis regulation was indicated by the treatment with an IDO inhibitor. However, pDCs have also been implicated in a disease-promoting role in the NOD mouse: IFN-α^{+} pDCs were increased in the pLNs of 4 week old NOD mice and an antibody to the IFN-α receptor was able to halt the diabetogenic process [156, 157]. Recently, it was shown that in the NOD pancreas at 3 weeks IFN-α expressing pDCs were present in the islets [158].

Aim of the thesis

Over the past years we developed the following two hypotheses:

1. The pancreas contains precursors for cDCs. Pancreatic DCs are not necessarily descendant from infiltrated monocytes or pre-cDCs, but can also be generated from these local precursors.
2. In autoimmune diabetes (such as in the NOD mouse) local pancreatic precursor cells are aberrant and generate pro-inflammatory and non-tolerogenic DCs, which accumulate at the islet edges to start the autoimmune insulitis.

These hypotheses are based on several observations done in the past decade in our group:

1. Local precursors for MΦs have been detected in the fetal pancreas of mice [33].
2. Treatment with clodronate-loaded liposomes causes a depletion of monocytes from the blood and of phagocytic cells (including MΦs and cDCs) from the pancreas. In the NOD mouse there is only late re-appearance (28 days post-injection) of cDCs and MΦs in the pancreas, at a time when these cells had already repopulated the circulation and the spleen (7 days post-injection) [138]. This indicates that the circulating monocytes are not likely the precursors of these pancreatic DCs and MΦs.
3. Furthermore, NOD mouse monocytes show a decreased in vivo migration towards the pro-inflammatory chemokines CCL2 and -3 [148]. In addition, the pro-inflammatory chemokine CCL2 that normally attracts classical monocytes to tissues is absent in the NOD pancreas [155].

Together these results suggest that the pancreas contains precursors not only for MΦs, but also for cDCs. In the NOD mouse BM precursors for DCs are abnormal in proliferation and apoptosis and generate pro-inflammatory DCs [149]. We thus extrapolate these observations to the pancreas precursors of the NOD mouse and assume that these are equally abnormal. To investigate our first hypothesis, the presence of precursors for cDCs was studied in the normal fetal and neonatal pancreas in mice and humans (see **Chapter 2** and **3**). We also investigated the fetal and neonatal pancreas of NOD mice, since we hypothesized that the NOD mouse pancreas contains abnormal local precursors giving rise to pro-inflammatory cDCs that accumulate around the islets of the NOD pancreas (see **Chapter 2**).

Apart from the studies on the presence of pancreatic cDC precursors, we investigated the composition of the DC population in the pancreas of NOD mice before and during the insulitis process. We focused on the cDC (see **Chapter 4**) and pDC population (see **Chapter 5**) in the pancreas of NOD mice.

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CHAPTER 2

ABNORMALITIES OF DENDRITIC CELL PRECURSORS IN THE PANCREAS OF THE NOD MOUSE MODEL OF DIABETES

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Abstract

The non-obese diabetic (NOD) mouse is a widely used animal model for the study of human diabetes. Before the start of lymphocytic insulitis, dendritic cell (DC) accumulation around islets of Langerhans is a hallmark for autoimmune diabetes development in this model. Previous experiments indicated that an inflammatory influx of these DCs in the pancreas is less plausible. Here we investigated whether the pancreas contains DC precursors and whether these precursors contribute to DC accumulation in the NOD pancreas.

Fetal pancreases of NOD and control mice were isolated followed by FACS using ER-MP58, Ly6G, CD11b and Ly6C. Sorted fetal pancreatic ER-MP58^{+} cells were cultured with GM-CSF and tested for DC markers and antigen processing. CFSE labeling and Ki-67 staining were used to determine cell proliferation in cultures and tissues.

Ly6C^{hi} and Ly6C^{low} precursors were present in fetal pancreases of NOD and control mice. These precursors developed into CD11c^{+}MHCII^{+}CD86^{+} DCs capable of processing DQ-OVA. ER-MP58^{+} cells in the embryonic and pre-diabetic NOD pancreas had a higher proliferation capacity.

Our observations support a novel concept that pre-diabetic DC accumulation in the NOD pancreas is due to aberrant enhanced proliferation of local precursors, rather than to aberrant "inflammatory infiltration" from the circulation.

Introduction

The non-obese diabetic (NOD) mouse is used as a spontaneous model to study the development of type 1 diabetes [1]. Lymphocytes accumulate around and in the islets of Langerhans in NOD mice from around 6 weeks of age onwards, which results in the destruction of β-cells followed by a decrease in insulin production leading to diabetes. Prior to T and B cell accumulation the number of dendritic cells (DCs) increases in the pancreas and concentrates around the islets (from the age of 5 weeks onwards) [2, 3]. DCs are potent antigen presenting cells capable of stimulating both naïve and memory T cells [4]. The observation that DCs are the first immune cells to increase in number in the NOD pancreas points to a crucial role for DCs in the initiation of the islet autoimmune reaction. Such a role was recently proven by the demonstration that a temporal depletion of DC totally abrogated the development of insulitis and diabetes in the NOD mouse model [5].

Early studies have shown that bone marrow (BM) precursors give rise to monocytes in blood, which circulate for a few days before they migrate into tissue where they develop into different types of DCs and macrophages. Blood monocytes can be subdivided into at least 2 subsets based on their Ly6C expression: classical and nonclassical monocytes. The classical monocytes, which are $Ly6C^{hi}$, are selectively recruited to inflamed tissues and lymph nodes and differentiate into inflammatory DCs [6]. The nonclassical monocytes, which are $Ly6C^{low}$, patrol the endothelium of the blood vessels and are required for rapid tissue invasion at the site of an infection [7]. $Ly6C^{low}$ monocytes are considered $CD11c^{-}$, but some studies have reported the expression of CD11c on these cells [6, 8]. Both types of monocytes are $F4/80^{+}$ and $CD86^{-}$ [6].

Data are accumulating on the presence of local tissue precursors for DCs and macrophages and the contribution of these precursors to DC and macrophage accumulation under pathological conditions. In organs, such as the skin and brain, local precursors for macrophages and Langerhans cells have been detected [9-11]. We earlier described the presence of local precursors for macrophages in the fetal pancreas of C57BL/6 mice [12]. However, little is known about the origin of the DCs that accumulate in the pre-diabetic NOD pancreas and the factors driving this accumulation. It is generally assumed that these cells are inflammatory in nature and infiltrate from the circulation. However, previous studies from our group suggest that the early accumulation of DCs in the pre-diabetic NOD pancreas can not only be explained by a massive influx of DCs and DC precursors from the blood. Firstly, pro-inflammatory chemokines that normally attract monocytic cells (CCL2 and CCL3) could not be detected in the pancreas at the time of DC accumulation [13]. Secondly, DCs and monocytes of NOD mice have an impaired migration towards pro-inflammatory chemokines in vivo and in vitro [13], although the contribution of other chemokines cannot be excluded. Finally, the depletion of phagocytic cells with clodronate resulted in a late re-

appearance of DCs in the NOD pancreas (28 days after depletion), while monocytes and DCs had already re-appeared in the blood and spleen 4 days after depletion. This late re-appearance suggests that pancreatic DCs are not only replenished from the circulation [14]. We therefore hypothesized that local precursors for DCs are present in the pancreas and that an enhanced proliferation and differentiation of these cells is responsible for the enhanced accumulation of pancreatic DCs initiating the islet autoimmune reaction.
In this study, the presence of local pancreatic precursors for DCs, their proliferative capacity and the actual generation of DCs from these pancreatic precursors was investigated in the fetal pancreas and the pre-diabetic pancreas of NOD and control mice.

Materials and methods

Animals

C57BL/6 mice were obtained from Charles River Laboratories (Maastricht, The Netherlands), BALB/c mice from Harlan (Horst, The Netherlands) and NOR/LTj mice from the Jackson Laboratory (Bar Harbor ME, USA). NOD/LTj mice were bred in our own facility under specified pathogen free conditions. Breedings were done from the age of 8 weeks and older. The appearance of the vaginal plug was noted as E0.5. Pregnant mice were sacrificed and embryos dissected at embryonic age of E15.5. BM cells were isolated from the femora from mice of 8 weeks. All mice were female and were supplied with water and standard chow ad libitum. Experimental procedures were approved by the Erasmus University Animal Ethical Committee.

Preparation of cell suspensions

Embryonic (E15.5) pancreas (pooled) and liver were isolated and micro-dissected from the stomach and digested with Collagenase Type 1 (1 mg/ml), hyaluronidase (2 mg/ml) (both Sigma Aldrich, St. Louis, MO, USA) and DNAse I (0.3 mg/ml) (Roche Diagnostics, Almere, The Netherlands) for 10 minutes at 37ºC. Embryonic pancreas and liver cells were flushed through a 70 µm filter and washed. Pancreases of 5 week old mice were isolated after a cardiac perfusion and cut into small pieces and digested with Collagenase Type 1, hyaluronidase and DNAse I for 40 minutes at 37ºC. Cells were flushed through a 70 µm filter and washed. Blood of 4 week old mice was collected in EDTA tubes using a heartpunction. Erythrocytes were lysed with $NHCL_2$ buffer and washed. Single-cell suspensions of BM were prepared as described previously [15]. All cells were resuspended in PBS containing 0.1% BSA and were ready for flow cytometry staining.

Flow cytometry

Single-cell suspensions from pancreas (E15.5 and 5 weeks) were labeled with mAbs. Antibodies used were ER-MP58-biotin (own culture), Ly6C-FITC (Abcam, Cambridge, UK), Ly6G-Pacific Blue (Biolegend, Uithoorn, The Netherlands), CD11b-allophycocyanin-Cy7, CD86-PE (both Becton Dickinson, San Diego, CA, USA), CD11c-allophycocyanin, CD11c-PE, CD11c-PE-Cy7, CD86-Pacific Orange, F4/80-PE-Cy5 (all eBiosciences, San Diego, CA, USA), MHC class II-PE (C57BL/6, clone M5/114, Becton Dickinson) and MHC class II-biotin (NOD clone 10.2.16, own culture). Afterwards cells were washed and incubated with streptavidin-allophycocyanin (Becton Dickinson). To detect proliferation, the cells were fixed in 2% paraformaldehyde, and permeabilized using 0.5% saponin. Subsequently, cells were incubated with Ki-67-FITC (Becton Dickinson) diluted in 0.5% saponin, washed and resuspended in 0.1% BSA. Cells suspensions were analyzed using a FACS Canto HTSII (Becton Dickinson) flow cytometer and FACS Diva and Flowjo software.

Endocytosis assay

Antigen processing was determined by measurement of the fluorescence upon proteolytic degradation of the self-quenched conjugate DQ-Ovalbumin [16]. Briefly, cells were resuspended in PBS with 2% FCS and 100 μg/ml DQ-Ovalbumin (Molecular Probes, Breda, The Netherlands) and incubated for 30 minutes at 37ºC. Cells were washed and incubated with CD11c-allophycocyanin and analyzed by flow cytometry.

Cell sort experiments

Cells from the embryonic pancreas (pooled), liver and adult BM were incubated with ER-MP58-biotin (own culture) and afterwards with streptavidin-allophycocyanin (Becton Dickinson). ER-MP58^{+} cells were sorted with FACSAria (Becton Dickinson). Subsequently, ER-MP58^{+} cells were cultured for 8 days on 0.5% gelatin coated wells (96 wells plate) in RPMI 1640 medium supplemented with 10% FCS, 50 μM beta-mercaptoethanol and 50 ng/ml GM-CSF (MT Diagnostics, Etten-Leur, the Netherlands). Finally cells were harvested with 2 mM EDTA.

CFSE labeling

To monitor the proliferation capability, cells from the embryonic pancreas (pooled), liver, adult BM and blood were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Sigma Aldrich) and incubated for 10 minutes at 37ºC. Cells were washed and incubated with ER-MP58-biotin and afterwards with streptavidin-allophycocyanin. ER-MP58^{+} cells were sorted with FACSAria and were cultured for 8 days with 50 ng/ml GM-CSF. Cells were harvested with 2 mM EDTA.

Immunofluorescence

Cryostat sections (6 μm) of E15.5 pancreases from C57BL/6 and NOD/LTj mice were prepared and fixed with cold methanol and acetone. Slides were incubated with guinea pig-anti-insulin (DAKO, Glostrup, Denmark) and rat-anti-ER-MP58 followed by rabbit-anti-guinea pig-FITC (Abcam) and goat-anti-rat-TexasRed (Southern Biotechnology Associates Inc., Birmingham, AL, USA). Finally, slides were mounted in Vectashield with DAPI (Vector Laboratories Inc., Burlingame, CA, USA).
Cryostat sections (6 μm) of 5 weeks old pancreases from C57BL/6, NOR/LTj and NOD/LTj mice were prepared and fixed with cold methanol and acetone. Slides were incubated with Ki-67-FITC and rat-anti-ER-MP58 followed by goat-anti-rat-TexasRed. Finally, slides were mounted in Vectashield with DAPI.

Statistical analysis

Data were analyzed by Mann-Whitney U test for unpaired data. All analyses were carried out using SPSS software (SPSS Inc., Chicago, IL, USA) and considered statistically significant if $p<0.05$.

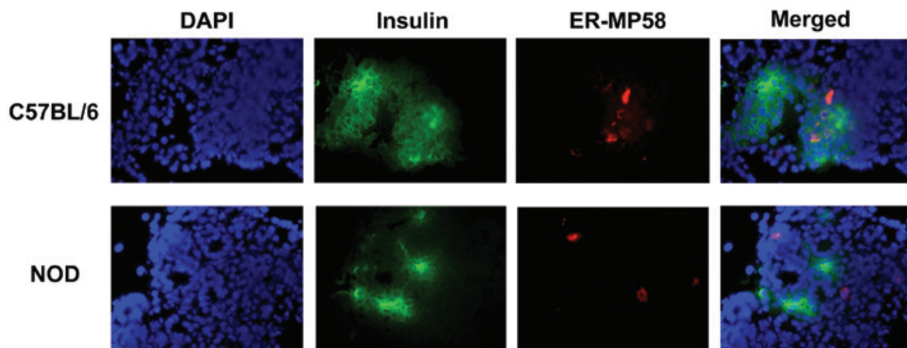

Figure 1. ER-MP58$^+$ cell population in E15.5 pancreas.

E15.5 pancreases of C57BL/6 (top) and NOD/LTj (bottom) mice were stained for insulin (green), ER-MP58 (red) and DAPI (blue) by immunofluorescence. Magnification 400x. Data shown are representative of 3 mice.

Results

Myeloid precursor populations in the fetal pancreas

The presence of precursors for DCs in the fetal pancreas was studied using the myeloid progenitor marker ER-MP58. ER-MP58 has previously been described by our laboratory as a marker for all myeloid progenitor cells in BM [17].

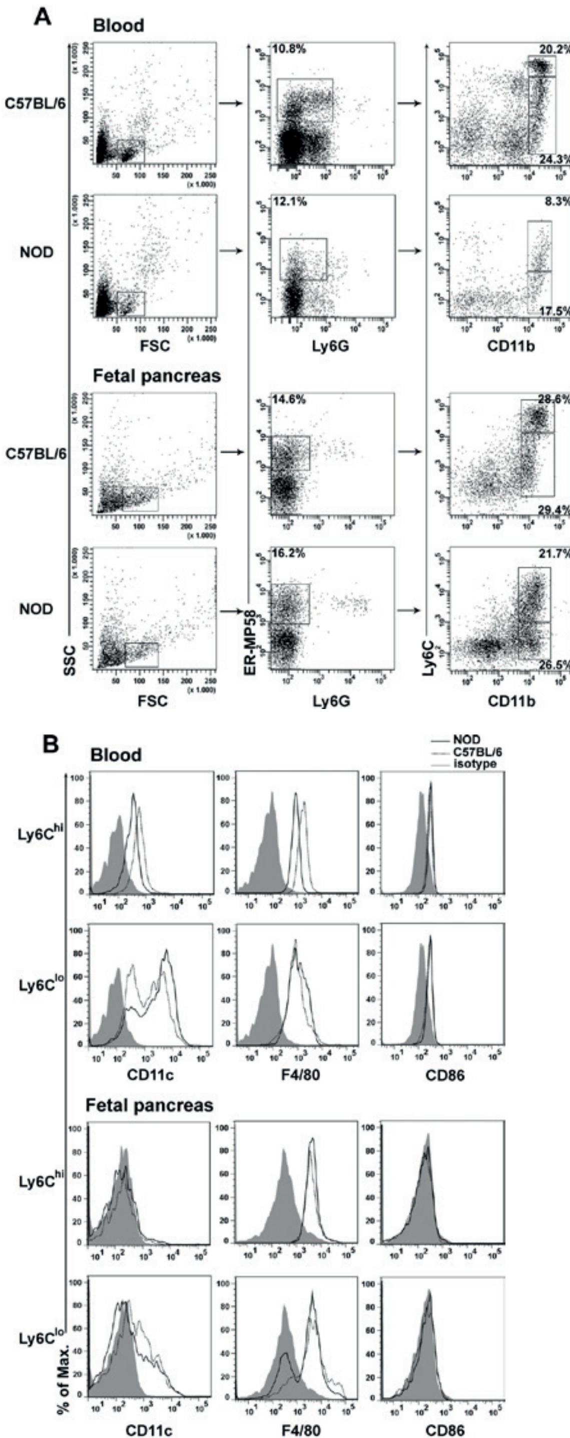

Figure 2. Presence of precursor populations in blood and fetal pancreas.

Flow cytometry analysis was performed on blood (top panels, 4 weeks) and E15.5 pancreases (bottom panels, pooled) of C57BL/6 and NOD mice. (A) SSC^{low} cells were gated for ER-MP58 and Ly6G expression. ER-MP58^{+}Ly6G^{-} cells were gated on CD11b and Ly6C expression. (B) Representative histograms show the expression of CD11c, F4/80 and CD86 on CD11b^{hi}Ly6C^{hi} and CD11b^{hi}Ly6C^{low} cells. Shaded histogram, isotype control; dotted line histogram, C57BL/6; solid line histogram, NOD. Data shown are representative of 6-10 experiments with 1 mouse (blood) or 10 embryos (fetal pancreas) per experiment.

To investigate the phenotype of this myeloid precursor in the pancreas a FACS staining was performed on fetal pancreas cells and compared to blood monocytes (4 weeks) from C57BL/6 and NOD/LTj mice. In the blood, SSC^{low} cells were gated on ER-$MP58^{+}Ly6G^{-}$ cells.
These cells were subdivided into two populations; $CD11b^{hi}Ly6C^{hi}$ (classical) and $CD11b^{hi}Ly6C^{low}$ (non-classical) monocytes (Fig.2A). In the fetal pancreas two precursor populations were present with a similar phenotype as blood monocytes. Due to a genetic abnormality of the Ly6C gene in NOD mice the expression of Ly6C is present, but significantly lower than in control mice [18].
The phenotype of the two monocyte populations was further characterized using Ab against CD11c, F4/80 and CD86. In blood, $Ly6C^{hi}$ monocytes were $CD11c^{low}F4/80^{+}CD86^{low}$ in both C57BL/6 and NOD mice (Fig.2B). $Ly6C^{low}$ blood monocytes expressed CD11c. Two $CD11c^{+}$ cell populations were observed: $CD11c^{low}$ and $CD11c^{hi}$. The $Ly6C^{low}$ blood monocyte population of NOD mice had more $CD11c^{hi}$ cells than in C57BL/6 mice. $Ly6C^{low}$ blood monocytes were $F4/80^{+}CD86^{low}$ in both strains. In the fetal pancreas $Ly6C^{hi}$ cells were $CD11c^{-}F4/80^{+}CD86^{-}$ in C57BL/6 and NOD mice. In the fetal pancreas $Ly6C^{low}$ cells were $F4/80^{+}CD86^{-}$ and expressed CD11c, although not that high as the $Ly6C^{low}$ blood monocytes. No differences were observed between C57BL/6 and NOD fetal pancreas. Thus, in the fetal pancreas two myeloid precursor populations ($Ly6C^{hi}$ and $Ly6C^{low}$) were present. These cells showed a similar expression of F4/80 as blood monocytes, but had a lower CD11c expression on $Ly6C^{low}$ cells and lacked CD86.

Isolated myeloid precursors from fetal pancreas differentiate into functional DCs

To show that ER-$MP58^{+}$ cells in the fetal pancreas are able to develop into $CD11c^{+}$ DCs, ER-$MP58^{+}$ cells were isolated by cell sorting followed by culture with GM-CSF. After culture for 8 days the generated cells displayed a typical DC appearance with dendrites (Fig.3A). More than 40% of these cells expressed CD11c and expressed MHCII and the co-stimulatory molecule CD86 (Fig.3B). The absolute number of generated $CD11c^{+}$ cells from cultured pancreatic ER-$MP58^{+}$ cells was significantly higher in NOD than in C57BL/6 (Fig.3C). The generated $CD11c^{+}$ cells from NOD and C57BL/6 were able to quench DQ-OVA showing the capability to process antigens (Fig.3D). No significant difference in the DQ-OVA expression was detected between NOD and C57BL/6.

Increased proliferation of myeloid precursors in NOD fetal pancreas

A property of precursors is their proliferative capacity; therefore the proliferation of precursors in the fetal pancreas was analyzed by flow cytometry using Ki-67. In NOD fetal pancreas the number of $Ly6C^{hi}Ki\text{-}67^{+}$ cells was significantly higher than in C57BL/6 (2.5-fold). No difference was found in the number of $Ly6C^{low}Ki\text{-}67^{+}$ cells between NOD and C57BL/6 (data not shown).

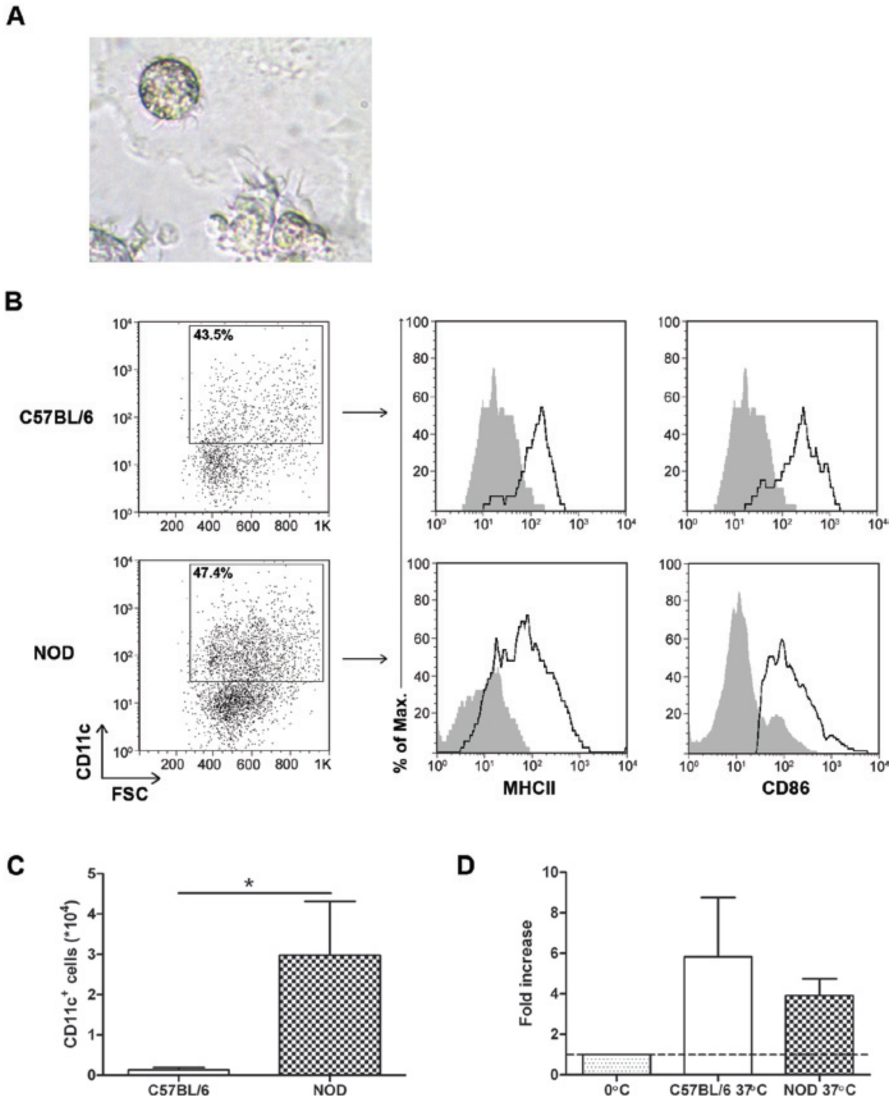

Figure 3. DC generation from ER-MP58⁺ cells from E15.5 pancreas.

ER-MP58⁺ cells were sorted from E15.5 pancreases of C57BL/6 and NOD mice and cultured for 8 days with GM-CSF. (A) Morphology of C57BL/6 DCs after 8 days of culture. Magnification 320x. (B) Cells were gated on CD11c and histograms show the expression of MHC class II and CD86 on CD11c⁺ cells. Shaded histogram, isotype control; solid line, marker-specific staining. (C) Graph shows the absolute number of generated CD11c⁺ cells corrected for the input cell number. (D) Generated CD11c⁺ cells from day 8 were tested for antigen processing by DQ-OVA. Antigen processing was determined by measurement of the fluorescence upon proteolytic degradation of the self-quenched conjugate DQ-Ovalbumin. Fold increase indicates the amount of the proteolytic degradation of DQ-OVA at 37°C compared to 0°C. Data are presented as mean + SEM, n = 5 experiments (C) and n = 3 experiments (D) with 10 embryos pooled per experiment. *$p < 0.02$ as determined by unpaired Mann-Whitney U test.

To determine the proliferative capacity of ER-MP58$^+$ cells in culture we used CFSE labeling. ER-MP58$^+$ cells from the fetal pancreas, fetal liver, adult BM and blood were labeled and cultured with GM-CSF. Microscopic evaluation on day 4 of the GM-CSF culture of ER-MP58$^+$ cells from the NOD fetal pancreas revealed increased cell numbers compared to C57BL/6 and BALB/c cultures (Fig.4A). After 2 days culture the CFSE signal on half of the ER-MP58$^+$ cells from the NOD fetal pancreas was decreased, in contrast to the C57BL/6 (Sup.Fig.1A). After 5 and 8 days culture the CFSE signal of ER-MP58$^+$ cells from the NOD fetal pancreas was dramatically decreased in line with a high proliferative activity (Fig.4B and Sup.Fig.1A). No such a decrease was detected in C57BL/6. Although a decrease of the CFSE signal was detected in the BALB/c fetal pancreas, the decrease was less compared to NOD. In the fetal liver as well as in the adult BM the majority of ER-MP58$^+$ cells showed a low CFSE signal, with no differences between NOD and controls. The number of CFSE^{low} cells in the culture of ER-MP58$^+$ cells from the NOD fetal pancreas was significantly higher compared to controls. Cells with at least 5 divisions were counted as CFSE^{low} cells (Fig.4C).
As monocytes in the peripheral blood also express ER-MP58 these cells were analyzed for their proliferative capacity too. The CFSE signal of day 8 cultures of ER-MP58$^+$ cells from the blood was not decreased, showing that ER-MP58$^+$ peripheral blood monocytes were not able to proliferate after GM-CSF stimulation (Sup.Fig.1B). In conclusion, myeloid precursors in the NOD fetal pancreas have a specific proliferation abnormality.

Increased proliferation of ER-MP58$^+$ cells in pre-diabetic NOD pancreas

DCs are the first cells that start to accumulate around the islets in the pancreas at 5 weeks of age in the pre-diabetic NOD mice. To investigate if this DC accumulation is preceded by an increased proliferation of local pancreatic precursors the pre-diabetic pancreas was studied for ER-MP58$^+$Ki-67$^+$ cells by immunofluorescence and FACS analysis. To assess if the proliferation abnormality in the NOD pancreas is a general phenomenon of the genetic background of these mice, the nonobese diabetic resistant mouse (NOR) was included as an extra control. In the NOD pancreas of 5 weeks of age the number of ER-MP58$^+$Ki-67$^+$ cells was significantly higher compared to C57BL/6 and NOR (Fig.5A and B). This was confirmed by FACS analysis of the pancreas of 5 week old NOD, NOR and C57BL/6 mice (Sup.Fig.2 and Fig.5C). No significant difference in the total number of ER-MP58$^+$ cells between NOD, NOR and C57BL/6 was detected (data not shown). Thus, proliferating myeloid precursors are present before the DC accumulation in the NOD pre-diabetic pancreas and this is not due to the genetic background of this mouse.

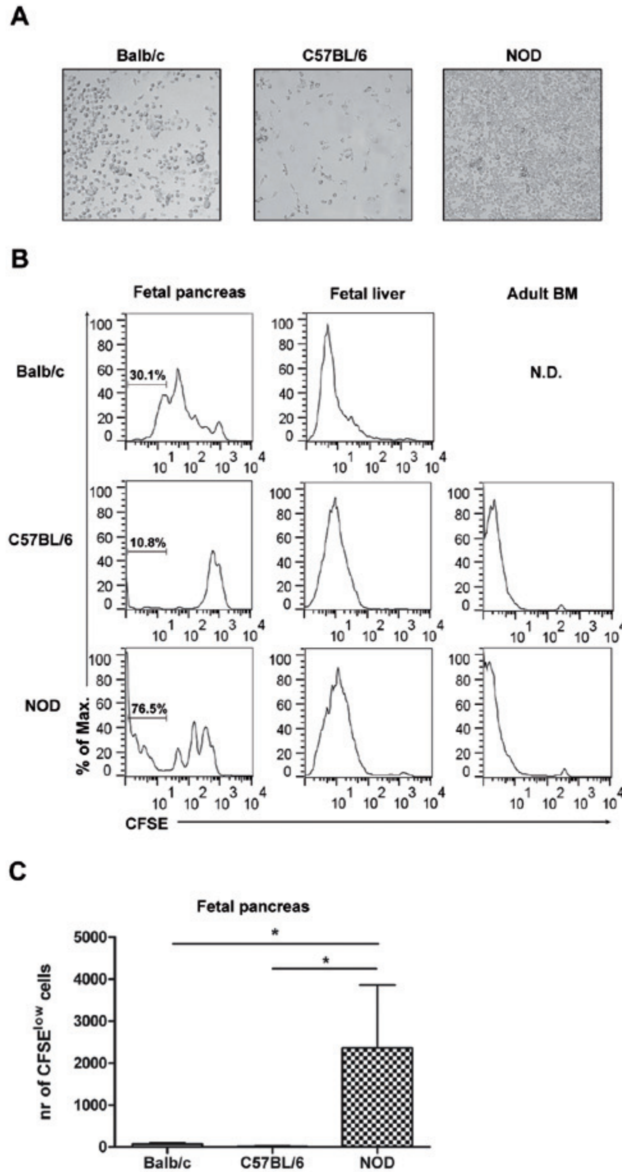

Figure 4. Proliferative capacity of ER-MP58⁺ cells from fetal pancreas.

Isolated cells from pooled E15.5 pancreases, E15.5 livers and BM (8 weeks) were labeled with CFSE and sorted on ER-MP58 expression. Sorted ER-MP58⁺ cells were cultured with GM-CSF for 8 days. (A) Culture of sorted ER-MP58⁺ cells from fetal pancreas on day 4. Magnification, 200x. (B) Representative histograms show the CFSE expression of ER-MP58⁺ sorted cells from the fetal pancreas (left), fetal liver (middle) and adult BM (right) on day 8. (C) The absolute numbers of $CFSE^{low}$ cells (cells with at least 5 divisions) on day 8 of the GM-CSF cultured ER-MP58⁺ cells from the fetal pancreas are shown. Data were corrected for input cell number. Data are presented as mean + SEM, n = 4 experiments. *$p < 0.03$ as determined by unpaired Mann-Whitney U test.

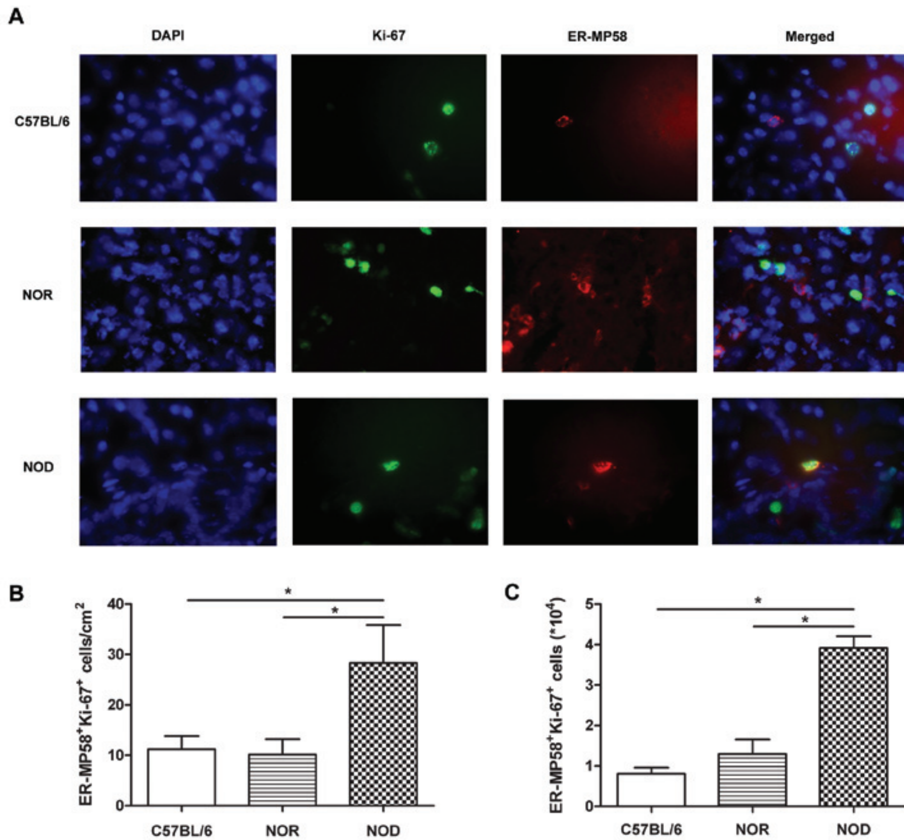

Figure 5. Proliferation of ER-MP58$^+$ cells in pre-diabetic pancreas.

(A) Pancreases from C57BL/6 (top), NOR (middle) and NOD (bottom) mice at 5 weeks of age were stained for Ki-67 (green), ER-MP58 (red) and DAPI (blue) by immunofluorescence. Magnification, 630x. (B) The mean number of ER-MP58$^+$Ki-67$^+$ cells per cm^2 is shown. (C) Flow cytometry analysis of the pancreas at 5 weeks shows the absolute number of ER-MP58$^+$Ki-67$^+$ cells. Data are presented as mean + SEM, n = 4-5 mice. *$p < 0.04$ as determined by the unpaired Mann-Whitney U test.

Discussion

We here show that ER-MP58$^+$Ly6G$^-$CD11b^{hi}Ly6C^{hi} and ER-MP58$^+$Ly6G$^-$CD11b^{hi}Ly6C^{low} precursors for myeloid DCs are present in the pancreas of C57BL/6 and NOD mice from embryonic (E15.5) age onwards. After sorting and culture in GM-CSF, these precursors have the potential to develop into CD11c$^+$MHCII$^+$CD86$^+$ DCs capable of processing antigens. Although the number of precursors is not increased in the NOD mouse pancreas, the cells have a higher proliferative capacity in the embryonic as well as in the pre-diabetic NOD pancreas. This abnormality was specific for the pancreas and did not occur in blood, liver and BM.

It is assumed that the autoimmune process in the NOD mouse starts with DC accumulation around 5 weeks of age. However, the presence of abnormal DC precursors in the fetal and pre-diabetic pancreas of NOD mice indicates that the autoimmune process in the NOD mouse starts much earlier. Several studies showed aberrancies already in the pre-diabetic NOD mice. An increased level of the extracellular matrix protein fibronectin was found in the early postnatal NOD pancreas, and is associated with an enhanced accumulation of macrophages and altered islet morphology [19]. In the early neonatal pancreas of NOD mice abnormalities in DC and macrophage populations were described [20].

ER-MP58 is a marker which is present on all myeloid progenitors. However, some non-myeloid cells can express this marker at low levels [17]. Isolated ER-MP58^{+} cells from the pancreas were used in cultures with GM-CSF and developed into DCs. Only cells of the myeloid lineage will respond to this growth factor [21].

BM cells from NOD mice have previously been shown by several groups to have reduced responses to GM-CSF [22, 23]. In contrast, myeloid precursors from NOD fetal pancreas showed an increased response to GM-CSF compared to C57BL/6. These cells had an increased proliferation and produced more DC, suggesting a proliferation and/or apoptotic defect in myeloid precursors in the NOD fetal pancreas and indicating towards an intrinsic abnormality of these cells. Interestingly, it has been described that NOD myeloid cells have a high GM-CSF expression [24]. This suggests that if the pancreatic precursors exhibit this phenotype as well, an autocrine loop driven by GM-CSF might contribute to the abnormal expansion and differentiation of the local pancreas DC precursors in the NOD mouse. However, a contribution of additional signals from the pancreatic tissue itself might explain why at specific ages waves of DC accumulation have been observed.

Our observations on the presence of abnormal local precursors in the NOD pancreas are suggestive for a new concept on the role of local pancreatic DC precursors in the development of diabetes. This proposed model differs from current paradigms of acute inflammation, where Ly6C^{hi} monocytes are recruited from the circulation to a site of pre-autoimmune injury to become DCs [25-27]. In our concept inflammation and organ-specific autoimmunity use different routes for accumulation of DCs in target organs-to-be and suggest that the accumulating DCs in the NOD pancreas are different from the well characterized TNF/iNOS-producing DCs (TIP-DCs) that are recruited from the peripheral blood to sites of inflammation.

A large body of research has been carried out on the development of DCs in various lymphoid tissues from bone-marrow precursors. The macrophage and DC precursor (MDP) for lymphoid tissue conventional DCs (cDCs), pDCs and monocytes is characterized as a cell expressing Lin^{-}c-kit^{hi}CD115^{+}CX$_{3}$CR1^{+}Flt3^{+} [8, 28]. Another distinct progenitor is called the common DC precursor (CDP) (Lin^{-}c-kit^{low}CD115^{+}Flt3^{+}) and is restricted to produce cDCs and pDCs, but not monocytes [29, 30]. Preliminary data showed that ER-MP58^{+} cells do not

express Flt3 and do not produce pDCs when cultured in the presence of Flt3 in the fetal and pre-diabetic pancreas. This suggests that our pancreas DC precursor is distinct from the MDP or CDP. We therefore assume that the local pancreatic precursor has a unique phenotype different from peripheral blood monocytes and precursors for cDCs in the BM.

Our study has limitations. One could argue that the local precursors are not present in the "pancreas-anlage" itself, but in the vicinity of this tissue in specialized blood-forming tissues, like the aorta-gonad-mesonephros (AGM) and the fetal liver. In this study the preparation method excludes these organs, which strongly argues in favor of a presence of the precursors in the fetal pancreas itself.

Secondly, the local pancreatic precursor could simply represent early seeded monocytes in the tissues. Indeed, the local pancreas DC precursor has a similar phenotype as blood monocytes, except for the lower CD11c expression on the Ly6C^{low} cells and is expressing ER-MP58, which is a marker for both myeloid precursors in the BM and peripheral blood monocytes [17]. Upon GM-CSF stimulation the local ER-MP58^{+} cells isolated from fetal pancreas displayed a high proliferative activity. Such a proliferation was not observed in cultures of ER-MP58^{+} monocytes isolated from NOD peripheral blood. It is known that blood monocytes are nondividing cells [25]. These data, the presence of ERMP58^{+} cells in the pancreas from embryonic live onwards and the observation of Ki-67^{+}ER-MP58^{+} cells in the pre-diabetic pancreas support our conclusion that this ER-MP58^{+} cell is a myeloid precursor cell distinct from a peripheral blood monocyte. However, the possibility that migrating blood monocytes are modified by the microenvironment of the pancreas and obtain a proliferative capacity cannot be excluded completely.

The proliferation/differentiation aberrancies of local NOD pancreatic DC precursors described here are very similar to the aberrancies previously found by us in DC precursors of the BM in the animal models of type 1 diabetes [31]. DC precursors in BM of NOD mice and BB-DP rats also show proliferation/differentiation abnormalities and from these precursors abnormal "steady state" DCs arise with a spontaneous high pro-inflammatory set point [31, 32]. These abnormal DCs have a high level of NFkB and a high acid phosphatase, high IL-12 and low IL-10 expression [33-36]. These DCs are incapable of sufficiently sustaining the proliferation of Treg-cell populations in the NOD mouse and BB-DP rat [37, 38]. It has been shown that correction of these DC abnormalities prevents the development of autoimmune diabetes [39, 40]. It is tempting to speculate that the locally generated DCs in the pancreas of NOD mice show a similar pro-inflammatory set point as their BM correlates and cannot sustain Treg cells sufficiently.

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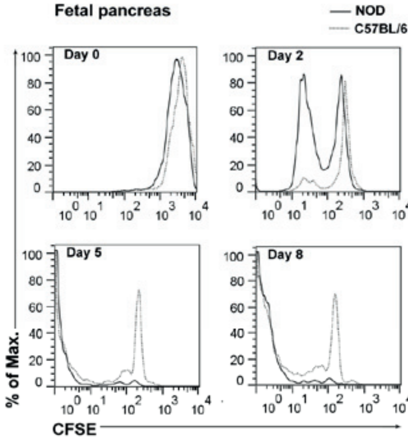
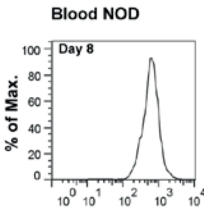
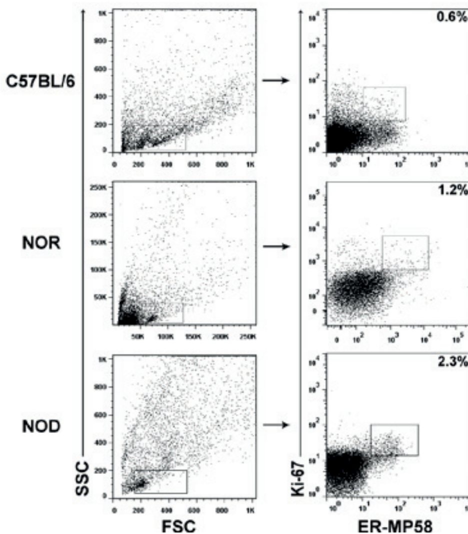
SUPPORTING INFORMATION





Supplemental Figure 1. Proliferative capacity of ER-MP58⁺ cells from fetal pancreas and blood.

Isolated cells from pooled E15.5 pancreases and blood (8 weeks) were labeled with CFSE and sorted on ER-MP58 expression. Sorted ER-MP58⁺ cells were cultured with GM-CSF for 8 days. (A) Histograms show the CFSE expression of sorted ER-MP58⁺ cells from the fetal pancreas on day 0, 2, 5 and 8. Dotted line histogram, C57BL/6; solid line histogram, NOD. (B) Histogram shows the CFSE expression of ER-MP58⁺ sorted cells from blood from the NOD mice on day 8. Data shown are representative of 3 experiments.



Supplemental Figure 2. Presence of proliferating ER-MP58⁺ cells in the pre-diabetic pancreas.

Flow cytometry analysis was performed on pancreas (5 weeks) of C57BL/6 and NOD mice. SSC^{low} cells were gated on the ER-MP58 and Ki-67 expression. Data shown are representative of 4-5 experiments.

CHAPTER 3

PROLIFERATING MONOCYTE-LIKE MYELOID PRECURSOR CELLS IN THE HUMAN FETAL AND ADULT PANCREAS

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Submitted

Abstract

Local proliferating monocyte-like precursors for dendritic cells and macrophages are present in the murine pancreas from the embryonic age onwards. For the human pancreas such data are not available. Therefore the presence and localization of proliferating monocyte-like precursors was investigated in the pancreas of fetuses, children and adults using immunofluorescence and flow cytometry.

In the fetal pancreas proliferating $CD14^{+}$ cells were located near the islets and outside von Willebrand factor^{+} vessels. The number of these cells was significantly higher as compared to the pancreas of children and adults.

This study shows for the first time the presence of local proliferating monocyte-like myeloid precursor cells in the human pancreas from the fetal stage onwards.

Introduction

The pancreas consists of two different tissue types which have different functions and morphology: exocrine and endocrine tissue (islets of Langerhans). During the development of the human fetal pancreas the formation of islets starts at 12 weeks of gestation and is completed around 26 weeks of gestation when the characteristic composition of postnatal islets is present [1]. Previously, we described the presence of peri- and intra-pancreatic lymphoid structures during human fetal pancreas development [2, 3]. Pancreatic lymphoid structures are present from 15 weeks of gestation onwards and intra-pancreatic lymphoid structures from 29 weeks of gestation onwards. These lymphoid structures contain different types of leukocytes, such as T cells, B cells, dendritic cells (DCs), macrophages and monocytes. Although T and B cells are mainly present in the lymphoid structures, macrophages and monocytes are more abundant in the parenchyma, which also contains low numbers of DCs. In the human circulation, monocytes can be divided into two major subsets: immature $CD14^{hi}CD16^{-}$ and mature $CD14^{+}CD16^{hi}$ monocytes [4, 5]. Immature monocytes are selectively recruited to inflamed tissues and lymph nodes and differentiate into the so called inflammatory DCs [6, 7]. These immature monocytes also contribute to the renewal of several resident subsets of macrophages and DCs, such as lung DCs and Langerhans cells in the skin [8-10]. The mature monocytes patrol in the steady state the blood vessels and extravasate during infection [11]. In addition, it has also been described that mature monocytes differentiate into DCs that induce T cell tolerance [12]. Recently, we showed that such infiltration is not the only route to replenish intra-pancreatic DC and macrophages, but that local proliferating precursors for DCs are present in the murine pancreas [13]. These precursors have a monocyte-like appearance, but are most likely not infiltrated blood monocytes, since it is known that blood monocytes are non-dividing cells [6].

Previously, we and others described that macrophages and DCs accumulate near islets in the pancreases of Type 1 diabetes (T1D) patients [2, 14-16] and of animal models of the disease [17-19]. The macrophage and DC accumulation around the islets in the pre-diabetic phase precedes the lymphocyte infiltration in the animal models and is thought to play a crucial role in the initiation of the disease [20]. We recently made it plausible that the early pre-diabetic DC accumulation in the NOD pancreas is due, at least in part, to an aberrant enhanced proliferation of the local monocyte-like precursors, since we showed that the local pancreatic monocyte-like cells in the NOD mouse showed an enhanced proliferation and the potential to develop into functional DCs [13].

For the human pancreas it is unknown whether such monocyte-like proliferating precursors are present. Such presence might be relevant to also gain more insight in the development of human type 1 diabetes. For this short report, we investigated the presence and localization of proliferating monocyte-like precursors in the human fetal pancreas and in the pancreas of children and adults.

Materials and methods

Tissue specimens

Frozen and fresh human fetal pancreatic samples between 20 and 36 weeks of gestation (Table 1) and frozen pancreas tissue from children (1-7 years) and adults (57-87 years) (Table 2) were used in this study.

Table 1. Overview of fetal patients used in the study

Age *(weeks)*	***Gender***	***COD***
20.4	Male	Abortion: holoprosencephaly
21.3	Male	Abortion: urinary obstruction
22.0	Male	Abortion: spina bifida
22.0	Male	Abortion: observed abnorm of heart, lungs, fingers
23.6	Male	Abortion: urinary obstruction
24.4	Male	Spontaneous delivery: died after 12 days, sepsis
34.3	Male	Abortion: Klinefelter syndrome
36.2	Male	Caesarean, fetal distress: myopathy, hydrops foetalis, died after 6 days

Age is given in weeks of gestation, COD: cause of death

Table 2. Overview of pediatric and adult patients used in the study

Age *(years)*	***Gender***	***COD***
1	Female	RS virus infection
2	Female	Unknown
7	Female	Cardiomyopathy
7	Male	Partial malrotation of intestine
57	Male	Urotheliumcellcarcinoma metastases, sepsis
62	Male	Amyloidosis
76	Male	Adenocarcinoma metastases, sepsis
87	Female	Nephritis, sepsis

COD: cause of death

Ethics statement

The frozen pancreatic samples were retrieved from the tissue bank of the Department of Pathology of the Erasmus MC University Medical Centre, Rotterdam, The Netherlands. The pancreas samples were obtained from autopsies, after death from causes other than pancreas disease. In all cases the pancreas was normally developed. The study was approved

by the Medical Ethics Committee and written informed consent was obtained from relatives. The study was conducted in compliance with all relevant Dutch laws and in agreement with international and scientific standards and guidelines.

Immunohistochemistry

Cryostat sections (6 µm) of the pancreases were prepared and fixed with cold methanol and acetone. Slides were incubated with guinea pig-anti-insulin (DAKO, Glostrup, Denmark) or mouse-anti-CD14 (BD Biosciences) followed by rabbit-anti-guinea pig-PO (DAKO) or goat-anti-mouse-PO (DAKO). Subsequently, slides were incubated with Nickel-DAB and counterstained with nuclear fast red (both Sigma Aldrich, St. Louis, MO, USA), followed by mounting in Entallan (Merck, Darmstadt, Germany).

Immunofluorescence

Cryostat sections (6 µm) of the pancreases were prepared and fixed with cold methanol and acetone. Slides were incubated mAbs. Antibodies used were CD14, CD16-FITC and Ki-67-FITC (all BD Biosciences), von Willebrand factor (vWF) (Abcam, Cambridge, United Kingdom) and insulin (DAKO) followed by rabbit-anti-mouse-TRITC (DAKO) or rabbit-anti-guinea pig-FITC (Abcam). Finally, slides were mounted in Vectashield with DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

Preparation of cell suspensions

Fetal pancreases were cut into small pieces and digested with collagenase type 1 (1 mg/ml), hyaluronidase (2 mg/ml) (both Sigma Aldrich, St. Louis, MO, USA) and DNAse I (0.3 mg/ml) (Roche Diagnostics, Almere, The Netherlands) for 30 minutes at 37ºC. Subsequently, cells were flushed through a 70 µm filter and washed. Cells were resuspended in PBS with 0.1% BSA followed by flow cytometric staining.

Flow cytometry

Single-cell suspensions from the fetal pancreas were labeled with CD45 beads (Miltenyi) and $CD45^+$ cells were sorted using AutoMACS (Miltenyi) and labeled with mAbs. Antibodies used were CCR2-PE (R&D Systems, Minneapolis, MN, USA), CD14-APC and CD16-FITC (all BD Biosciences). Afterwards cells were washed and resuspended in 0.1% BSA and DAPI (Sigma Aldrich) to detect dead cells. Cells were measured on a FACS Canto HTSII (Becton Dickinson) flow cytometer and analyzed using FACS Diva and Flowjo software.

Statistical analysis

Data were analyzed by Mann-Whitney U test for unpaired data. All analyses were carried out using SPSS software (SPSS Inc., Chicago, IL, USA) and considered statistically significant if $p<0.05$.

Results

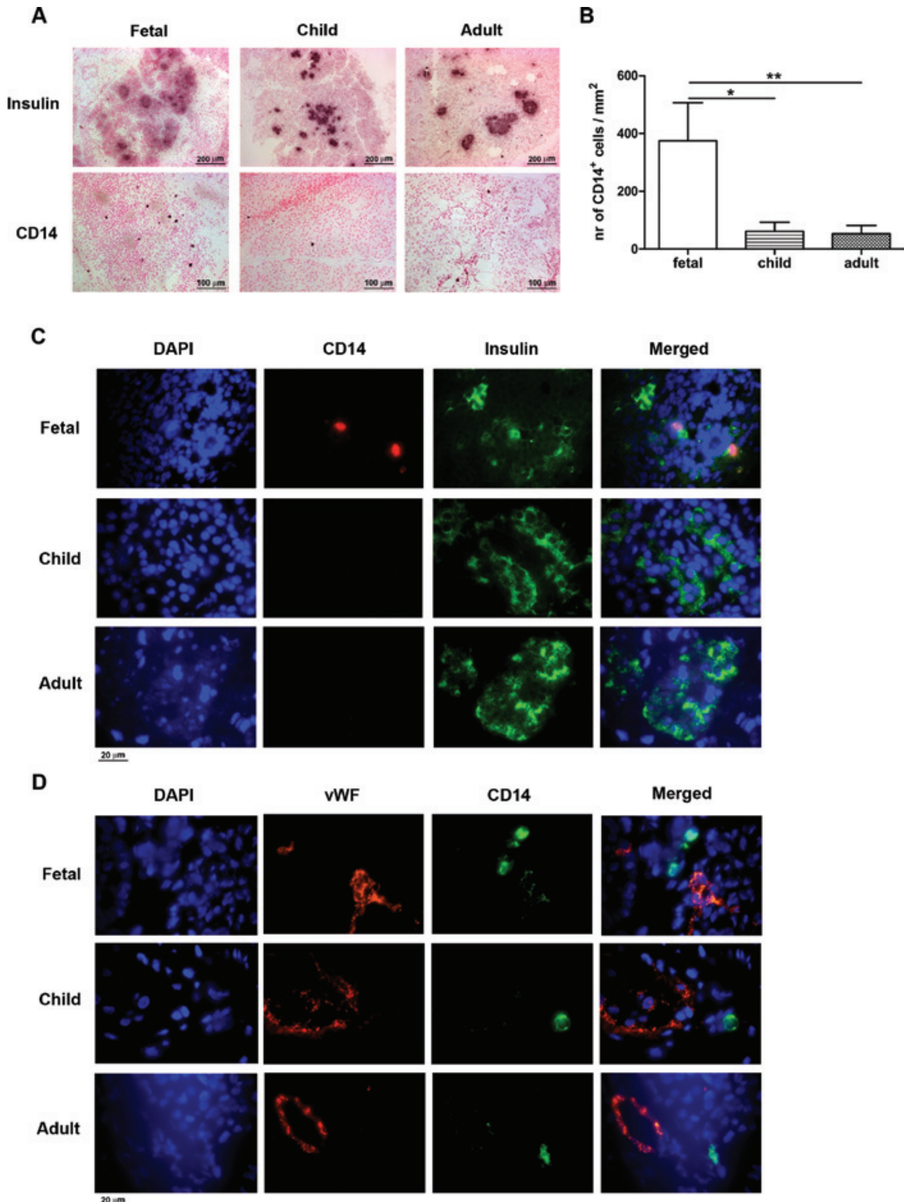

Figure 1. Presence of CD14⁺ cells in the human fetal pancreas.

Pictures represent the insulin (magnification 100x) and CD14 expression (magnification 250x) in the human pancreas (A). Bar graph shows the number of CD14⁺ cells/mm² in the pancreas (B). Pictures represent CD14 (red), insulin (green) and DAPI (blue) expression in the human pancreas, magnification 630x (C). Data are presented as mean + SEM, n = 4-8 samples, *p < 0.03 and **p < 0.001 as determined by unpaired Mann-Whitney U test.

$CD14^+$ cells were present in the fetal pancreas as well as in the pancreas of children and adults (Fig.1A). In the fetal pancreas the number of $CD14^+$ cells was significantly higher as compared to the pancreas of children and adults (Fig.1B). They were located in close vicinity to $insulin^+$ islets (Fig.1C) and outside vWF^+ vessels (Fig.1D) and in lymphoid structures (data not shown). Interestingly, in the pancreas of children and adults $CD14^+$ cells were not detected in the vicinity of islets (Fig.1C).

To investigate the phenotype of $CD14^+$ cells in the fetal pancreas flow cytometry staining was performed on sorted $CD45^+$ cells from the fetal pancreas of 20 weeks of gestation and older. Cells were stained with DAPI to exclude dead cells and viable cells were gated as $DAPI^-$ cells (Fig.2A). Two monocytic cell populations were present in the fetal pancreas: a major $CD14^+CD16^-$ population and a minor $CD14^+CD16^+$ population (Fig.2B and C). The chemokine receptor CCR2 was expressed by $CD14^+CD16^-$ monocytic cells, whereas $CD14^+CD16^+$ monocytic cells were $CCR2^{low}$ (Fig.2D).

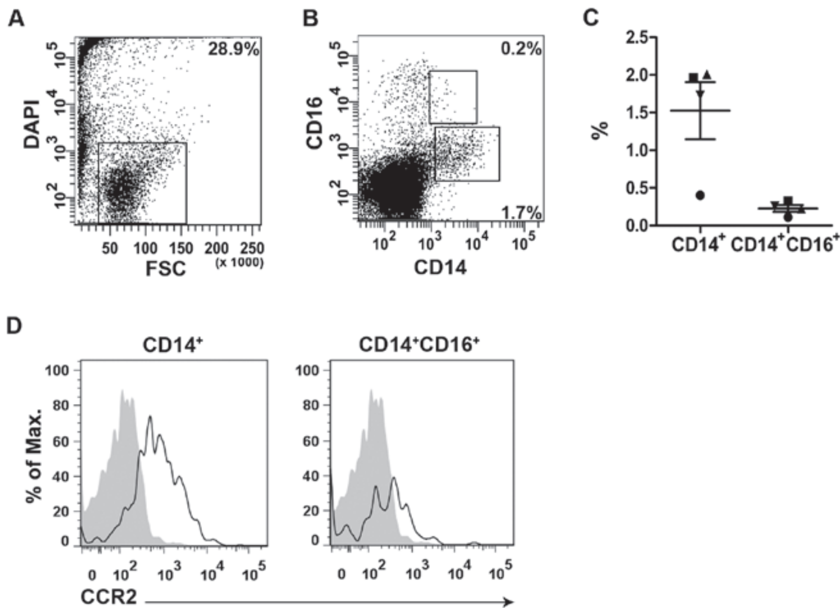

Figure 2. Presence of $CD14^+$ and $CD14^+CD16^+$ monocytic cells in the human fetal pancreas.

The presence of monocytic cells in the fetal pancreas (20 weeks and older) was determined by flow cytometry. Dot plot shows the DAPI expression on sorted $CD45^+$ cells from the human fetal pancreas (A). Dot plot shows the CD14 and CD16 expression on $CD45^+DAPI^-$ cells from the fetal pancreas (B). Graph represents the percentage of $CD14^+$ and $CD14^+CD16^+$ cells (C). Histograms represent the CCR2 expression on $CD14^+$ and $CD14^+CD16^+$ cells (D). Data shown are representative of 4 samples and presented as average ± SEM.

Proliferation of CD14⁺ monocytic cells in the human pancreas was investigated using Ki-67 expression and detection by immunofluorescence. CD14⁺Ki-67⁺ monocytic cells were detected in the pancreas of fetuses, children and adults (Fig.3A). In the fetal pancreas the percentage of proliferating CD14⁺ monocytic cells was significantly higher as compared to the pancreas of children and adults (Fig.3B).

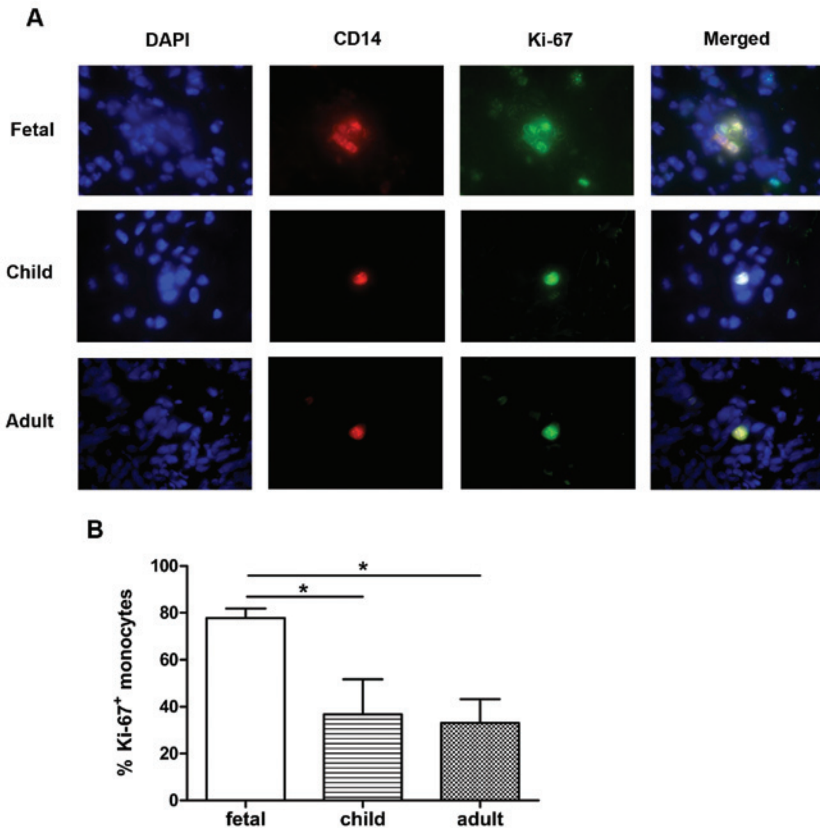

Figure 3. Presence of proliferating CD14⁺ monocytic cells in the human pancreas.

Pictures represent CD14 (red), Ki-67 (green) and DAPI (blue) expression in the human pancreas, magnification 630x (A). Bar graph represents the percentage of CD14⁺Ki-67⁺ cells of the total CD14⁺ monocytic cells (B). Data are presented as mean + SEM, n = 4-8 samples, *$p < 0.04$ as determined by unpaired Mann-Whitney U test.

Discussion

In this study we showed that proliferating CD14⁺ monocyte-like cells were present in the human pancreas, particularly in the fetal stage and close to the islets. This is in accord with our previous expressed view that the progeny of these cells, the macrophages and DCs, are

involved in the morphological and functional development of the islets [21, 22].
A limitation of our study is that the obtained material was heterogeneous regarding age and cause of death. In addition, some of the fetuses had lived for several days before they died and this may have also influenced results. Taken these limitations into account, it is striking that our observation of proliferating monocyte-like cells was consistent for all specimen studied.
It is known that blood monocytes are non-dividing cells [6]. Therefore, our data indicate that pancreatic proliferating monocyte-like cells are distinct from peripheral blood monocytes. This is supported on the one hand by our observation that proliferating monocyte-like cells are located outside the vessels, on the other hand by a recent study where a dual origin of tissue macrophages was proposed. One subset was found to be derived from a local yolk sac (YS)-derived precursor and the other subset from circulating precursors that derived from hematopoietic stem cells (HSCs) [23]. Interestingly, these YS-derived macrophages were proliferating in most fetal tissues, including the pancreas. These data are in line with our present observation of proliferating monocyte-like cells in the pancreas and the hypothesis that these cells are distinct from peripheral blood monocytes. However, the possibility that migrating blood monocytes are modified by the microenvironment of the pancreas and obtain a proliferative capacity cannot be excluded.
In conclusion, the presence of proliferating monocyte-like cells in the normal human pancreas has strengthened our concept that also in human local pancreatic myeloid precursors may give rise to local resident macrophages and DCs in the steady state pancreas. The contribution of local monocyte-like cells and their progeny in the development of human T1D remains speculative and needs to be established.

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The authors would like to thank the Juvenile Diabetes Research Foundation (no. 5-2006-31) for supporting this study. For the collection of pancreas tissues we would like to thank and P. Riegman from the Erasmus MC tissue bank and N. van der Graaf from the department of Pathology.

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CHAPTER 4

REDUCED NUMBERS OF DENDRITIC CELLS WITH A TOLEROGENIC PHENOTYPE IN THE PRE-DIABETIC PANCREAS OF NOD MICE

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Abstract

The nonobese diabetic (NOD) mouse is a widely used animal model of autoimmune diabetes. Prior to the onset of lymphocytic insulitis, dendritic cells (DCs) accumulate at the islet edges. Our recent work indicated that these DCs may derive from aberrantly proliferating local precursor cells. Since CD8α^{+} DCs play a role in tolerance induction in steady state conditions, we hypothesized that the autoimmune phenotype might associate with deficiencies in CD8α^{+} DCs in the pre-diabetic NOD mouse pancreas.

We studied CD8α^{+} DCs in the pancreas and pancreas-draining lymph nodes (pLNs) of NOD and control mice focusing on molecules associated with tolerance induction (CD103, Langerin, CLEC9A, CCR5). mRNA expression levels of tolerance-modulating cytokines were studied in pancreatic CD8α^{+} DCs of NOD and control mice.

In the NOD pancreas the frequency of CD8α^{+}CD103^{+}Langerin^{+} cells was significantly reduced compared to control mice. NOD pancreatic CD8α^{+}CD103^{+}Langerin^{+} DCs expressed reduced levels of CCR5, CLEC9A and IL-10 as compared to control DCs. These alterations in the CD8α^{+}CD103^{+}Langerin^{+} DC population were not present in pLNs.

We demonstrate local abnormalities in the CD8α^{+} DC population in the pre-diabetic NOD pancreas. These data suggest that abnormal differentiation of pancreatic DCs contributes to loss of tolerance, hallmarking the development of autoimmune diabetes.

Introduction

The nonobese diabetic (NOD) mouse is used as a spontaneous model to study the development of type 1 diabetes [1]. NOD mice show infiltrations with T, B and NK lymphocytes around and in the islets of Langerhans from 10 weeks of age onwards. This finally ends with the destruction of the β-cells leading to diabetes. From the age of 5 weeks onwards, prior to the accumulation of lymphocytic cells, the number of dendritic cells (DCs) and macrophages increases in the exocrine pancreas and these antigen presenting cells concentrate, cluster and accumulate at the islet pole and around the islets [2-3]. Our recent work has indicated that these DCs may derive from aberrantly proliferating local precursor cells in the fetal and pre-diabetic pancreas of NOD mice [4]. The observation that DCs are the first immune cells accumulating in the NOD pancreas and concentrating at the islet edges suggests a key role for DCs in the initiation of the autoimmune response against pancreatic islets. Indeed, temporal depletion of DCs totally abrogates the development of insulitis and diabetes in the NOD mouse model [5].

Recently, DC populations involved in tolerance induction have been identified and aberrations in these tolerogenic DCs may contribute to the development of autoimmunity [6]. In the mouse cells within the CD8α^{+} DC subset are thought to be involved in tolerance induction during steady state [7]. CD8α^{+} DCs in the pancreas draining lymph nodes (pLNs) cross-present self-antigen to cytotoxic T cells and induce tolerance in a mouse model of diabetes in which ovalbumin is expressed by beta cells in the pancreas [8]. Interestingly, treatment of NOD mice with fms-like tyrosine kinase 3 ligand (Flt3L) enhances splenic CD8α^{+} DCs numbers and induces protection against diabetes [9].

Other surface molecules expressed by DC populations in lymphoid and non-lymphoid organs which are suggested to be associated with tolerogenic functions are CD103, Langerin, CLEC9A and CCR5. CD103^{+} DCs and their function are well characterized in mucosal murine tissues and secondary lymphoid organs [10-12]. In the small intestine these DCs are potent inducers of gut-homing receptors on T cells [13]. CD103^{+} DCs isolated from the gut and mesenteric lymph nodes can drive the differentiation of Foxp3-expressing regulatory T cells via a TGF-β dependent mechanism [14]. CD8α^{+}CD103^{+}Langerin^{+} DCs isolated from the spleen were predominantly involved in phagocytosis of apoptotic cells and presented cell-associated antigens to T cells, in order to induce self-tolerance [15].

CLEC9A, a C-type lectin expressed on CD8α^{+} DCs, recognizes a signal on necrotic cells. Loss of CLEC9A specifically reduces cross-presentation of dead-cell-associated antigens and decreases the immunogenicity of necrotic cells, but does not impair the uptake of necrotic cell material by CD8α^{+} DCs [16].

With regard to CCR5, in a mouse model for experimental autoimmune encephalitis, oral tolerance could be induced in wild-type, but not in CCR5$^{-/-}$ mice [17]. Furthermore, in CCR5$^{-/-}$ mice increased levels of IL-12 were observed in the gut associated lymphoid tissue. It was

recently shown that the development of diabetes was accelerated in $CCR5^{-/-}$ NOD mice [18]. A previous study of our group revealed that DCs isolated from NOD salivary glands lack CCR5, in contrast to their counterparts in control mice [19]. Collectively these studies point to a pivotal role for $CCR5^{+}$ DCs in tolerance induction.

For the present study we hypothesized that during the development of NOD islet autoimmunity the local pancreatic DC population in charge of preserving local tolerance is aberrant in the pre- and post-lymphocyte infiltration period. Currently, data on the presence of tolerogenic $CD8\alpha^{+}$ DCs during the development of spontaneous diabetes in the NOD pancreas are lacking. Therefore, we studied the $CD8\alpha^{+}$ DCs in the pancreas, LNs, lungs and blood of 4 and 10 week old NOD and control mice using flow cytometry. We focused on the expression of molecules associated with tolerance induction i.e. CD103, Langerin, CLEC9A and CCR5. In addition, we studied the mRNA expression levels of IL-10, TGF-β, TNF-α and IL-12p40 in isolated pancreatic $CD8\alpha^{+}$ DCs of NOD and control mice.

Materials and methods

Animals

C57BL/6 and NOD/shiLTj female mice were obtained from Charles River Laboratories (Maastricht, The Netherlands), BALB/c mice from Harlan (Horst, The Netherlands) and NOR/LTj mice from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were bred and housed under specific pathogen-free conditions. Experimental procedures were approved by an independent certified Animal Ethical Committee.

Preparation of cell suspensions

Pancreases (4, 5 and 10 weeks of age) and lungs (4 weeks age) were isolated after a cardiac perfusion and cut into small pieces and digested with collagenase type 1 (1 mg/ml), hyaluronidase (2 mg/ml) (both Sigma Aldrich, St. Louis, MO, USA) and DNAse I (0.3 mg/ml) (Roche Diagnostics, Almere, The Netherlands) for 40 minutes at 37ºC. Subsequently, cells were flushed through a 70 µm filter and washed. pLNs and inguinal LNs (iLNs) were isolated from 10 weeks old mice and flushed through a 70 µm filter and washed. Blood of 4 weeks old mice was collected in EDTA tubes after a cardiac punction. Erythrocytes were lysed with NH_4Cl buffer and washed. All cells were resuspended in PBS containing 0.1% BSA and were ready for flow cytometric staining.

Flow cytometry

Single-cell suspensions from pancreas and lungs were labeled with CD45 beads (Miltenyi, Leiden, The Netherlands) and $CD45^{+}$ cells were sorted with the AutoMACS (Miltenyi) and labeled with mAbs. Single-cell suspensions from pLNs and iLNs (10 weeks) and blood (4 weeks) were labeled with mAbs. Antibodies used for extracellular staining were CD11c-

PE-Cy7, CD8α-Pacific Blue, CD103-allophycocyanin, Langerin-FITC, CCR5-PE, CLEC9A-PE and CCR7-PE-Cy7 (all eBiosciences, San Diego, CA, USA). Afterwards cells were washed and resuspended in 0.1% BSA / 0.5% paraformaldehyde. To detect intracellular Langerin, CCR5 and CLEC9A, cells were fixed in 4% paraformaldehyde, and permeabilized using 0.5% saponin. Subsequently, cells were incubated with Langerin-FITC, CCR5-PE or CLEC9A-PE diluted in 0.5% saponin, washed and resuspended in 0.1% BSA. Cells suspensions were analyzed using a FACS Canto HTSII (Becton Dickinson) flow cytometer and FACS Diva and Flowjo software.

Cytokine measurement by flow cytometry

Single-cell suspensions from pancreas (4 weeks) were labeled with CD11c beads (Miltenyi) and CD11c^{+} cells were sorted with the AutoMACS (Miltenyi) and cultured with and without 100 ng/ml LPS (Sigma Aldrich), including Goligstop (Becton Dickinson), for 5 hours. Cells were harvested and incubated with anti-CD16/CD32 (Biolegend) to block Fc-receptors. Antibodies used for extracellular staining were CD11c-PE-Cy7, CD8α-Pacific Blue, CD103-allophycocyanin, Langerin-FITC. To detect intracellular IL-10, TNF-α and TGF-β, cells were fixed in 4% paraformaldehyde, and permeabilized using 0.5% saponin. Subsequently, cells were incubated with IL-10-PerCP-Cy5, TNF-α-PerCP-Cy5 and TGF-β-PE (all eBiosciences) diluted in 0.5% saponin, washed and resuspended in 0.1% BSA. Cells suspensions were analyzed using a FACS Canto HTSII (Becton Dickinson) flow cytometer and FACS Diva and Flowjo software.

Quantitative-PCR (Q-PCR)

To purify leukocytes single-cell suspensions from pancreas (5 weeks) were labeled with CD45 beads, sorted using AutoMACS and labeled with CD11c-PE-Cy7 and CD8α-Pacific blue. CD11c^{+}CD8α^{+} cells were sorted with FACSAria (Becton Dickinson). Subsequently, the cells were directly dissolved in Extraction Buffer (Applied Biosystems, Foster City, CA, USA) and RNA was isolated using the Arcturus PicoPure kit (Applied Biosystems). RNA was reversed-transcribed into cDNA and amplified with the WT-Ovation RNA amplification System (NuGEN, Bemmel, The Netherlands). cDNA was purified using the QIAquick PCR purification kit (Qiagen). Finally, Q-PCR was performed as previously described in detail [20] for the following genes: IL-10, TGF-β, TNF-α, IL-12p40, Casp3, Casp6, Bcl-2, Bcl-XL, cdk1, cdk2 and cdk4 (all Applied Biosystems).

Statistical analysis

Data were analyzed by Mann-Whitney U test for unpaired data. All analyses were carried out using SPSS software (SPSS Inc., Chicago, IL, USA) and considered statistically significant if $p<0.05$.

Results

Reduced numbers of CD8α$^+$CD103$^+$Langerin$^+$ DCs in the NOD pancreas at 4 and 10 weeks of age.

To investigate the phenotype of pancreatic DCs a flow cytometry staining was performed on sorted CD45$^+$ cells from the pancreas of NOD, C57BL/6, BALB/c and nonobese diabetic resistant (NOR) mice. The NOR mouse is a NOD-related MHC-syngenic strain, neither developing diabetes nor massive insulitis [21]. Pancreatic CD45$^+$ cells were studied at 4 weeks of age at the start of DC accumulation (but prior to lymphocytic infiltration) and at 10 weeks when DCs and lymphocytes have accumulated particularly at the islet edges in the NOD pancreas.

We observed that a small population of CD8α$^+$CD11c$^+$ DCs was present in the pancreas of 4 and 10 weeks old mice in all strains (Fig.1A), and differences were not observed in the percentages of CD8α$^+$CD11c$^+$ DCs between NOD and control strains. The percentage of CD103$^+$Langerin$^+$ cells within the CD8α$^+$CD11c$^+$ population was decreased in the NOD pancreas of 4 and 10 weeks of age compared to controls (Fig.1B). No significant differences were detected in the absolute number of CD45$^+$ cells in the pancreas of 4 weeks between the strains (Fig.1C). However, the absolute number of CD8α$^+$CD103$^+$Langerin$^+$ DCs and the percentage of CD8α$^+$CD103$^+$Langerin$^+$ DCs from the total CD11c$^+$ DC population were significantly reduced in the NOD pancreas of 4 weeks old mice compared to controls (Fig.1D, E). CD8α$^+$CD103$^+$ DCs in the NOD and C57BL/6 pancreas expressed intracellular Langerin, but differences were not detected between the mouse strains (Sup.Fig.1A). At 10 weeks of age the absolute number of CD45$^+$ cells in the NOD pancreas was significantly increased compared to the C57BL/6 and NOR pancreas (Fig.1F). At this age, the absolute number of CD8α$^+$CD103$^+$Langerin$^+$ DCs and the percentage of CD8α$^+$CD103$^+$Langerin$^+$ DCs from the total CD11c$^+$ DC population were both significantly reduced in the NOD pancreas compared to controls (Fig.1G, H).

To investigate whether the reduced number of CD8α$^+$CD103$^+$Langerin$^+$ DCs in the NOD pancreas is due to increased cell death or an aberrant proliferation the following genes were studied using Q-PCR: Casp3 and Casp6 (pro-apoptotic), Bcl-2 and Bcl-XL (anti-apoptotic), Cdk1, Cdk2 and Cdk4 (proliferation). All genes showed a lower mRNA level in CD8α$^+$ DCs from the NOD pancreas as compared to the C57BL/6, but no significant differences were detected (data not shown). This indicated that these genes did not contribute to the reduced number of CD8α$^+$CD103$^+$Langerin$^+$ DCs in the NOD pancreas.

Finally, the CD8α$^-$ DC population in the pancreas was CD103$^+$Langerin$^-$ and no differences in the CD103 and Langerin expression were detected between the mouse strains (data not shown).

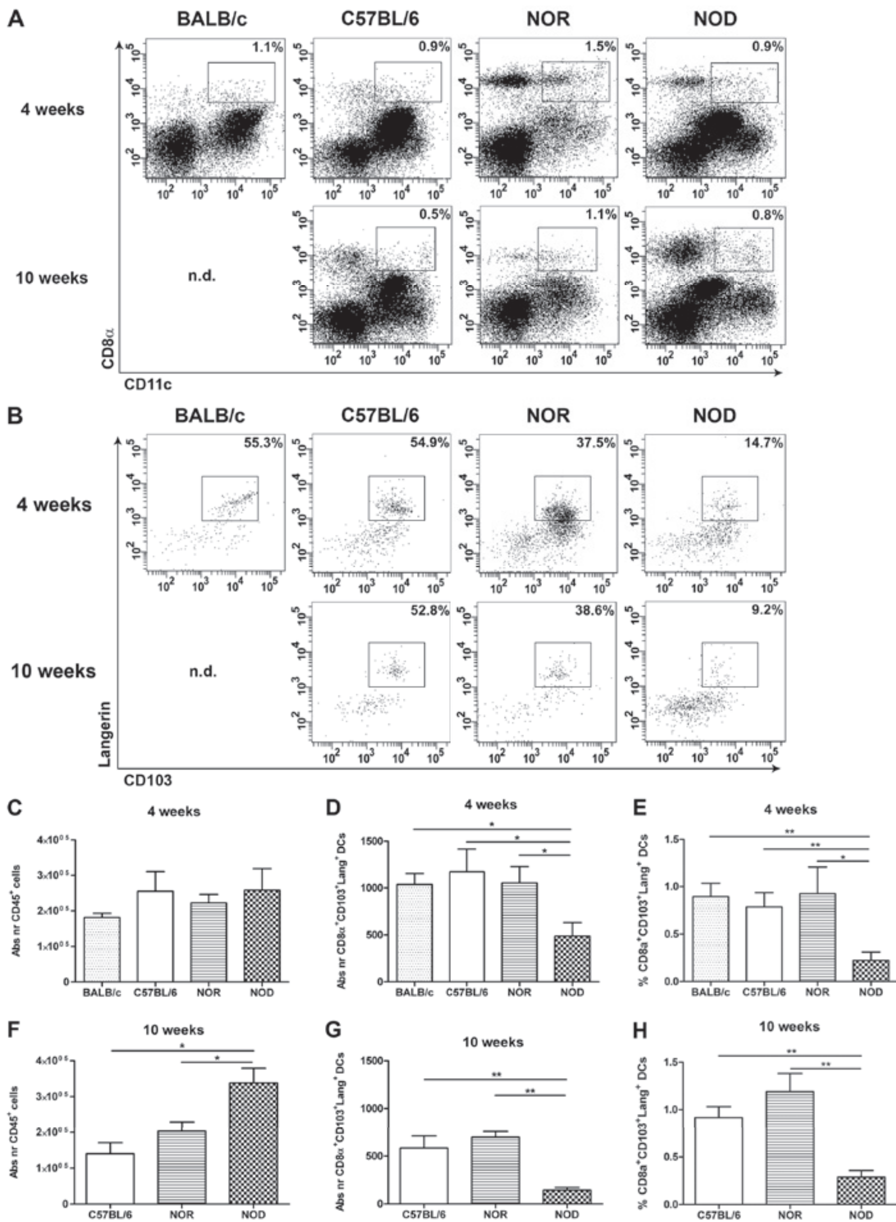

Figure 1. Presence of CD8α⁺CD103⁺Langerin⁺ DCs in the pancreas.

Flow cytometric analysis was performed on CD45⁺ cells isolated from the pancreas of BALB/c, C57BL/6, NOR and NOD mice (4 and 10 weeks). Dot plots show CD11c and CD8α expression on CD45⁺ cells from the pancreas (n.d. = not determined) (A). Dot plots show CD103 and Langerin (extracellular) expression on CD11c⁺CD8α⁺ cells from the pancreas (B). Bar graphs show the absolute number of CD45⁺ cells, the absolute number of CD8α⁺CD103⁺Langerin⁺ DCs and the percentage of CD8α⁺CD103⁺Langerin⁺ DCs of the total CD11c⁺ population at 4 (C-E) and 10 weeks (F-H) of age. Data are presented as average + SEM, n=5-9 experiments, * $p < 0.02$ and ** $p < 0.01$ as determined by unpaired Mann-Whitney U test.

Decreased expression of CLEC9A, CCR5 and IL-10 in CD8α$^+$CD103$^+$Langerin$^+$ DCs in the NOD pancreas

Studying additional molecules on CD8α$^+$CD103$^+$Langerin$^+$ DCs we observed that the CLEC9A expression was significantly decreased on these cells isolated from the NOD pancreas compared to C57BL/6 and NOR (Fig.2A, B). The CCR5 expression was significantly decreased on CD8α$^+$CD103$^+$Langerin$^+$ DCs from NOD as well as NOR mice as compared to C57BL/6 mice (Fig.2A, C). Staining for the intracellular presence of CLEC9A and CCR5 revealed a significant decrease of intracellular CCR5 expression in CD8α$^+$CD103$^+$Langerin$^+$ DCs from the NOD pancreas compared to C57BL/6 and no difference in the intracellular CLEC9A expression (Sup.Fig2). The CD8α$^+$CD103$^+$Langerin$^+$ DC subset in the pancreas expressed CCR7 at a similar level in NOD and controls, suggesting a comparable capacity for LN migration (Fig.2A, D). Like the CD8α$^+$ DCs, CD8α$^-$ DCs in the NOD pancreas had a significant decreased CLEC9A expression as compared to C57BL/6 and NOR (data not shown). Both NOD and NOR showed a significantly lower CCR5 expression as compared to C57BL/6 (same as CD8α$^+$ DCs). CD8α$^-$ DCs expressed CCR7 and no significant differences were detected on the CCR7 expression between the mouse strains.

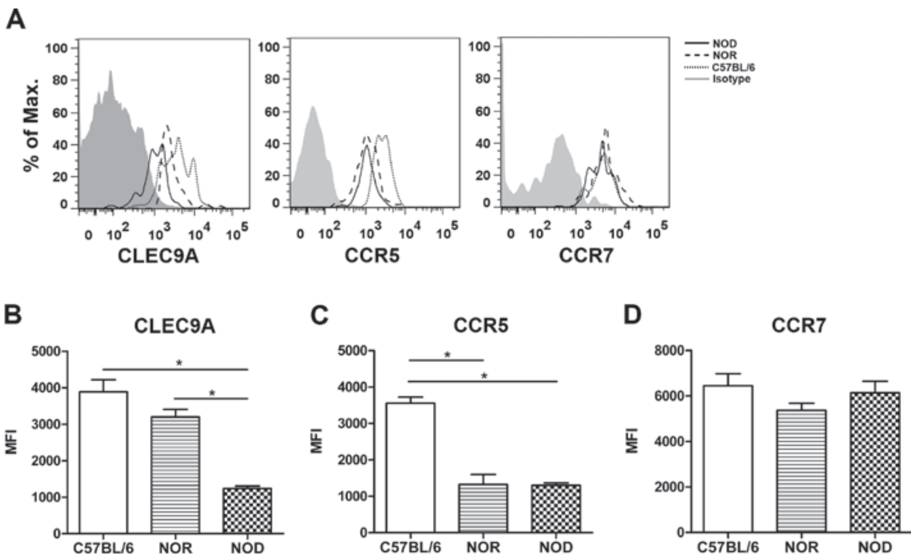

Figure 2. Phenotype of CD8α$^+$CD103$^+$Langerin$^+$ DCs in the pancreas.

Flow cytometric analysis was performed on CD45$^+$ cells isolated from the pancreas of C57BL/6, NOR and NOD mice of 4 weeks. Histograms show the CLEC9A, CCR5 and CCR7 expression on CD8α$^+$CD103$^+$Langerin$^+$ DCs (A). Bar graphs show the mean MFI of CLEC9A (B), CCR5 (C) and CCR7 (D) on CD8α$^+$CD103$^+$Langerin$^+$ DCs. Data are presented as average + SEM, n=4-5 experiments, * $p < 0.03$ and ** $p < 0.01$ as determined by unpaired Mann-Whitney U test.

In addition, the mRNA expression level of the cytokines IL-10, TGF-β, TNF-α and IL-12p40 was studied in freshly isolated CD8α+ DCs from the pre-lymphocytic NOD pancreas of 5 weeks of age. Expression of IL-10 in CD8α+ DCs from the NOD pancreas was hardly detectable and significantly reduced as compared to the level in C57BL/6 pancreatic DCs (Fig.3A). Low levels of TGF-β and TNF-α were detected in CD8α+ DCs from the NOD and C57BL/6 pancreas and differences between the two mouse strains were not statistically significant (Fig.3B-C). IL-12p40 expression was not detected neither in NOD or C57BL/6 pancreas CD8α+ DCs (data not shown). In contrast to CD8α+ DCs, CD8α- DCs in the pancreas had a lower IL-10 mRNA level and no significant differences were detected between NOD and C57BL/6. The TGF-β and TNF-α mRNA level of pancreatic CD8α- DCs showed no significant differences between NOD and C57BL/6.
IL-10, TGF-β and TNF-α protein levels in CD8α+ DCs from the pancreas were studied using flow cytometry. Sorted CD11c+ cells from the C57BL/6 and NOD pancreas of 4 weeks were cultured with and without LPS for 5 hours. After culture, CD8α+CD103+ Langerin+ DCs from the C57BL/6 as well as the NOD expressed TGF-β and TNF-α (Sup.Fig.3). No differences were observed between the mouse strains and with or without LPS. IL-10 expression was not detected due to a high background staining.

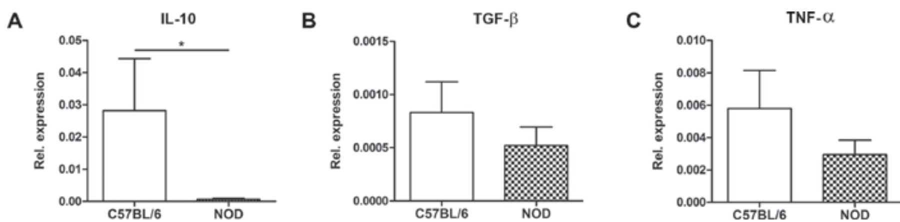

Figure 3. Expression of cytokine mRNA levels by DCs in the pancreas.

CD8α+ DCs from C57BL/6 and NOD pancreas (5 weeks) were isolated. The level of IL-10, TGF-β and TNF-α was measured in CD8α+ DCs by Q-PCR (A-C). Data are presented as average + SEM, n=4 experiments, with 10 mice pooled per experiment, * $p < 0.03$ as determined by unpaired Mann-Whitney U test.

Normal numbers of CD8α+CD103+Langerin+ DCs in the pLNs

To investigate whether CD8α+CD103+Langerin+ DCs in LNs have alterations, like in these pancreatic DCs, the pLNs and iLNs from 10 weeks old NOD and C57BL/6 mice were studied. CD8α+ DCs were detected in both LNs and a population of the cells expressed CD103 (Fig.4A, B). In contrast to DCs in the pancreas, DCs in LNs did not express Langerin on their cell membrane, but only intracellular (data not shown). In pLNs of NOD and NOR mice the total cell number and the absolute cell number of CD8α+CD103+Langerin+ DCs appeared significantly increased as compared to the C57BL/6 pLNs, likely explained by the ongoing autoimmune response in the pancreas (Fig.4C, D).

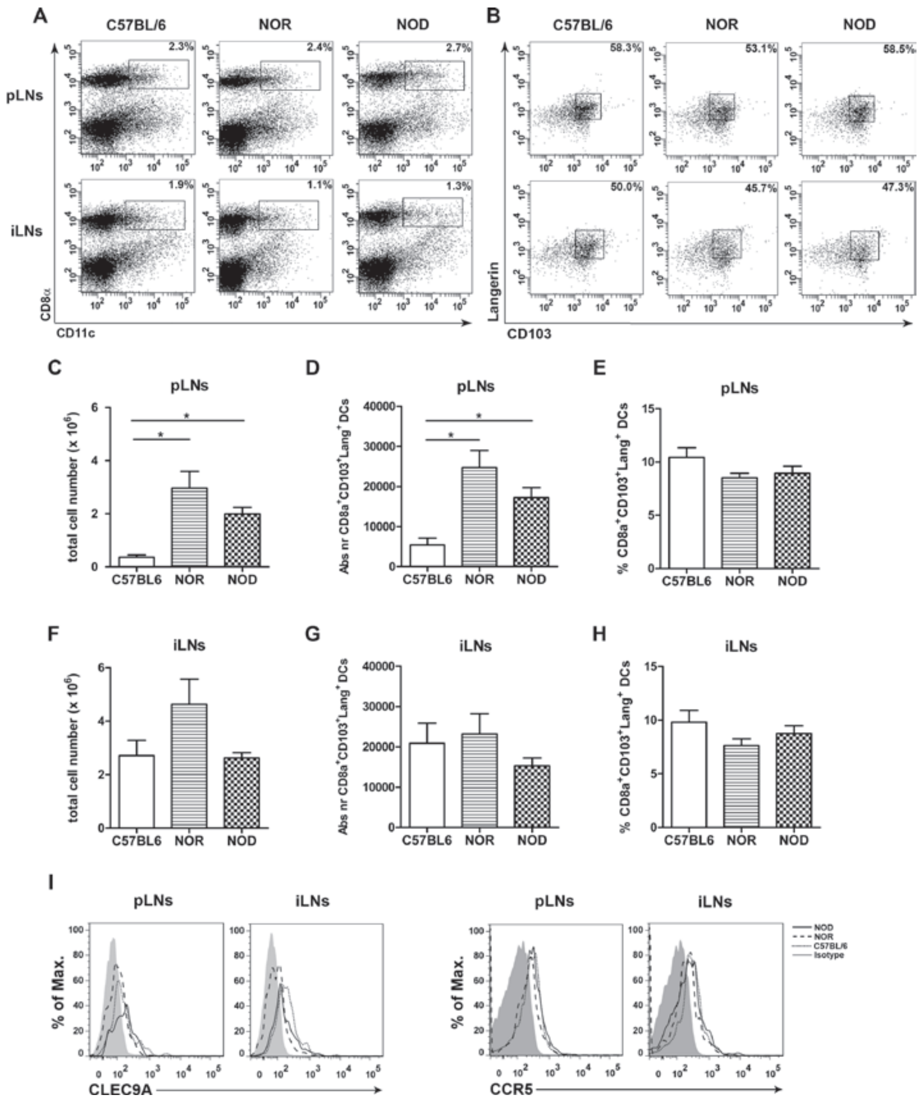

Figure 4. Phenotype of DCs in pLNs and iLNs.

Flow cytometric analysis was performed on cells from the pLNs and iLNs of C57BL/6, NOR and NOD mice of 10 weeks. Dot plots show CD11c and CD8α expression (A) and CD103 and intracellular Langerin expression on CD8α⁺ DCs (B). Bar graphs show the total cell number, the absolute cell number of CD8α⁺CD103⁺Langerin⁺ DCs and the percentage of CD8α⁺CD103⁺Langerin⁺ DCs of the total CD11c⁺ population in pLNs (C-E) and iLNs (F-H). Histograms represent the CLEC9A and CCR5 expression on CD8α⁺CD103⁺Langerin⁺ DCs (I). Data are presented as average + SEM, n=4 mice, * $p < 0.03$ as determined by unpaired Mann-Whitney U test.

However, no differences in the percentages of CD8α$^+$CD103$^+$Langerin$^+$ DCs of the total CD11c$^+$ DC population between NOD and control pLNs were detected (Fig.4E). In contrast, in iLNs the total cell number, the absolute cell number of CD8α$^+$CD103$^+$Langerin$^+$ DCs and the percentgae of CD8α$^+$CD103$^+$Langerin$^+$ DCs of the total CD11c$^+$ DC population showed no significant differences between NOD and control mice (Fig.4F-H).
CD8α$^+$CD103$^+$Langerin$^+$ DCs in the pLNs and iLNs did express CLEC9A and CCR5, but at a much lower level as compared to pancreas DCs. Significant differences in CLEC9A and CCR5 expression between NOD and control mice were not detected (Fig.4I).

NOD lung and blood CD8α$^+$CD103$^+$ DCs are present in normal numbers and lack expression of Langerin

To study whether the abnormalities of the CD8α$^+$ DC population as detected in the NOD pancreas are presented systemically or locally, blood and lungs of three mouse strains were analyzed using flow cytometry. In the lungs as well as the blood CD8α$^+$CD103$^+$ DCs were present in all strains, but extracellular or intracellular Langerin expression could not be detected (Sup.Fig.4, 5 and Sup.Fig.1B, C). Significant differences in CD8α$^+$CD103$^+$ DC numbers in the blood and lungs were not detected between the mouse strains. Furthermore, the expression of CCR5 and CLEC9A on the CD8α$^+$CD103$^+$ DCs in the lungs as well as the blood was low and differences were not detected between the mouse strains (data not shown). These results indicate that the abnormalities of the CD8α$^+$ DC population are only present in the NOD pancreas DC population.

Discussion

This observational study shows that the pancreas of wild type mice contains a population of CD8α$^+$CD103$^+$Langerin$^+$ DCs which express CLEC9A and CCR5. The expression of Langerin and CLEC9A suggest that this population is involved in the uptake of cell-associated antigens from endocrine and exocrine cells. It is known that CD8α$^+$ DCs have the capacity to produce high levels of IL-12 upon inflammatory stimulation [22-23], while in steady state these DCs do not [24]. This is in line with our observation that CD8α$^+$ DCs in the C57BL/6 pancreas do not express IL-12 mRNA, but have high mRNA expression levels of IL-10 and express CCR5. This phenotype suggests that this subset of DCs is involved in processing cell-bound auto-antigens to induce tolerance.
Interestingly, we observed that particularly this presumed tolerogenic subset of CD8α$^+$CD103$^+$Langerin$^+$ DCs was significantly decreased in the NOD pancreas. Moreover, the CD8α$^+$CD103$^+$Langerin$^+$ DCs demonstrated reduced expression of CLEC9A and CCR5 and diminished mRNA levels for IL-10 as compared to non-diabetic controls. These data suggest that reduced numbers of CD8α$^+$CD103$^+$Langerin$^+$CLEC9A$^+$CCR5$^+$ DCs in the pancreas are

associated with loss of tolerance in the NOD mouse model of diabetes.
A clear limitation of our study is that the $CD8\alpha^+$ DC population in the pancreas was too small to isolate enough cells to perform functional studies such as suppressor assays. Such studies are a prerequisite before a definite conclusion can be drawn regarding the actual tolerogenic properties of this pancreas DC population.
In our study we isolated DCs from the total pancreas. These DC preparations included intra-islet DC population studied by others [25-27]. However, we showed earlier that in the pre-diabetic NOD pancreas DCs first accumulate near and around the islets of Langerhans [2]. Furthermore, data in the literature indicate that all intra-islet DCs are $CD8\alpha^-$ [27].
It is previously demonstrated by other investigators that depletion or enrichment of DCs can both abrogate diabetes in NOD mice. Depletion experiments are targeting the total $CD11c^+$ cell population [5]. Therefore, no effect of the depletion of the minute $CD8\alpha^+$ population can be expected. Treatment of NOD mice with Flt3L enhances splenic $CD8\alpha^+$ DCs numbers and induces protection against diabetes [9]. This study showed that these splenic $CD8\alpha^+$ DCs had a tolerogenic function and protects against diabetes. No data on the pancreatic $CD8\alpha^+$ DCs are supplied in this study, but the Flt3L treatment is likely to enhance the $CD8\alpha^+$ pancreatic DC population as well. Increased numbers of this tolerogenic DC population might overcome the defects we describe here and result in protection.
It is assumed that the autoimmune process in the NOD mouse starts with DC accumulation around 5 weeks of age. Recently we showed the presence of proliferating DC precursors in the fetal and pre-diabetic pancreas of NOD mice, indicating that the autoimmune process in the NOD mouse starts much earlier [4]. This data on proliferating precursors and our present results on $CD8\alpha^+$ DC abnormalities in the NOD pancreas point to an intrinsic abnormality of these cells, which can lead to a loss of tolerance and finally in auto-immune diabetes. Whether the abnormal precursors for DCs in the NOD pancreas give rise to the abnormal $CD8\alpha^+$ DCs we describe here remains to be established.
In pLNs of NOD mice increased numbers of $CD8\alpha^+CD103^+Langerin^+$ DCs were present, which correlates with a significantly larger size of the pLNs due to the ongoing autoimmune response. The reduction of $CD8\alpha^+$ DCs in the NOD pancreas can be explained by an enhanced migration of these DCs to pLNs. However, pLNs of NOR mice showed also an increased number of $CD8\alpha^+CD103^+Langerin^+$ DCs, whereas in the pancreas of NOR mice no reduction of $CD8\alpha^+CD103^+Langerin^+$ DCs was detected. Furthermore, monocytes and DCs from NOD mice had an increased adhesion to fibronectin and a decreased migration towards the pro-inflammatory chemokines CCL2 and -3 [28-30]. In addition, NOD mice show an increased number of mature circulating monocytes [31]. Taken this together, the increased adhesion and the decreased migration of NOD monocytes may contribute to the reduced $CD8\alpha^+$ DC numbers in the NOD pancreas, rather than an enhanced migration towards the pLNs.
In the pLNs of control mice the $CD8\alpha^+CD103^+$ DCs showed a reduced Langerin and CLEC9A

surface expression as compared to their peripheral counterparts. This suggests that after traveling to the draining LN the altered make up of these molecules reflects the altered function of LN DCs as compared to that of peripheral DCs, namely that uptake of cellular material is of less importance in the LN as it is in the periphery (while antigen presentation has gained in importance). This is in line with the observation that lectins are internalized from the cell surface upon maturation [32].

In the NOD mouse the defects in expression of CD103, Langerin, CLEC9A and CCR5 as observed in pancreas CD8α$^+$ DCs was no longer detectable in the pLNs. It thus seems that the pancreas environment is of importance to bring out the alterations in the CD8α$^+$ subset of tolerogenic DCs specialized in taking up cellular auto-antigens. This alteration is overcome after a maturation of DCs when travelling to the draining LN. In line with this, in a previous study by our group we observed no clear phenotypic or functional abnormalities in DCs isolated from NOD mouse LNs and spleen, while such abnormalities could easily be detected when studying DCs generated from NOD BM precursors or NOD circulating monocytes [31, 33]. In these studies DC precursors isolated from the BM of NOD mice showed proliferation/differentiation abnormalities while in vitro these precursors gave rise to DCs with a spontaneous high pro-inflammatory profile [34-35]. These abnormal DCs have a high level of NFkB, high IL-12 and low IL-10 expression [36-39] and such DCs are incapable of sufficiently sustaining the proliferation of Treg cell populations [40]. Collectively, these data suggest that in particular immature DC populations in peripheral tissues are defective in tolerance induction in the NOD mouse model, while mature DC populations in lymphoid organs have overcome these defects. However, this notion needs confirmation in further functional studies.

In conclusion, we demonstrate for the first time local abnormalities in the CD8α$^+$ DC population in the pre-diabetic NOD pancreas. These data support the earlier expressed concept of a key role for abnormally differentiated myeloid cell populations in the loss of tolerance in the development of autoimmune diabetes.

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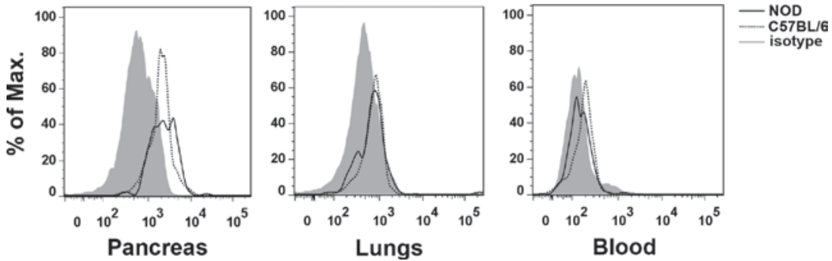
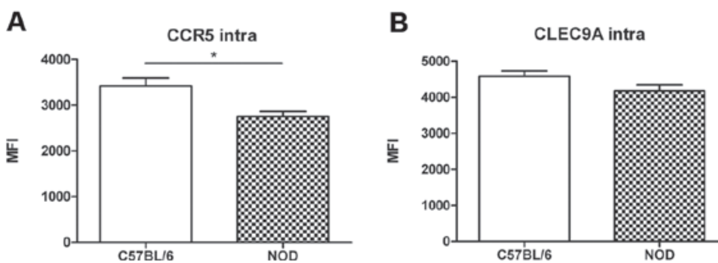
Supplemental figures



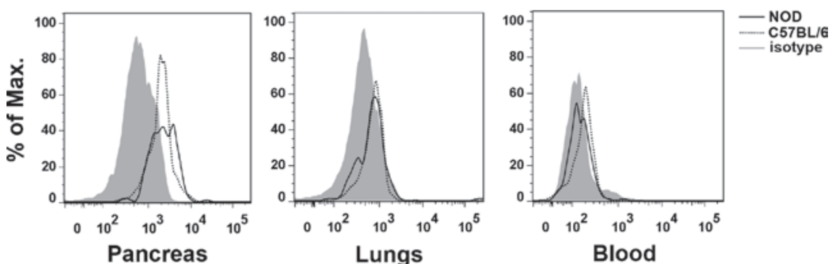
Sup Fig. 1. Intracellular Langerin expression on CD8α$^+$ DCs in the pancreas.

Flow cytometry was performed on CD45$^+$ cells isolated from the pancreas and lungs and on blood cells of C57BL/6 and NOD mice of 4 weeks. Histograms show the intracellular expression of Langerin on CD8α$^+$CD103$^+$ DCs at 4 weeks of age of the pancreas (A), lungs (B) and blood (C). Data shown are representative of 5 mice.



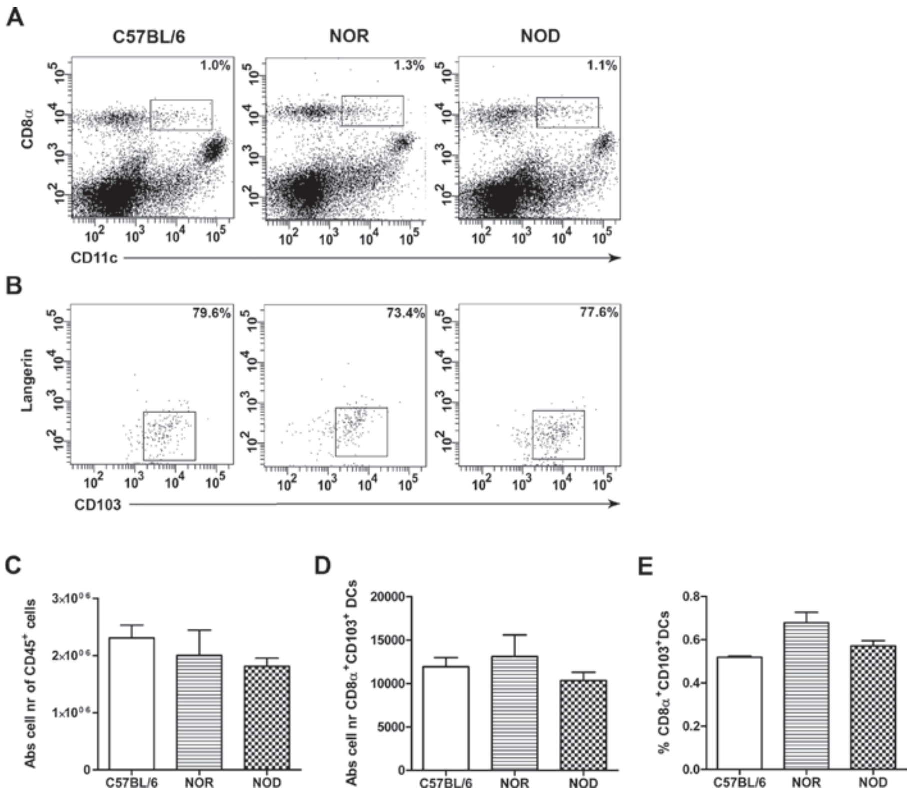
Sup Fig. 2. Intracellular CCR5 and CLEC9A expression on CD8α$^+$CD103$^+$Langerin$^+$ DCs in the pancreas.

CD45$^+$ cells isolated from the pancreas of C57BL/6 and NOD mice (4 weeks) were analyzed using flow cytometry. Bar graphs show the mean MFI of CCR5 (A) and CLEC9A (B) on CD8α$^+$CD103$^+$Langerin$^+$ DCs 4 weeks of age. Data are presented as average + SEM, n=5 experiments, * $p < 0.03$ as determined by unpaired Mann-Whitney U test.



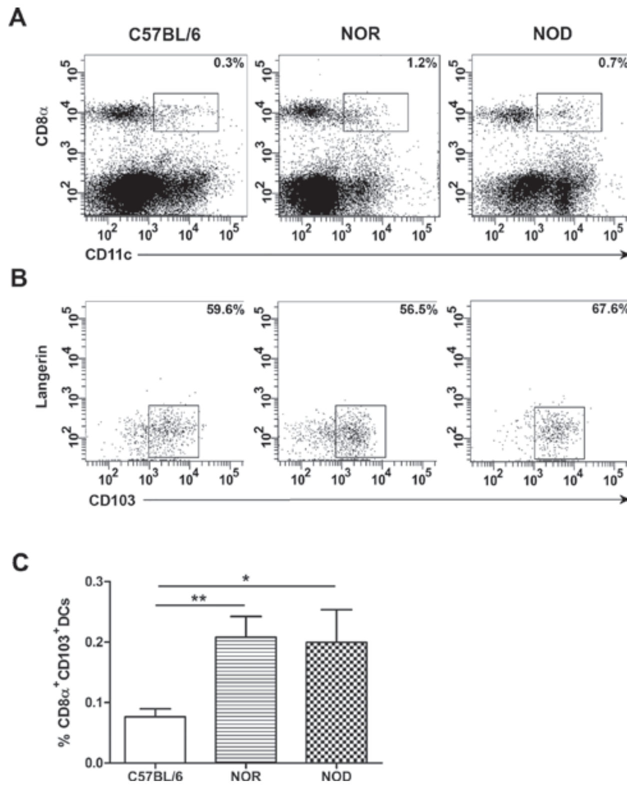
Sup Fig.3. Expression of IL-10, TGF-β and TNF-α on CD8a$^+$ DCs from the pancreas.

CD11c$^+$ cells were isolated from the pancreas (4 weeks) of C57BL/6 and NOD mice and were stimulated with LPS (including Golgistop) for 5 hours. CD11c$^+$CD8$^+$CD103$^+$Langerin$^+$ cells were characterized using flow cytometry as described. Histograms represent the IL-10, TGF-β and TNF-α expression. Data shown are representative of 2 experiments, with 2 pooled mice per experiment.



Sup.Fig.4. Phenotype of CD8a⁺ DCs in lungs.

Flow cytometric analysis was performed on $CD45^+$ cells isolated from the lungs of C57BL/6, NOR and NOD mice of 4 weeks. Dot plots show CD11c and CD8α expression (A) and CD103 and extracellular Langerin expression on $CD8\alpha^+$ DCs (B). Bar graphs show the total cell number (C), the absolute cell number of $CD8\alpha^+CD103^+$ DCs (D) and the percentage of $CD8\alpha^+CD103^+$ DCs of the total $CD11c^+$ population (E). Data are presented as average + SEM, n=4 mice, * $p < 0.03$ as determined by unpaired Mann-Whitney U test.



Sup.Fig.5. Phenotype of CD8a$^+$ DCs in the blood.

Flow cytometric analysis was performed on cells from blood of C57BL/6, NOR and NOD mice of 4 weeks. Dot plots show CD11c and CD8α expression (A) and CD103 and extracellular Langerin expression on CD8α$^+$ DCs (B). Bar graphs show the percentage of CD8α$^+$CD103$^+$ DCs of the total CD11c$^+$ population (E, H). Data are presented as average + SEM, n=4 mice, * $p < 0.03$ as determined by unpaired Mann-Whitney U test.

CHAPTER 5

THE KINETICS OF PLASMACYTOID DENDRITIC CELL ACCUMULATION IN THE PANCREAS OF THE NOD MOUSE DURING THE EARLY PHASES OF INSULITIS

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Abstract

In non-obese diabetic (NOD) mice that spontaneously develop autoimmune diabetes, plasmacytoid dendritic cells (pDCs) have a diabetes-promoting role through IFN-α production on one hand, while a diabetes-inhibiting role through indoleamine 2,3-dioxygenase (IDO) production on the other. Little is known about the kinetics and phenotype of pDCs in the NOD pancreas during the development of autoimmune diabetes.

While para/peri-insular accumulation of conventional dendritic cells (cDCs) could be observed from 4 weeks of age onwards in NOD mice, pDCs only started to accumulate around the islets of Langerhans from 10 weeks onwards, which is concomitant with the influx of lymphocytes. NOD pancreatic pDCs showed a tolerogenic phenotype as assessed by their high expression of IDO and non-detectable levels of IFN-α and MxA. Furthermore, expression of the pDC-attracting chemokines CXCL10 and CXCL12 was significantly increased in the NOD pancreas at 10 weeks and the circulating pDC numbers were increased at 4 and 10 weeks.

Our data suggest that a simultaneous accumulation of IDO^+ pDCs and lymphocytes in the pancreas in 10 weeks old NOD mice, which may reflect both an immunogenic influx of T cells as well as a tolerogenic attempt to control these immunogenic T cells.

Introduction

The nonobese diabetic (NOD) mouse model is a widely used animal model of type 1 diabetes (T1D) [1] and characteristically develops lymphocyte accumulations around the islets of Langerhans from the age of 10 weeks onwards [2, 3]. These cellular accumulations subsequently progress to infiltration of the islets and finally destruction of the insulin-producing beta cells. Prior to the lymphocyte accumulation, an increased influx of conventional dendritic cells (cDCs) into the NOD pancreas from 4 weeks onwards can be observed and these cDCs concentrate in and around the islets [4, 5]. Previously, we showed in a depletion study using clodronate-loaded liposomes, that these early accumulating mDCs are essential for the recruitment of lymphocytes into the NOD pancreas [6]. A study by Saxena et al. [7] using conditional knock-out mice confirmed these observations and showed that an early temporal depletion of mDCs totally abrogated the development of insulitis and diabetes in the NOD mouse model.
In addition to mDCs, distinct and vital roles for pDCs have also been described to be important in the development and progression of diabetes [8, 9]. In mice pDCs are characterized as $CD11b^{-}CD11c^{low}B220^{+}PDCA\text{-}1^{+}Siglec\text{-}H^{+}$ cells, and these cells express the chemokine receptors CCR5, -7, CXCR3 and -4 [9]. pDCs are classically known as important mediators of antiviral immunity through their ability to produce large quantities of type I interferons (IFN) upon viral infection and stimulation of the appropriate toll-like receptors (TLRs) [8]. While virally activated pDCs act as immunogenic cells, resting or alternatively activated pDCs have been described to have tolerogenic activity [10, 11]. Interestingly, an aberrant chronic pDC activation and secretion of type I IFN has been found in systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjögren's Syndrome, and it is thought to play an important role in the pathogenesis of these systemic autoimmune disorders [12-15].
In the development of autoimmune diabetes in NOD mice, both a pathogenic and tolerogenic role for pDCs has been described. pDCs were found to be pathogenic and contribute to disease development through IFN-α production on one hand [7, 16, 17], while depletion of pDCs on the other led to an acceleration of insulitis and a loss of local indoleamine 2,3-dioxygenase (IDO) [7, 18]. It has been suggested that the aggressiveness of the NOD insulitis is controlled by local tolerogenic pDCs and production of IDO, which could induce deletion of self-reactive cells by depleting the cells of tryptophan or could stimulate the development of regulatory T cells through the production of kynurenine products [19]. pDCs could also act in a tolerogenic manner through interaction between the programmed death ligand 1 (PD-L1) on pDCs and PD-1 on T cells, which delivers inhibitory signals to T cells [20]. However, whether this pathway plays a role in the tolerogenic function of pDCs in NOD autoimmune diabetes has not yet been addressed. In fact, little is known about the kinetics of pDC accumulation in the NOD pancreas during the spontaneous development of the autoimmune insulitis process.

Here we investigated the presence and localization of pDCs in the NOD pancreas during the early phases of insulitis (4 and 10 weeks). We analyzed their expression of IDO and PD-L1 as well as local IFN production, both by protein expression and by induction of the IFN-inducible gene myxovirus (influenza) resistance A (MxA). In addition, we studied the pancreatic expression of chemokines that are important for the attraction of pDCs from the circulation and enumerated pDCs in the circulation of the NOD mouse in the early phases of the insulitis process.

Materials and methods

Animals

C57BL/6 and NOD/shiLTj female mice were obtained from Charles River Laboratories (Maastricht, The Netherlands) and NOR/LTj mice from the Jackson Laboratory (Bar Harbor, ME, USA). Mice of 4, 10 and 20 weeks of age were used and housed under specific pathogen-free conditions.

Ethics statement

Sampling of the mice was approved by the Animal Experiments committee of the Erasmus MC (Dierexperimentencommissie (DEC), which is the ethical committee installed and officially recognized as required by the Dutch Law on Experimental Animals, the Dutch analogue for the IACUC). The approval number is: DEC#2334, dated June 15, 2011. The study was conducted in compliance with all relevant Dutch laws and in agreement with international and scientific standards and guidelines.

Immunohistochemistry

Cryostat sections (6 μm) of pancreases of C57BL/6, NOR and NOD mice were prepared and fixed with cold methanol and acetone. Slides were incubated with rat-anti-Siglec-H (both eBiosciences, San Diego, CA, USA) or rat-anti-IDO (Biolegend, San Diego, CA, USA) followed by rabbit-anti-rat-PO (DAKO, Glostrup, Denmark). Subsequently, slides were incubated with Nickel-DAB and counterstained with nuclear fast red (both Sigma Aldrich, St. Louis, MO, USA), followed by mounting in Entallan (Merck, Darmstadt, Germany). Insulitis was evaluated by the analysis of at least 50 islets according the following scale: 0, no infiltrating cells; 1, few infiltrating cells peri-insular; 2, large numbers of infiltrating cells around the islet and 3, large numbers of infiltrating cells in the islet.

Immunofluorescence

Cryostat sections (6 µm) of pancreases of C57BL/6 and NOD mice of were prepared and fixed with cold methanol and acetone. Slides were incubated with rat-anti-IDO followed by goat-anti-rat-TexasRed (Southern Biotechnology Associates Inc., Birmingham, AL, USA) and rat-anti-Siglec-H-FITC (eBiosciences). Finally, slides were mounted in Vectashield with DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

Preparation of cell suspensions

Pancreases were isolated after a cardiac perfusion and cut into small pieces and digested with collagenase type 1 (1 mg/ml), hyaluronidase (2 mg/ml) (both Sigma Aldrich, St. Louis, MO, USA) and DNAse I (0.3 mg/ml) (Roche Diagnostics, Almere, The Netherlands) for 40 minutes at 37ºC. Subsequently, cells were flushed through a 70 µm filter and washed. Blood was collected in EDTA tubes after a cardiac punction. Erythrocytes were lysed with NH_4Cl buffer and cells washed with PBS. All cells were resuspended in PBS containing 0.1% BSA followed by flow cytometric staining.

Flow cytometry

Single-cell suspensions from pancreas were labeled with CD45 beads (Miltenyi, Leiden, The Netherlands) and $CD45^+$ cells were sorted using AutoMACS (Miltenyi) and labeled with mAbs. Single-cell suspensions from blood were labeled with mAbs. Antibodies used were B220-Pacific Blue, CCR5-PE, CCR7-PE-Cy7, CXCR3-PE, CXCR4-APC, CD11b-APC-Cy7, CD80-PE-Cy5, CD86-APC, PDCA-1-FITC and PD-L1-PE (all eBiosciences, San Diego, CA, USA). Afterwards cells were washed and resuspended in 0.1% BSA / 0.5% paraformaldehyde, followed by analysis on a FACS Canto HTSII (Becton Dickinson) flow cytometer and FACS Diva and Flowjo software.

Protein expression determination

Pancreas lysates of C57BL/6, NOR and NOD mice were prepared by homogenization of the pancreas in ice-cold PBS supplemented with protease inhibitor cocktail (Life Technologies, Paisley, UK). The lysates were sonicated twice for 30 seconds and centrifuged at 1000g at 4°C for 10 minutes. The supernatant was collected and stored at -80°C. The protein concentration in the pancreas lysates was determined using the Bradford method (Bio-rad Laboratories GmbH, München, Germany).
The pancreas lysates were tested for CXCL10 protein expression using a cytometric bead array according to the manufacturer's protocol (eBiosciences). Briefly, a mixture of beads coated with antibodies against CXCL10 was incubated with the lysate or standard mixture.

A biotin-conjugated second antibody mixture was added followed by streptavidin-PE. Samples were analyzed using a FACS Canto HTSII (Becton Dickinson) and FlowCytomix Pro Software (eBiosciences). CXCL9, -11 and -12 (R&D Systems, Minneapolis, MN, USA) and IFN-α (PBL Interferon source) protein expression were determined using ELISA kits according to the manufacturers protocol.

Statistical analysis

Data were analyzed by Mann-Whitney U test for unpaired data. All analyses were carried out using SPSS software (SPSS Inc., Chicago, IL, USA) and considered statistically significant if $p<0.05$.

Results

pDCs accumulate around the islets in the NOD pancreas concomitant with lymphocytes, but later than mDCs

The localization of mDCs and pDCs in the pancreas of NOD, C57BL/6, and nonobese diabetic resistant (NOR) mice of 4 and 10 weeks was studied by immunohistochemistry. The NOR mouse is a NOD-related MHC-syngeneic strain, and often used as a control strain for the NOD mouse model. NOR mice do not develop diabetes and only have a mild lymphocytic peri-insulitis [21].

From 4 weeks onwards $CD11c^+$ mDCs were observed to accumulate around the islets in the NOD pancreas (Fig.1A and B). The peri-islet accumulation of $CD11c^+$ mDCs was also observed in the NOR pancreas, although at reduced numbers. Siglec-H^+ pDCs were detectable in the exocrine pancreas of NOD mice from 4 weeks onwards, but significant differences were not detected between NOD and control strains. At 10 weeks the lymphocytic para/peri-insulitis had started in the NOD mouse, at that time mDCs had become more numerous and pDCs also had started to accumulate at the islet edges (Fig.1A). The number of Siglec-H^+ pDCs was significantly higher in the NOD pancreas as compared to NOR and C57BL/6 at 10 and 20 weeks (Fig.1C). In the NOD pancreas the levels of mDCs and pDCs at 20 weeks were not significantly different from the NOD levels at 10 weeks.

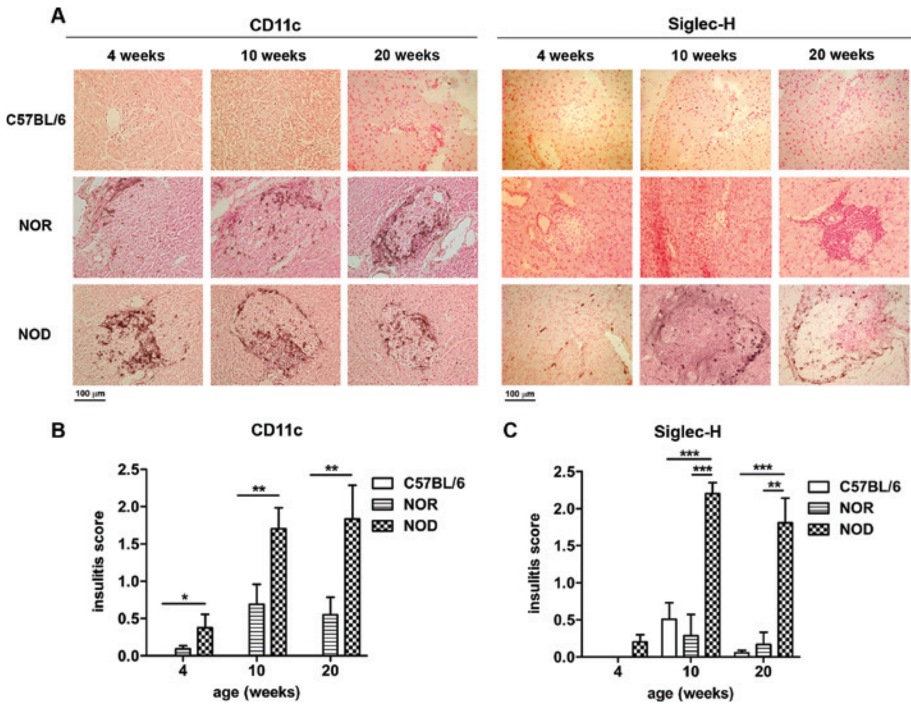

Figure 1. Accumulation of mDCs and pDCs in the NOD pancreas.
The localization of mDCs and pDCs in the C57BL/6, NOR and NOD pancreas at the age of 4, 10 and 20 weeks was determined by immunohistochemical detection. Pictures show CD11c and Siglec-H expression in the pancreas of mice from 4, 10 and 20 weeks of age, magnification 200x (A). Bar graphs represent the mean insulitis score of $CD11c^{+}$ (B) and Siglec-H^{+} cells (C) in the pancreas. Data are presented as average + SEM, n=5 mice, * p< 0.04, ** p< 0.01, *** p< 0.001 as determined by the unpaired Mann-Whitney U test.

In addition to the immunohistochemistry, flow cytometry was performed on sorted $CD45^{+}$ cells from the pancreas of 4 and 10 week old C57BL/6, NOR and NOD mice. As Siglec-H was not detectable after the pancreatic digestion (data not shown), pDCs were identified by PDCA-1 staining. No differences in $CD11b^{-}$PDCA-1^{+} pDCs were observed between the strains at 4 weeks of age (Fig.2A-C). However, at 10 weeks both the percentage and absolute number of $CD11b^{-}$PDCA-1^{+} pDCs was significantly increased in NOD mice compared to controls (Fig.2A, D and E). All strains showed similar expression of B220 in the $CD11b^{-}$ PDCA-1^{+} pDC population (Fig.2F), but CD80 and CD86 were significantly increased in NOD mice (Fig.2F-H), suggesting increased level of activation.

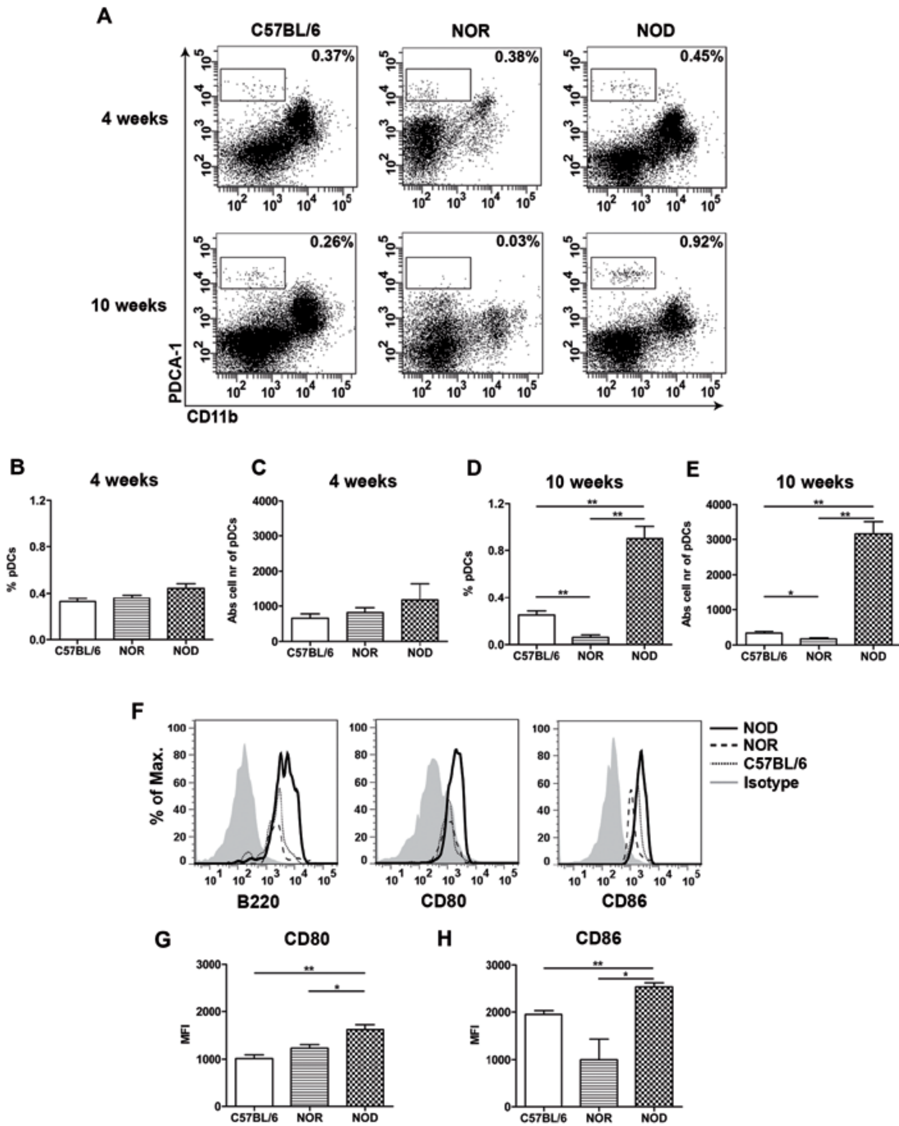

Figure 2. Increased pDC numbers in the NOD pancreas at 10 weeks of age.

The presence of pDCs in the C57BL/6, NOR and NOD pancreas at the age of 4 and 10 weeks was determined by flow cytometry. Dot plots show the CD11b and PDCA-1 expression on sorted CD45$^+$ cells from the pancreas (A). Bar graphs represent the percentage and the absolute number of CD11b$^-$ PDCA-1$^+$ cells in the pancreas at 4 (B-C) and 10 weeks (D-E). Histograms represent the B220, CD80 and CD86 expression on pDCs (CD11b$^-$PDCA-1$^+$ cells) in the pancreas of 10 weeks (F). Bar graphs represent the geometric MFI of CD80 (G) and CD86 (H) on pDCs. Data are presented as average + SEM, n=5-6 mice, * $p < 0.04$, ** $p < 0.01$ as determined by the unpaired Mann-Whitney U test.

Enhanced IDO, but decreased PD-L1 and absent IFN-α expression of NOD pancreatic pDCs

As NOD pancreatic pDCs expressed increased levels of co-stimulatory molecules, immunohistochemical analysis of both tolerogenic and activational markers was performed. In 4 week old NOD mice low numbers of IDO^{+} cells were detected near blood vessels in the exocrine pancreas. From 10 weeks onwards, when pDCs were found to accumulate at the islet edges, IDO^{+} cells were also detected both around and in the islets (Fig.3A). Immunofluorescent analysis confirmed that IDO^{+} cells were Siglec-H^{+} pDCs (Fig.3B).

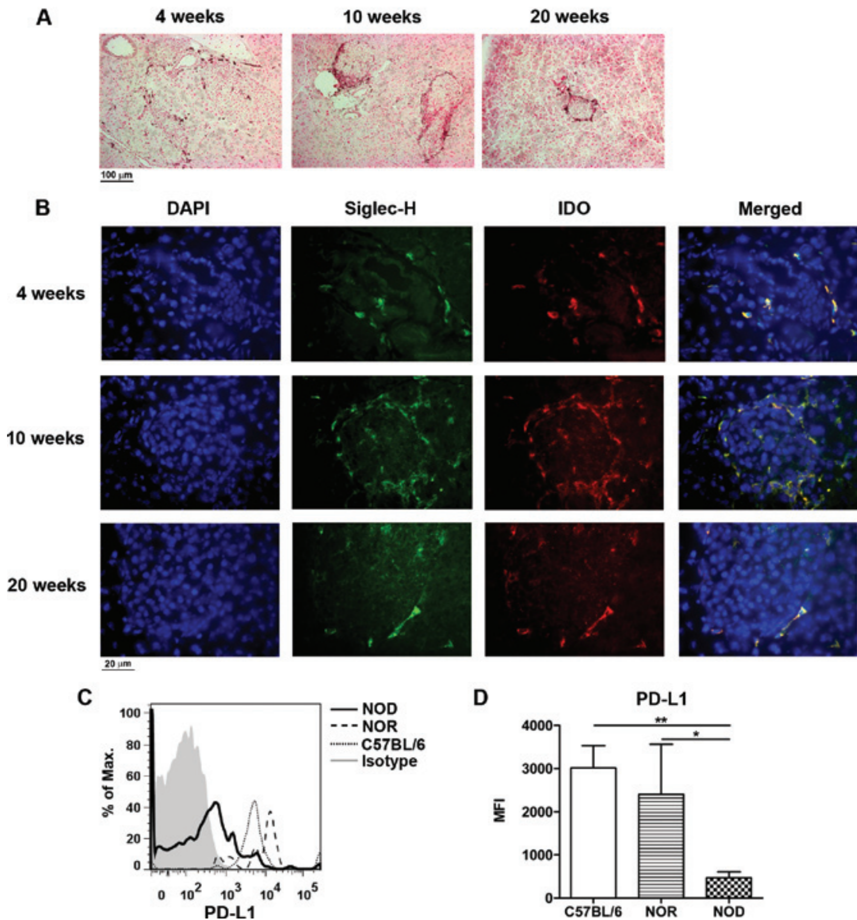

Figure 3. Enhanced IDO and a decreased PD-L1 expression in NOD pancreas pDCs.

Pictures show the immunohistochemical detection of IDO in the pancreas of NOD mice at 4, 10 and 20 weeks of age, magnification 200x (A). The pancreas of NOD mice was stained for Siglec-H (green), IDO (red) and DAPI (blue) by immunofluorescence, magnification 400x (B). Histogram represents the PD-L1 expression on pDCs (CD11b^{-}PDCA-1^{+} cells) in the pancreas of C57BL/6, NOR and NOD mice of 10 weeks of age (C). Bar graph represents the geometric MFI of PD-L1 on pDCs (D). Data are presented as average + SEM, n=5 mice, * $p< 0.04$, ** $p< 0.01$ as determined by the unpaired Mann-Whitney U test.

In the pancreas of C57BL/6 and NOR mice IDO$^+$ cells were not detected (data not shown). In contrast, NOD pancreatic pDCs showed significantly decreased expression of the tolerogenic ligand PD-L1 compared to control strains (Fig.3C and D). As pDCs are important producers of high amounts of IFN-α upon TLR activation by viruses and immune complexes, IFN-α expression in the pancreas was analyzed. No expression of IFN-α protein and the IFN-induced gene MxA mRNA was detected in the pancreas of all mouse strains at 4, 10 and 20 weeks of age (data not shown).

High expression of CXCL10 and CXCL12 in the NOD pancreas at the time of pDC accumulation

To investigate which chemokines and chemokine receptors might be important for the attraction of the pDCs, flow cytometry was performed on pancreatic pDCs. pDCs in the pancreas of all strains expressed similar levels of CXCR3 (Fig.4A), but NOD pDCs expressed increased levels of CXCR4 (Fig.4A and B). No expression of CCR5 or CCR7 was detected in all three strains (data not shown).

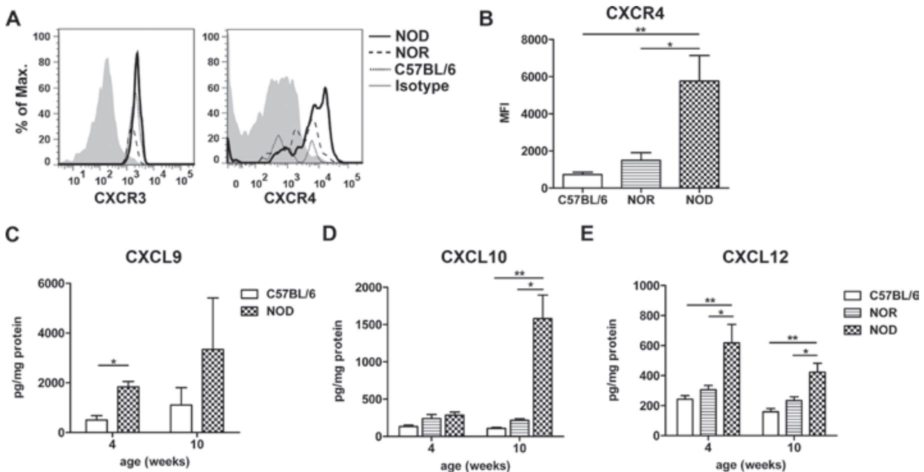

Figure 4. Expression of pDC receptors and chemokines in the pancreas.

Histograms represent the CXCR3 and CXCR4 expression on pDCs (CD11b$^-$PDCA-1$^+$ cells) in the pancreas of 10 weeks of age (A). Bar graph represents the geometric MFI of CXCR4 on pDCs in the pancreas of 10 weeks of age (B). Bar graphs represent the protein level of CXCL9 (C57BL/6 and NOD), CXCL10 and CXCL12 (C57BL/6, NOR and NOD) in pancreas lysates of 4 and 10 weeks (C-E). Data are presented as average + SEM, n=5-10 mice, * $p< 0.04$, ** $p< 0.01$ as determined by the unpaired Mann-Whitney U test.

As pancreatic pDCs expressed CXCR3 and CXCR4, the protein expression of their ligands CXCL9, CXCL10, CXCL11 (for CXCR3) and CXCL12 (for CXCR4) was assessed. CXCL9 and CXCL12 protein levels were significantly increased in the NOD pancreas at 4 weeks of age

(Fig.4C and E), although CXCL9 levels did not reach statistical significance at 10 weeks of age. CXCL10 protein expression levels were only significantly increased in the NOD pancreas at 10 weeks of age (Fig.4D) and no significant differences were found in the CXCL11 protein expression between the strains (data not shown).

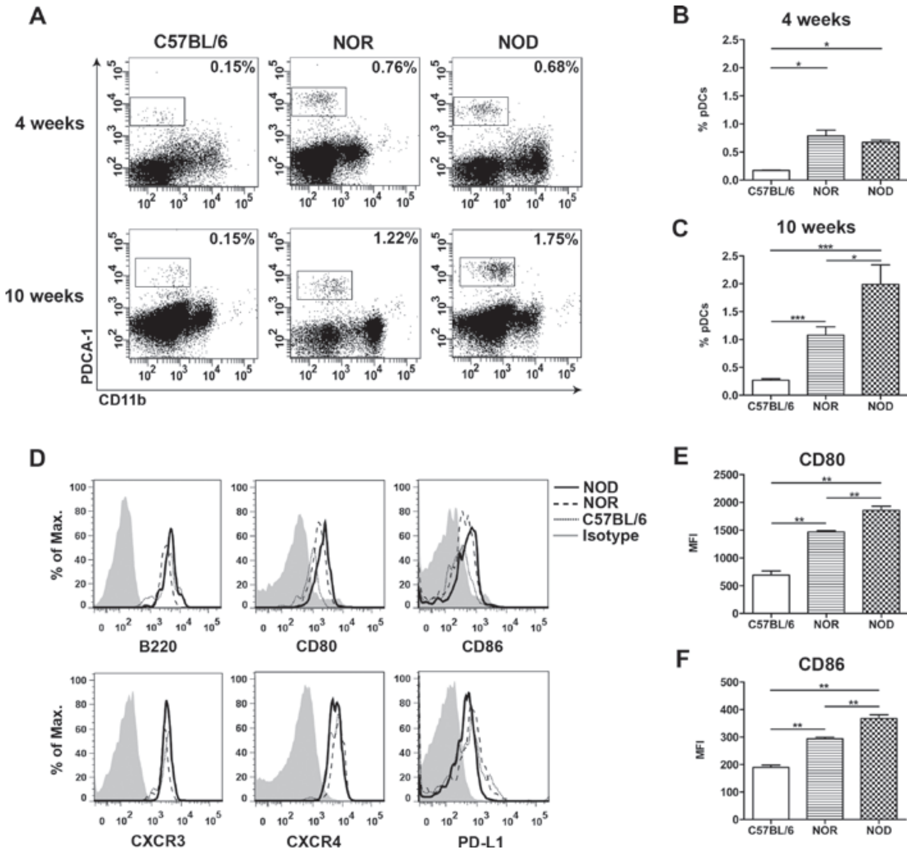

Figure 5. Increased percentage of pDCs in blood of NOR and NOD mice.

The presence of pDCs in the blood of C57BL/6, NOD and NOR of 4 and 10 weeks was determined by flow cytometry. Dot plots show the CD11b and PDCA-1 expression (A). Bar graphs represent the percentage of pDCs ($CD11b^{-}PDCA\text{-}1^{+}$ cells) at 4 weeks (B) and 10 weeks (C). Histograms represent the B220, CD80, CD86, CXCR3, CXCR4 and PD-L1 expression on pDCs in the pancreas of 10 weeks (D). Bar graphs represent the MFI of CD80 (E) and CD86 expression (F) on pDCs at 10 weeks. Data are presented as average + SEM, n=4-10 mice, * p< 0.04, ** p<0.01, *** p< 0.001 as determined by the unpaired Mann-Whitney U test.

Increased numbers of pDCs in the circulation of NOD mice

In peripheral blood, both an increase and reduction in the numbers of circulating pDCs have been described in the various phases of type 1 diabetes (T1D) development in humans [18,

22, 23]. Numbers of pDCs in the blood of C57BL/6, NOR and NOD mice were studied by flow cytometry at 4 and 10 weeks of age. At both ages the percentage of $CD11b^{-}PDCA\text{-}1^{+}$ pDCs in the blood of NOD and NOR mice was significantly increased as compared to C57BL/6 (Fig.5A-C). Furthermore, NOD mice showed an increased percentage of pDCs compared to NOR mice at 10 weeks of age (Fig.5A and C). pDCs were $B220^{+}$, $CXCR3^{+}$, $CXCR4^{+}$ and PD-L1^{low}, expressed at similar levels in all 3 strains (Fig.5D). However, the NOD and NOR pDCs expressed significantly higher levels of the co-stimulatory molecules CD80 and CD86 at 4 (data not shown) and 10 weeks of age compared to C57BL/6 mice (Fig.5D-F), suggesting increased activation state. Moreover, circulating pDCs in NOD mice also expressed significantly higher levels of CD80 and CD86 compared to NOR mice at 4 (data not shown) and 10 weeks of age (Fig.5D-F).

Discussion

This study shows that pDCs with a tolerogenic IDO^{+} profile accumulate at the islet edges in the NOD pancreas at 10 weeks of age. This is at the same time that lymphocytes can be observed to accumulate around the islets [2, 3]. In contrast, the accumulation of immunogenic disease-promoting mDCs occurred earlier from 4 weeks of age onwards. pDCs in the NOD pancreas have a partial tolerogenic profile by expressing IDO, although the PD-L1 expression was lower. Furthermore, no expression of IFN-α and the IFN-induced gene MxA is detected in the NOD pancreas. The pDC increase is accompanied by an increased expression of CXCL10 and -12 in the NOD pancreas.

Saxena et al. [7] described a role for IDO in the down-regulation of the NOD insulitis using an IDO inhibitor, but the actual IDO-producing cells were not identified. Here, we show that pDCs in the NOD pancreas express IDO, identifying a potential local tolerogenic role for these cells. However, pDCs have also been implicated in a disease-promoting role in the NOD mouse: IFN-α^{+} pDCs were increased in the pancreas-draining lymph nodes (pLNs) of 4 week old NOD mice and an antibody to the IFN-α receptor was able to halt the diabetogenic process [24, 25]. In our hands we did not detect significant numbers of pDCs in the NOD pancreas as early as 4 weeks, although we did not analyze pLNs. Furthermore, levels of IFN-α and the interferon-inducible gene MxA were not detectable in both NOD and control mouse strains. Our data would therefore not support an immunogenic function for pancreatic pDCs in the NOD mouse and we favour a tolerogenic role for pancreatic pDCs. Collectively, the literature data and our findings suggest that pDCs in the pLNs early in the process have a different and opposite function (i.e. disease-promoting) as compared to those infiltrating the pancreas at later stages of the disease (disease-inhibiting). While IDO-expressing pDCs were only found in the NOD pancreas, we found a reduced expression level of PD-L1 on these cells compared to pancreatic pDCs in control strains. Interestingly, endocrine cells of inflamed

islets of 10 week old pre-diabetic NOD mice also express PD-L1 (but not PD-L2) at 10 weeks of age, and blocking PD-L1 increased insulitis severity and diabetes development in NOD mice [26]. This suggests that PD-L1 may be involved in mediating apoptosis of autoreactive effector T cells in the peri-insulitis, but it remains to be investigated whether this is due to expression of PD-L1 by tolerogenic pDCs or by endocrine cells under immune attack.

A limitation of our study is that the number of pDCs in the pancreas was too low to perform functional studies. Such studies would have strengthened our observations. Another limitation is that the digestion method for the pancreas influenced the expression of Siglec-H. Siglec-H is selectively expressed on pDCs and certain macrophage subsets in the spleen and considered the best marker for pDCs [27, 28]. Since Siglec-H could not be used for the flow cytometric analysis we used PDCA-1 in combination with other phenotypic markers, like CD11b and B220, to distinguish pDCs. PDCA-1, also known as bone marrow stromal antigen-2, is selectively expressed on pDCs, but is also up regulated on other cell types upon type I IFN or IFN-γ stimulation [29]. Despite the reduced specificity of PDCA-1, both immunohistochemistry and flow cytometric analysis suggested an accumulation of pDCs in the NOD pancreas at the time of lymphocyte insulitis, but at a later stage than mDCs. The mean insulitis score of Siglec-H^{+} pDCs was higher as compared to CD11c^{+} mDCs likely due to the fact that pDCs express low levels of CD11c and the limited sensitivity of this staining.

With regard to the chemokines attracting pDCs, recent studies showed that the expression of CXCL10 alone was not sufficient for pDC recruitment, but that the co-expression of CXCL10 and CXCL12 synergistically was required to induce pDC migration [30, 31]. We observed that the expression of both CXCL10 and CXCL12 was elevated in the NOD pancreas at 10 weeks of age. In addition, the receptor for CXCL12, CXCR4, was significantly increased on pDCs in the NOD pancreas and CXCR3, the receptor for CXCL10, was normally expressed. These findings are supportive of synergistic function of CXCL10 and CXCL12 in the recruitment and retention of pDCs in the NOD pancreas. Moreover, in the pancreas of NOR mice CXCL10 and CXCL12 were not increased and pDC infiltration was not observed. It has been shown that beta cells express CXCL10 during the insulitis in LCMV-infected mice [32] and purified human and rat islet cells have been found to produce CXCL10 upon stimulation with IFN-γ or IL-1β [33]. It is therefore likely that the endocrine cells are the source of these pDC-attracting chemokines, but this needs further investigations.

Several reports have shown reduced pDC numbers in the circulation of patients with recent onset T1D (within 3 months) as well as in patients with long standing T1D (more than 5 years) [22, 23]. Interestingly, a recent study showed an increased frequency of pDCs in the blood of T1D patients at the time of diagnosis, which declined after disease onset [18]. This observation is in line with our data showing an increased pDC number in the blood of NOD mice with active insulitis and points to the putative importance of distinguishing the

different phases of disease development in humans and animal models with regard to pDC frequencies in the circulation. Perhaps high numbers of circulating pDCs might be an early sign of an existing (pre-)diabetic insulitis process.

In summary, our present and past studies on the kinetics of the accumulation of the various subsets of DCs in the NOD pancreas during the development of diabetes reveal the following pattern of accumulation:

1) From 4 weeks onwards mDCs start to accumulate in the NOD pancreas. It is our hypothesis that this accumulation of mDCs is (at least in part) due to an aberrant proliferation of local pancreatic precursors for mDCs [34]. Retention of activated and maturing mDC population in the pancreas might be due to an aberrant expression in the pancreas of the lymphoid tissue-specific CCR7 ligands CCL19 and CCL21, as described previously by us [35]. These mDCs are local drivers of the immunogenic effector response towards islet antigens. In addition, a recent study used magnetic resonance imaging (MRI) to visualize local effects of pancreatic-islet inflammation to predict the onset of diabetes in NOD mice [36]. They show that autoimmune diabetes is set at an early age in these mice. In pLNs, mDCs but also IFN-α-producing pDCs are drivers of the immunogenic effector response towards islet antigens.

2) From 10 weeks onwards pDCs and lymphocytes accumulate in the NOD pancreas, possibly attracted by CXCL10 and CXCL12 expression. The infiltrating pDCs express IDO and are meant to dampen the insulitis development in an attempt to halt the insulitis process.

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CHAPTER 6

GENERAL DISCUSSION

General Discussion

Summary of data

1. For the study on DC precursors in the normal pancreas of mice and humans as well as for the NOD pancreas we established:

    - That cDC precursors were present in the fetal pancreas of C57BL/6 and NOD mice. These cells had a monocyte-like phenotype, with CD11b, Ly6C and F4/80 expression.
    - That monocyte-like cells isolated from the fetal pancreas of wild-type and NOD mice develop into functional cDCs upon GM-CSF stimulation, but not upon Flt3L stimulation.
    - That in the NOD mouse model the monocyte-like cDC fetal precursor cells showed an enhanced proliferation in vivo, and in vitro upon GM-CSF stimulation, as compared to the same cells in C57BL/6 and NOR mice.
    - That in the human pancreas proliferating $CD14^+$ monocyte-like cells could be found located near the islets and outside the blood vessels. The number of these monocyte-like cells in the fetal pancreas was significantly higher as compared to the pancreas of children and adults.

2. For the study on the characterization of mature cDC populations in the NOD pancreas we established:

    - That reduced numbers of (tolerogenic) $CD8\alpha^+CD103^+Langerin^+$ cDCs were present in the NOD pancreas at 4 and 10 weeks of age as compared to the C57BL/6 and NOR pancreas.
    - That these $CD8\alpha^+$ cDCs in the NOD pancreas had a reduced expression of IL-10, CLEC9A and CCR5 as compared to $CD8\alpha^+$ cDCs in the C57BL/6 and NOR pancreas.
    - That these alterations in the $CD8\alpha^+$ cDC population in the NOD pancreas were not present in pancreas-draining lymph nodes (pLNs), lungs and circulation of NOD mice.

3. For the study on the kinetics of pDCs in the NOD pancreas we established:

    - That pDCs started to accumulate around the islets in the NOD pancreas after and much later than cDCs. The pDCs accumulated at the same time and together with the lymphocytes from 10 weeks of age onwards (para- and peri-insulitis). pDC accumulation in the C57BL/6 and NOR pancreas was not observed.

- That the pDCs in the NOD pancreas showed an enhanced indoleamine 2,3-dioxygenase (IDO) expression, but a decreased PD-L1 expression as compared to C57BL/6 and NOR pancreatic pDCs at 4, 10 and 20 weeks of age.
- That IFN-α protein and MxA mRNA expression was not detected in the NOD pancreas at any age.
- That a significantly higher expression of the chemokines CXCL10 and CXCL12 was present in the NOD pancreas at the time of pDC accumulation as compared to the C57BL/6 and NOR pancreas.
- That the number of pDCs (at 4 and 10 weeks of age) was increased in the circulation of NOD and NOR mice as compared to C57BL/6, but in the NOD mouse the highest.

Our studies on the kinetics of the accumulation of the various subsets of monocyte-like cells and DCs in the NOD pancreas during the development of diabetes therefore lead to the following conclusions:

1. The presence of proliferating monocyte-like cells in the normal pancreas of mice and humans has strengthened our concept that these cells give rise to local resident macrophages (MΦs) and cDCs. In addition, the pre-diabetic cDC accumulation in the NOD pancreas is due to an aberrant enhanced proliferation of these local monocyte-like cells, rather than to an inflammatory infiltration of monocytes from the circulation.
2. The reduced number of tolerogenic $CD8α^+CD103^+Langerin^+CCR5^+CLEC9A^+IL\text{-}10^+$ cDCs in the NOD pancreas might contribute to the loss of tolerance, hallmarking the development of autoimmune diabetes.
3. After the cDC accumulation IDO^+ pDCs accumulate in the NOD pancreas at the same time as lymphocytes (at 10 weeks of age), possibly attracted by the co-expression of CXCL10 and CXCL12. These infiltrating tolerogenic pDCs are likely to dampen the insulitis development in an attempt to halt the already initiated insulitis process.

The DC and MΦ population in general, and in the pancreas specifically, is very heterogeneous and plastic. One of the drawbacks of our study is that the use of cell-surface markers may lead to inconsistent results depending on the method of measurement and the localization of the cells under study. Therefore, it would be an advantage if classifications could be used other than based on cell surface markers to type the cells. Recently, a more reliable identification of the cells can be made using the expression of lineage-specifying transcription factors which are required for their development. The transcription factor PU.1 and STAT-3 and -5 are necessary for early DC development [1-5]. The transcription factor inhibitor of DNA binding-2 (Id2) plays an important role in the development of cDCs, whereas pDC development is mainly dependent on E2-2 [6-8]. The development of $CD8^-$ DCs from the CDP is driven by RelB and interferon regulatory factor 4 (IRF4) expression [9-

11]. The development of $CD8^+$ and $CD103^+$ DCs is driven by IRF8 and basic leucine zipper transcription factor 3 (Batf3) expression [10, 12]. The different precursors, DC subsets and their transcription factors are summarized in **Figure 1**. Using these transcription factors in future experiments it will be possible to get a more precise characterization of the local myeloid precursors and the DC subsets in the pancreas.

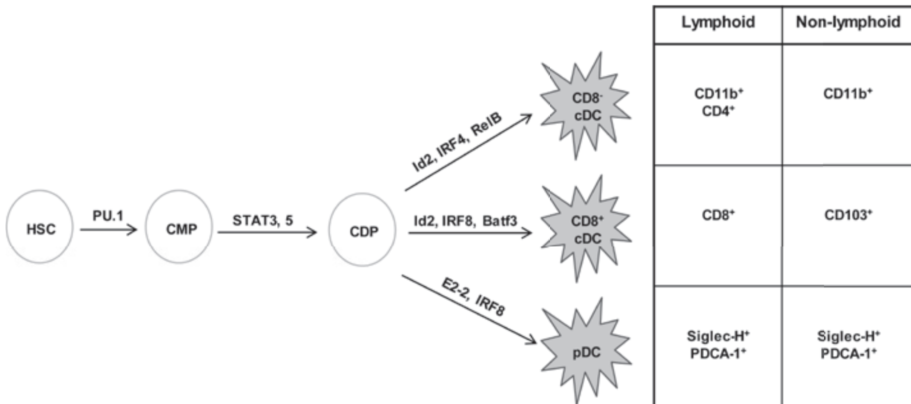

Figure 1. Development of DC subsets, their developmental regulators and surface markers in the mouse.

What is the origin of the monocyte-like cDC precursor in the pancreas?

After our studies the question arises what the origin of the monocyte-like cells in the pancreas is. The local pancreatic monocyte-like cells we observed could represent early seeded monocytes in the pancreas. Indeed, the monocyte-like cells have a similar phenotype as blood monocytes, such as the expression of CD11b, Ly6C and F4/80. But dissimilar from blood monocytes, the monocyte-like cells isolated from fetal NOD pancreas displayed a high proliferative activity upon GM-CSF stimulation. Such a proliferation was not observed in cultures of monocytes isolated from NOD peripheral blood (Chapter 2) and blood monocytes are described to be non-dividing cells [13]. These data and the observation of proliferating monocyte-like cells in the pre-diabetic pancreas, make us confident to conclude that the monocyte-like precursor cells are distinct from the peripheral blood monocytes.

In support of our view, a recent study revealed that some myeloid cells develop in the embryo before the appearance of hematopoietic stem cells (HSCs) [14]. This study showed that $CD11b^+F4/80^{hi}CX_3CR1^{hi}$ tissue MΦs derive from the yolk sac (YS) and are distinct from the HSC-derived MΦs, which are $CD11b^{hi}F4/80^{low}CX_3CR1^{low}$. They also found that the transcription factor Myb was required for HSC development and $CD11b^{hi}$ monocytes and $CD11b^{hi}$ MΦs, but not for YS-derived $F4/80^{hi}$ MΦs. These YS-derived $F4/80^{hi}$ MΦs were proliferating in most fetal tissues, and - important for our study - also in the pancreas.

In analogy with the YS-derived MΦs our pancreatic monocyte-like cells expressed CD11b

and F4/80 and were CX_3CR1^{hi} (preliminary data). The Ly6C expression on the YS-derived MΦs was not described, but these MΦs were $Flt3^{low}$, like our pancreatic monocyte-like cells.
Thus, we now assume that the pancreatic monocyte-like cells are YS-derived precursors, distinct from the HSCs from the bone marrow (BM).
In addition, there is evidence that another myeloid cell population, i.e. the microglia in the brain, derives from local proliferating progenitors [15]. This suggests that maintenance and local expansion of microglia in the brain are dependent on the self-renewal of these cells. Similar to the proliferation of monocyte-like cells in the NOD pancreas preliminary data show an enhanced proliferation of microglia in NOD brain (unpublished results). The proliferation capacity of the monocyte-like cells in the pancreas suggests that these cells might be self-renewal cells, like the microglia in the brain. However, the possibility that migrating monocytes from the circulation are modified by the microenvironment of the NOD pancreas and obtain proliferative capacity cannot be excluded and remains thus subject for further study.

Do proliferating monocyte-like cells contribute to the $CD8\alpha^+$ and/or $CD8\alpha^-$ cDC accumulation in the NOD pancreas?

Although our present data suggest that the cDC accumulation in the NOD pancreas is due to an aberrant enhanced proliferation of local monocyte-like cells (rather than to an inflammatory infiltration into the pancreas), the actual evidence for this view is limited.
It has been described that $CD8\alpha^+$ cDCs are mainly generated by Flt3L stimulation [16]. Our pancreatic monocyte-like cells have a low expression of the Flt3 receptor and do not give rise to cDCs and pDCs upon Flt3L stimulation in vitro (unpublished results). Therefore, it is not likely that pancreatic monocyte-like cells are capable of developing into $CD8\alpha^+$ cDCs. They probably give rise to $CD8\alpha^-$ cDCs, which are the accumulating cDCs in the NOD pancreas. The $CD8\alpha^+$ cDCs would in such a view derive from infiltrating monocytes or pre-cDCs from the circulation.

Do indeed the $CD8\alpha^-$ cDCs derive from the local monocyte-like precursor cells?

To answer this question several experimental approaches are possible. First, the use of transplantation experiments where NOD/CD45.2 BM is transferred into irradiated NOD mice might be performed. Using this approach the origin of accumulating infiltrating cells is normally determined. However, since cDCs are already present in the fetal and neonatal NOD pancreas (long before BM transplantation) an explicit answer cannot be given whether the accumulating cDCs derive from local proliferating monocyte-like cells.
Secondly, the use of conditional Myb knockout (KO) mice may give an answer whether the monocyte-like cells give rise to $CD8\alpha^-$ cDCs in the pancreas. The transcription factor Myb is required for HSC development of $CD11b^{hi}$ monocytes and MΦs, but not for YS-derived $F4/80^{hi}$

MΦs as described earlier [14]. Although this study described the distinct development of MΦs it may apply for cDCs as well, but further study is needed. If the pancreatic monocyte-like cells are derived from the YS these monocyte-like cells should be Myb-independent. Upon construction of conditional Myb-KO-NOD mice the following hypothesis can be addressed:
Myb-KO-NOD mice still develop autoimmune diabetes due to Myb independent accumulating cDCs derived from YS-derived monocyte-like cells.

Why does the NOD pancreas have low numbers of tolerogenic CD8α$^+$CD103$^+$Langerin$^+$ cDCs?

As indicated above, we assume that the CD8α$^+$ cDCs in the pancreas of NOD mice derive from infiltrating circulating monocytes or pre-cDCs. The lack of CD8α$^+$CD103$^+$Langerin$^+$ cDCs in the NOD pancreas might then be due to an impaired migration of circulating monocytes into the pancreas. Indeed, previous data showed that NOD mouse monocytes show a decreased migration in vivo towards the pro-inflammatory chemokines CCL2 and -3 [17]. In addition, the pro-inflammatory chemokine CCL2 that normally attracts monocytes was absent in the NOD pancreas [18].
Another explanation could be that the CD8α$^+$CD103$^+$Langerin$^+$ cDCs in the NOD pancreas have an enhanced migration to the pLNs. Indeed, in pLNs of NOD mice increased numbers of CD8α$^+$ cDCs were present, which correlates with a significantly larger size of the pLNs due to the ongoing autoimmune response. However, pLNs of NOR mice showed also an increased number of CD8α$^+$ cDCs, whereas in the pancreas of NOR mice a reduction of these cDCs was not detected. Therefore, it is unlikely that the reduced CD8α$^+$ cDC numbers in the NOD pancreas can be explained by an enhanced migration of pancreatic CD8α$^+$ cDCs towards the pLNs.
Finally, other possibilities for the lack of CD8α$^+$CD103$^+$Langerin$^+$ cDCs in the NOD pancreas might be: an increased cell death or an aberrant differentiation. However, no evidence was found for an increased apoptosis or reduced proliferation by studying genes which are involved in these pathways (data not shown). It is possible that in the NOD pancreas an aberrant differentiation of CD8α$^+$ cDCs results in a skewing towards CD8α$^-$ cDCs. To investigate this hypothesis blood monocytes can be labeled, traced and their development into CD8α$^+$ and/or CD8α$^-$ cDCs in the pancreas can be investigated.

Why do pancreatic pDCs in NOD mice produce elevated levels of the tolerogenic enzyme IDO?

We showed that pDCs in the NOD pancreas expressed the tolerogenic enzyme IDO at 4, 10 and 20 weeks of age, but not in the pancreas of C57BL/6 and NOR mice. Furthermore, it

has been described that depletion of pancreatic pDCs in BDC2.5/NOD mice (when insulitis is present) led to an acceleration of insulitis and a loss of local IDO in the pancreas [19]. Therefore, we like to believe that the accumulating IDO^{+} pDCs in the NOD pancreas have a tolerogenic function and are likely to dampen the insulitis development in an attempt to halt the insulitis process in later stages.

What is the trigger for the IDO production by pDCs in the NOD pancreas?

It has been described that IDO can be induced by a number of cytokines, such as IFN-γ, IFN-α, IL-1β, TNF-α and TGF-β either alone or in combination [20].
IFN-γ is the predominant cytokine implicated in the induction of IDO and represents the principal transcriptional regulator of the IDO-encoding gene (*Ido1*) [21-23]. Several studies described that IFN-γ has a limited role in the diabetes development in NOD mice [24-26]. In addition, a study demonstrated that IDO activity was not inducible by IFN-γ in splenic DCs from NOD mice [27]. However, in these studies the relationship between IFN-γ and IDO production by pancreatic pDCs was not investigated. Furthermore, when CTLA-4 binds to CD80/86 on pDCs, the latter molecules will be downregulated and cause IDO activation dependent on IFN-γ [28-30]. In contrast, pDCs in the NOD pancreas showed an increased expression of CD80 and -86. In conclusion, these data suggest that not IFN-γ but other factors are likely to be involved in the IDO production by pancreatic pDCs in NOD mice.
IDO can also be induced by IFN-α through the activation of Toll Like Receptor 9 (TLR9) and it has been shown that activation of TLR9 in vivo can protect mice from experimental autoimmune diabetes in an IDO-dependent fashion [31]. In addition, diabetic TLR9-deficient mice developed a more robust disease, accompanied by lack of IDO induction in pLNs [31, 32]. Another molecule which is involved in the production of IDO via IFN-α is the receptor CD200R1. The engagement of CD200R1 by CD200-expressing cells results in IFN-α release by the pDCs and consequent activation of IDO [33]. It remains unclear whether this pathway is involved in the diabetes development in NOD mice. Recently, a study showed that in the NOD pancreas a transient expression of IFN-α, produced by pDCs, was detected in the islets at 3-4 weeks of age [34]. However, in our study on pDCs in the NOD pancreas (Chapter 5) we did not detect noteworthy IFN-α protein or MxA mRNA expression in the NOD pancreas at 4, 10 and 20 weeks of age. It has to be noted that we studied the whole pancreas, while the study of Diana, et al [34] analyzed isolated infiltrating cells from the islets.
The inflammatory cytokines IL-1β and TNF-α act synergistically with IFN-γ to induce IDO [20, 35]. It has been shown that IL-1β [36], TNF-α (unpublished results) and IFN-γ [37] are upregulated in the NOD pancreas. Although IFN-γ seems not to have a major role in the IDO induction in NOD mice, it is likely that these three cytokines act synergistically to induce the IDO production in NOD pancreatic pDCs. Furthermore, a recent study revealed that the expression of the chemokine CXCL10 in islets, which was elevated in the NOD pancreas at

the time pDCs start to accumulate, was induced by IFN-γ and TNF-α [38]. The activation of TLR4 via lipopolysaccharide (LPS) can also induce IDO by a TNF-α-dependent mechanism, but is IFN-γ-independent [35].

Induction of the IDO gene (*Ido1*) expression is strongly enhanced by TGF-β [39], but it remains to be investigated whether TGF-β plays a role in the IDO induction in the NOD pancreatic pDCs.

In conclusion, several mechanisms are involved in the IDO induction and further study is needed to determine which cytokines are the driving forces behind the IDO production by pancreatic pDCs in NOD mice. Here we assume that it is a concerted action of various pro-inflammatory cytokines.

Concluding and renewed hypothesis

Collectively our data lead to the following renewed hypothesis as given in the scheme in **Figure 2**.

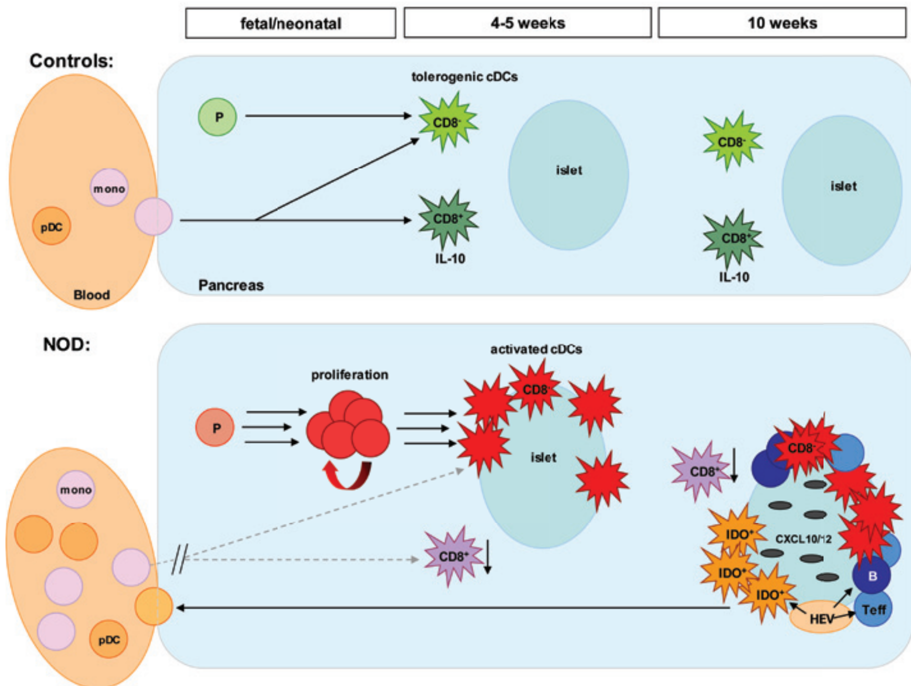

Figure 2. Hypothetical scheme of the early phase of diabetes development in the pancreas of NOD mice.

In the pancreas of control mice monocyte-like precursors (P) hardly proliferate and are capable of replenishing the CD8α$^-$ cDCs present in the exocrine and endocrine pancreas. This population is also replenished from the few circulating monocytes/pre-cDCs migrating from the circulation to the pancreas. These infiltrating cells probably also develop into CD8α$^+$ tolerogenic cDCs, which express IL-10. It must be noted that in fact the accumulation of CD8α$^-$ cDCs and pDCs is nearly or completely absent in the pancreas of control mice (C57BL/6 and NOR).
In the fetal and neonatal NOD pancreas monocyte-like precursor cells show an enhanced proliferation, which results in a local expansion of these cells (red arrow). We hypothesize that this enhanced proliferation of monocyte-like precursor cells results in the accumulation of CD8α$^-$ cDCs at 4-5 weeks of age. At the same time, circulating NOD monocytes have a decreased migration (dotted arrows) towards the pancreas. This results in an elevated number of monocytes in the circulation and in a reduced number of CD8α$^+$ tolerogenic cDCs in the NOD pancreas at 4-5 and 10 weeks of age. This tips the balance towards auto-immunization and the start of the para- and peri-insulitis process.
In the NOD circulation increased pDC numbers are present as compared to C57BL/6 and NOR mice, at the start of para- and peri-insulitis process. At 10 weeks of age these pDCs are recruited via high endothelial venules (HEVs) into the NOD para- and peri-insulitis, together with effector T cells and B cells, most likely attracted by the chemokines CXCL10 and -12. The accumulating pDCs in the NOD pancreas express the tolerogenic enzyme IDO and are instrumental in dampening a further destructive insulitis development.
In the end stage, DCs, MΦs and lymphocytes infiltrate from the islet edges into the core of the islets and at that time beta cells vanish, which lead to massive loss of insulin-producing cells and overt diabetes.

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ADDENDUM

SUMMARY
SAMENVATTING
ABBREVIATIONS
DANKWOORD
CURRICULUM VITAE
PORTFOLIO
PUBLICATIONS

Summary

Antigen presenting cells (APCs), like dendritic cells (DCs), are positioned at the interface of innate and adaptive immunity. They are crucial for the innate and adaptive immune response against infections and for maintaining immune tolerance to self-tissues. DCs arise from a common myeloid progenitor (CMP) in the bone marrow (BM), which develops into the macrophage (MΦ) and DC precursor (MDP). The MDP gives rise to monocytes or via the common DC precursor (CDP) to conventional DCs (cDCs) or plasmacytoid DCs (pDCs). cDCs sample antigens in tissues and subsequently migrate to local lymph nodes (LNs) via the afferent lymphatics and develop into mature or semi-mature cDCs. Semi-mature cDCs are thought to induce tolerance and mature cDCs primarily induce immunity and have a high expression of co-stimulatory molecules and MHC class II. pDCs are found in the BM, blood, secondary lymphoid organs and re-enter the blood before homing to other lymphoid organs in both steady-state and inflammatory conditions. Unlike cDCs, which enter LNs via the afferent lymphatics, pDCs enter LNs through high endothelial venules (HEVs). pDCs have to be activated to start releasing massive amounts of type I interferon and/or take up and present antigens.

Besides the generation of DCs via the CDP, monocytes can also give rise to cDCs, the so-called monocyte-derived DCs. Mouse blood monocytes are divided into two major subsets: immature/classical Ly6C^{hi}CD43^{+} (CD14^{hi}CD16^{-} in humans) and mature/nonclassical Ly6C^{low}CD43^{hi} monocytes (CD14^{+}CD16^{hi} in humans). Classical Ly6C^{hi} monocytes are thought to diapedese to inflamed tissues and to differentiate into the so called inflammatory TNF-α/iNOS producing DCs (TipDCs). Classical monocytes can also contribute to the renewal of several resident subsets of MΦs and DCs, such as mucosal DCs and lung DCs, or develop into nonclassical Ly6C^{low} monocytes. The nonclassical Ly6C^{low} monocytes patrol blood vessels in the steady state and are also capable to extravasate during inflammation. It has also been described that Ly6C^{low} monocytes differentiate into tolerogenic DCs to induce T cell tolerance.

When the immune system fails to recognize its own constituent parts as 'self' and respond against its own cells and tissues, it is called autoimmunity. Type 1 diabetes (T1D) is an autoimmune disease and is characterized by autoimmune-mediated destruction/silencing of pancreatic beta cells resulting in insulin deficiency. The onset of T1D is associated with infiltration of the islets of Langerhans by mononuclear cells (insulitis). To study autoimmune diabetes the nonobese diabetic (NOD) mouse is a widely used animal model. cDC accumulation around the islets from 4-5 weeks of age onwards is a hallmark for autoimmune diabetes development in the NOD mouse. From 10 weeks onwards T and B cells surround the islets and finally these cells infiltrate the islets and the beta cells vanish. This leads to massive loss of insulin production and overt diabetes.

Over the past years we developed the following two hypotheses:

1. The pancreas contains precursors for cDCs. Pancreatic DCs are not necessarily descendant from infiltrated monocytes or pre-cDCs, but can also be generated from these local precursors.
2. In autoimmune diabetes (such as in the NOD mouse) local pancreatic precursor cells are aberrant and generate pro-inflammatory and non-tolerogenic cDCs, which accumulate at the islet edges to start the autoimmune insulitis.

These hypotheses are based on several observations done in the past decade in our group:

1. Local precursors for MΦs have been detected in the fetal pancreas of mice.
2. Treatment with clodronate-loaded liposomes causes a depletion of monocytes from the blood and of phagocytic cells (including MΦs and cDCs) from the pancreas. In the NOD mouse there is only late re-appearance (28 days post-injection) of cDCs and MΦs in the pancreas, at a time when these cells had already repopulated the circulation and the spleen (7 days post-injection). This indicates that the circulating monocytes are not likely the precursors of these pancreatic DCs and MΦs.
3. Furthermore, NOD mouse monocytes show a decreased in vivo migration towards the pro-inflammatory chemokines CCL2 and -3. In addition, the pro-inflammatory chemokine CCL2 that normally attracts monocytes to tissues is absent in the NOD pancreas.

Together these results suggest that the pancreas contains precursors not only for MΦs, but also for cDCs. In the NOD mouse BM precursors for DCs are abnormal in proliferation and apoptosis and generate pro-inflammatory DCs. We thus extrapolate these observations to the pancreas precursors of the NOD mouse and assume that these are equally abnormal.

In **Chapter two** we show that local precursors for cDCs, with a monocyte-like appearance, are present near the islets in the murine pancreas from embryonic (E15.5) age onwards. In in vitro experiments we showed that the monocyte-like precursor cells of the murine pancreas did develop into cDCs. In **Chapter three** we describe that proliferating monocyte-like cells were also present in the human pancreas and - similar to the mouse - located near the islets and outside blood vessels in fetal, neonatal and adult pancreas. In the human fetal pancreas the number of monocyte-like cells was significantly higher as compared to the pancreas of children and adults. In the NOD mouse pancreas the monocyte-like cells had a higher proliferative capacity in the embryonic as well as in the pre-diabetic pancreas as compared to the C57BL/6 and NOR pancreas (**Chapter two**). The presence of proliferating monocyte-like cells in the normal pancreas of mice and humans strengthen our concept that these cells are precursors for local resident MΦs and cDCs in the pancreas. The higher proliferation

of the monocyte-like precursors in the NOD mouse pancreas let us believe that the pre-diabetic cDC accumulation around the NOD islets might be due to an aberrant enhanced proliferation of the local cDC precursors, rather than to an aberrant 'inflammatory infiltration' of cDCs from the circulation.

In **Chapter four**, we describe a significant reduction in the presumed tolerogenic subset of CD8α$^+$CD103$^+$Langerin$^+$ cDCs in the NOD pancreas as compared to the C57BL/6 and NOR pancreas. In addition, these tolerogenic cDCs had a reduced expression of CCR5, CLEC9A and IL-10. This indicates that a lack of the tolerogenic CD8α$^+$ cDC subset is a hallmark of the NOD para/peri-insulitis and might be an important factor in the loss of tolerance towards islet antigens.

In **Chapter five**, we showed that after the initial accumulation of CD8α$^-$ cDCs in the NOD pancreas (at 4-5 weeks) pDCs start to accumulate around the islets of Langerhans from 10 weeks onwards together with effector T- and B lymphocytes. In the C57BL/6 and NOR pancreas pDC accumulation was not observed. Furthermore, pancreatic pDCs in NOD mice of 4, 10 and 20 weeks of age expressed the tolerogenic enzyme IDO. In the NOD pancreas IDO$^+$ pDCs and lymphocytes were possibly attracted by the co-expression of CXCL10 and -12. These infiltrating tolerogenic pDCs are likely to dampen the insulitis development in an attempt to halt the insulitis process. It is unlikely that these cells are involved in a massive production of IFN-α, since we were unable to find noteworthy levels of IFN-α and the interferon-inducible gene MxA in the NOD pancreas.

Finally, the results of the different chapters are integrated in **Chapter six**. The intrinsic proliferative abnormalities of the pancreatic monocyte-like precursors of the cDCs and the abnormal apportioning between pro- and anti-inflammatory cDCs in the para/peri-insulitis probably represent key abnormalities in the break of tolerance and the early pathogenesis of T1D. It remains puzzling how the monocyte-like cells and the cDCs in the NOD pancreas get activated and which key elements are playing a role in this activation. In particular manipulations in the NOD mouse model, such as mimics of bacterial and viral infections, diets inducing activation of mononuclear phagocytes and chronic stress, might be helpful to unravel the complicated mononuclear phagocyte-endocrine cell interactions in the development, prevention and treatment of T1D.

Samenvatting

In het dagelijks leven worden we blootgesteld aan allerlei ziekteverwekkers, zoals virussen, bacteriën en schimmels. Desondanks worden we niet vaak ziek. Dit komt door de beschermende werking van ons immuunsysteem, dat ziekteverwekkers (pathogenen) en veranderde lichaamseigen cellen herkent en opruimt. Het immuunsysteem kan worden onderverdeeld in aangeboren en verworven afweer. De aangeboren afweer ruimt pathogenen op zonder dat er geheugen wordt opgebouwd. Naast anatomische barrières (b.v. de huid en slijmlaag van de darmen) zijn diverse celtypen, zoals, macrofagen (MΦs) en granulocyten onderdeel van de aangeboren afweer. Deze cellen komen als eersten in actie wanneer de anatomische barrières worden doorbroken. MΦs (letterlijk 'grote eters') en granulocyten herkennen pathogenen en eten deze op, ook wel 'fagocyteren' genoemd. Dendritsche cellen (DCs) zijn cellen die net als MΦs kunnen fagocyteren en vervolgens een belangrijke rol spelen bij het activeren van de verworven afweer. Dit doen DCs door pathogenen op te nemen en vervolgens kleine stukjes van pathogenen (antigenen) op de buitenkant van hun celmembraan te presenteren. Als DCs een bepaald pathogeen hebben opgenomen migreren ze via lymfevaten naar de lymfeklieren waar de antigenen worden gepresenteerd aan lymfocyten. Dit leidt tot activatie van de lymfocyten en vervolgens tot het onschadelijk maken van het pathogeen. Tevens vindt er vorming van geheugencellen plaats zodat er snel gereageerd kan worden bij een volgende ontmoeting met het pathogeen.
Naast het bestrijden van infecties spelen DCs ook een rol bij het opbouwen van tolerantie voor lichaamseigen stoffen (tolerantie is een specifiek **niet** reageren tegen lichaamseigen stoffen als bijvoorbeeld insuline). DCs nemen ook lichaamseigen stoffen op, maar raken daardoor niet of maar weinig geactiveerd. Deze DCs zijn juist in staat om een speciale groep van lymfocyten te activeren die specifiek zijn voor deze lichaamseigen stoffen. Deze lymfocyten reageren dan met het maken van ontstekingsonderdrukkende stoffen. Dit zijn de zogenaamde T regulator cellen.

DCs ontstaan uit een myeloïde voorlopercel (precursor) in het beenmerg, die via een tussenstap als MΦ/DC precursor kan ontwikkelen tot monocyt of via de DC precursor tot conventionele DCs (cDCs) of plasmacytoïde DCs (pDCs). cDCs zijn de eerder genoemde DCs die migreren door de lymfevaten naar de lymfeklieren om vervolgens immuniteit of tolerantie te induceren. pDCs zijn belangrijk voor de afweer tegen virussen en bevinden zich in het beenmerg, bloed en in de lymfoïde organen (zoals milt, lymfeklieren) en migreren via het bloed naar deze lymfoïde organen. Anders dan cDCs, die de lymfeklieren via de lymfevaten bereiken, komen pDCs via de zogenaamde hoog-endotheliale venules vanuit het bloed de lymfeklier binnen. pDCs produceren na activatie grote hoeveelheden type I interferonen (IFN) en kunnen antigenen presenteren aan lymfocyten.

Naast de ontwikkeling van DCs via de DC precursor, kunnen cDCs ook ontstaan uit monocyten, de zogenaamde monocyt-afkomstige DCs. Monocyten in het bloed van de muis kunnen worden onderverdeeld in twee groepen: immature Ly6C^{hi}CD43^{+} (CD14^{hi}CD16^{-} in mensen) en mature Ly6C^{low}CD43^{hi} monocyten (CD14^{+}CD16^{hi} in mensen). Immature Ly6C^{hi} monocyten verlaten de bloedbaan om naar een ontstekingshaard in het weefsel te gaan en rijpen daar uit tot de 'inflammatoire' TNF-α/iNOS producerende DCs (Tip-DCs). Immature monocyten dragen ook bij aan het in stand houden van verschillende populaties antigeen-presenterende cellen die onder gezonde omstandigheden in de weefsels aanwezig zijn (MΦs en cDCs in de longen en darmen). De mature Ly6C^{low} monocyten patrouilleren langs de wand van de bloedvaten en zijn in staat om tijdens een infectie het weefsel in te gaan. Deze mature monocyten kunnen tevens uitgroeien tot tolerogene cDCs en vervolgens tolerantie genereren.
Als het immuunsysteem zich richt tegen lichaamseigen cellen en deze gaat aanvallen, spreken we van een auto-immuunziekte. Type 1 diabetes (T1D) is zo'n auto-immuunziekte waarbij immuuncellen de insuline-producerende bèta cellen in de eilandjes van Langerhans in de alvleesklier (pancreas) vernietigen waardoor er een insuline tekort ontstaat dat uitendelijk tot diabetes zal leiden. Bij mensen treden er pas symptomen op in de laatste fase, wanneer de insuline-producerende bèta cellen al zijn verdwenen. Hierdoor is het moeilijk om de vroege fase van T1D te onderzoeken.
Om het vroege ontstaan van T1D te bestuderen wordt het non-obese diabetes (NOD) muis model gebruikt. Deze muizen krijgen spontaan auto-immuun diabetes. De ophoping van cDCs rond de eilandjes in de NOD pancreas (insulitis), vanaf de leeftijd van 4-5 weken, is karakteristiek voor de ontwikkeling van auto-immuun diabetes in deze muis. Vanaf 10 weken gaan lymfocyten rondom de eilandjes ophopen en uiteindelijk zullen deze cellen de eilandjes in gaan en de bèta cellen vernietigen. Dit leidt tot een massaal verlies van insuline productie capaciteit en dit resulteert in diabetes.

De afgelopen jaren hebben we de volgende hypotheses onderzocht:

1. Niet alleen het beenmerg maar ook de pancreas bevat precursors voor cDCs.
2. Bij auto-immuun diabetes (zoals bij de NOD muis) zijn de lokale precursors in de pancreas afwijkend.

Deze hypotheses zijn gebaseerd op verschillende studies die de afgelopen tien jaar binnen onze groep zijn uitgevoerd:

1. Lokale precursors voor MΦs zijn in de foetale pancreas van muizen gevonden.
2. Behandeling met clodronaat liposomen zorgt voor een verwijdering van monocyten en andere fagocyterende cellen (MΦs en cDCs) uit het bloed en organen, zoals de pancreas. Wanneer NOD muizen worden behandeld met clodronaat liposomen

verdwijnen de MΦs en cDCs uit het bloed en de pancreas. Na 7 dagen keren deze cellen weer terug in het bloed, terwijl ze pas na 28 dagen terugkeren bij de eilandjes in de pancreas. Deze verlate terugkeer is in tegenspraak met het feit dat monocyten in het bloed de precursors zouden zijn voor de cDCs en MΦs in de NOD pancreas.

3. NOD monocyten vertonen in vivo een verlaagde migratie van de bloedvaten naar de weefsels. Belangrijke stoffen voor deze migratie zijn de pro-inflammatoire chemokinen CCL2 en CCL3. CCL2 is niet aanwezig in de NOD pancreas. Ook dit geeft aan dat het onwaarschijnlijk is dat de monocyten in het bloed de precursors zijn voor de cDCs en MΦs in de NOD pancreas.

Samengevat suggereren deze resultaten dat de pancreas precursors voor MΦs en voor cDCs bevat. Aangezien de precursors voor DCs in het beenmerg van de NOD muis afwijkend zijn (deling en apoptose) en pro-inflammatoire DCs genereren, ontwikkelden wij daarnaast de hypothese dat ook de precursors in de NOD pancreas afwijkend zijn. Deze afwijkende precursors in de NOD pancreas zouden dan voornamelijk bijdragen tot de ontwikkeling van diabetes.

In **Hoofdstuk twee** laten we zien dat vanaf de foetale (E15.5) leeftijd lokale precursors voor cDCs, met een monocyt-achtig uiterlijk, aanwezig zijn bij de eilandjes van Langerhans in de pancreas van de muis. In vitro experimenten tonen aan dat deze monocyt-achtige precursors uit de foetale pancreas zich kunnen ontwikkelen tot cDCs.
In **Hoofdstuk drie** beschrijven we dat ook in de humane pancreas bij de eilandjes van Langerhans delende monocyt-achtige cellen aanwezig zijn. In de foetale humane pancreas was het aantal van deze monocyt-achtige cellen significant hoger vergeleken met de pancreas van kinderen en volwassenen. In zowel de foetale als de neonatale pre-diabetische NOD pancreas hebben de monocyt-achtige precursors een hogere delingscapaciteit vergeleken met precursors uit de pancreas van muizen die geen diabetes ontwikkelen (C57BL/6 en NOR muizen) (**Hoofdstuk twee**). De verhoogde deling van de monocyt-achtige precursors in de NOD pancreas geeft aan dat de ophoping van cDCs rond de NOD eilandjes mogelijk het resultaat is van deze afwijkende verhoogde deling van de lokale monocyt-achtige precursors en niet zoals verondersteld werd van een inflammatoire infiltratie van monocyten of pre-cDCs uit het bloed.

In **Hoofdstuk vier** en **Hoofdstuk vijf** bestuderen en karakteriseren we de verschillende DC populaties, die in de NOD pancreas aanwezig zijn.
In **Hoofdstuk vier** beschrijven we een significante verlaging van de tolerogene $CD8\alpha^+CD103^+Langerin^+$ cDC populatie in de NOD pancreas vergeleken met de C56BL/6 en NOR pancreas. Deze cDCs hebben een verlaagde expressie van CCR5, CLEC9A en IL-10.

Dit zou kunnen betekenen dat de verminderde aanwezigheid van deze tolerogene cDC populatie in de NOD pancreas een rol speelt bij het verlies aan tolerantie voor antigenen van de eilandjes.
In **Hoofdstuk vijf** tonen we aan dat tegelijkertijd met de ophoping van lymfocyten pDCs rond de NOD eilandjes beginnen op te hopen (op een leeftijd van 10 weken). Dit is later dan de ophoping van CD8α^{-} cDCs in de NOD pancreas, die op 4-5 weken begint. In de pancreas van muizen die geen diabetes ontwikkelen (C57BL/6 en NOR muizen) wordt geen pDC ophoping waargenomen. De pDCs in de NOD pancreas van 4, 10 en 20 weken produceren het tolerogene enzym indoleamine 2,3-dioxygenase (IDO). Daarnaast kunnen pDCs ook grote hoeveelheden IFN-α produceren. Wij hebben echter geen aanwijzingen gevonden dat de pDCs in de NOD pancreas IFN-α produceren. In de NOD pancreas worden de pDCs en lymfocyten mogelijk aangetrokken door de chemokinen CXCL10 en CXCL12, waarvan we in de NOD pancreas op 10 weken een verhoogde expressie hebben aangetoond. Er zijn aanwijzingen dat de ophopende IDO^{+} pDCs in de NOD pancreas wellicht een remmend effect op het insulitis proces hebben. Dit is blijkbaar niet voldoende om de uiteindelijke ontwikkeling van auto-immuun diabetes te voorkomen.
Ten slotte zijn de resultaten en conclusies van de verschillende hoofdstukken geïntegreerd in **Hoofdstuk zes**. De abnormale deling van de monocyt-achtige precursors voor cDCs en de afwijkende samenstelling van de pro- en anti-inflammatoire cDC populaties in de NOD pancreas tonen aan dat onze hypotheses juist zijn. Waarschijnlijk leveren deze abnormaliteiten een bijdrage aan de ontwikkeling van T1D in de pre-diabetische pancreas van de NOD muis. Verder onderzoek moet aantonen welke factoren de monocyt-achtige precursors, cDCs en pDCs in de NOD pancreas activeren. Meer inzicht in de gecompliceerde interacties van DCs en endocriene cellen gedurende de ontwikkeling van T1D (bijvoorbeeld door specifieke manipulaties in de NOD muis) zullen leiden tot het ontrafelen van het ontstaan van T1D en uiteindelijk tot een genezing dan wel preventie van de ziekte.

Abbreviations

AGM	aorta-gonad-mesonephros
APC	antigen presenting cell
BB-DP rat	biobreeding diabetes-prone rat
BM	bone marrow
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CFSE	carboxyfluorescein succinimidyl ester
cDC	conventional dendritic cell
CDP	common dendritic cell precursor
CLP	common lymphoid precursor
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
Flt3L	fms-like tyrosine kinase 3 ligand
GM-CSF	granulocyte macrophage-colony stimulation factor
HEV	high endothelial venules
HSC	hematopoietic stem cells
ICAM	intracellular adhesion molecule
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
iLN	inguinal lymph node
KO	knock out
LPS	lipopolysaccharide
M-CSF	macrophage-colony stimulation factor
mDC	myeloid dendritic cell
MDP	macrophage and dendritic cell precursor
MHC	major histocompatibility complex
MΦ	macrophage
NFkB	nuclear factor kappa B
NOD	nonobese diabetic
NON	nonobese non-diabetic
NOR	nonobese diabetic resistant
PDCA	plasmacytoid dendritic cell antigen

pDC	plasmacytoid dendritic cell
PD-L1	programmed death ligand 1
pLN	pancreas draining lymph node
SCID	severe combined immunodeficiency
SLE	systemic lupus erythematosus
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TGF	transforming growth factor
TIP-DC	TNF/iNOS-producing dendritic cell
TLR	toll-like receptor
TNF	tumor necrosis factor
YS	yolk sac

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Rotterdam in de trein gezeten. Jij bent inmiddels al een aantal jaren geleden gepromoveerd en jij bent voor mij een voorbeeld geweest. Je bent super ver gekomen en ik weet zeker dat je een mooie toekomst tegemoet gaat. Dank je voor de leuke tijd en ook al zitten we nu niet meer samen in de trein, de vriendschap zal blijven bestaan.

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Lieve Gerwin, je bent mijn broer en ook jij weet hoe het is om van onderaan te moeten beginnen. Wij hebben samen maar mooi laten zien hoe ver je kunt komen met doorzettingsvermogen. Wij hebben samen altijd zo veel lol en vaak hoeven we maar naar elkaar te kijken en dan weten we genoeg. Tijdens mijn afstudeerpraatje op de HLO had je zo goed opgelet dat je nog steeds weet waar het over ging: in situ hybridisatie (of was het in snitu hydribidibidibatie). Ik hoop dat je nu weer goed gaat opletten, het gaat namelijk over andere dingen: het moeilijkste woord is indoleamine 2,3-dioxygenase, of te wel IDO.

Lieve pap (vatur), jij hebt altijd in mij geloofd en mij ook de kracht gegeven om door te gaan. Jij was degene die zei dat de prijzen pas aan het eind worden uitgereikt. En dat klopt. Hoe lang en zwaar het soms kan zijn het is altijd goed gekomen. Daarom ga ik ook met vertrouwen de toekomst tegemoet.

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Jo

Curriculum Vitae

Personal information

Name	Jojanneke Maria Cecilia Welzen-Coppens
Born	July 9, 1980 in Roosendaal
Nationality	Dutch

Work and education

2007-2012	PhD thesis: Development of type 1 diabetes – Monocytes and dendritic cells in the pancreas. Immunology Department, Erasmus MC, Rotterdam
2002-2007	Research Technician Immunology Department, Erasmus MC, Rotterdam
2002	Internship, BSc thesis: Changes in molecular composition of bladder muscle tissues caused by outlet obstruction. Urology Department, Erasmus MC, Rotterdam
1999-2002	BSc / ing. - Higher Laboratory Education, Histology & Laboratory Animal Science Hogeschool West-Brabant, Etten-Leur
1996-1999	Middle Laboratory Education, Histology Baronie College, Breda
1992-1996	MAVO Norbertus College, Roosendaal

Additional certificates

Radiation Safety and Hygienic, level 5B certificate
Laboratory Animal Science, article 12 certificate

PhD Portfolio

	Year	ECTS
General Academic skills		
Basistraining didactiek	2007	0.65
Management voor promovendi en Postdocs	2008	1.0
English Biomedical Writing	2009	1.4
Photoshop CS3 Workshop	2010	0.3
Loopbaan Oriëntatie	2011	0.85
In-dept courses		
Medische Immunologie	2006	1.0
Molecular Immunology	2008	3.0
Neuro-Immuno-Endocrinology	2008	0.85
Symposia and conferences		
NVVI, Lunteren	2007	1.0
EMDS, Brescia, Italië - Poster	2008	1.0
NVVI, Noordwijkerhout - Poster	2008	1.0
Molecular Medicine Day, Rotterdam - Poster	2009	0.3
EMDS, Brussel, België - Poster	2011	1.0
IDS, Victoria, Canada - Poster	2012	1.0
Teaching		
Vaardigheidsonderwijs Immunologie, Geneeskunde 2de jaars studenten	2006 t/m 2012	8.0
Snuffelstage VWO studenten	2007	1.8
Begeleiden studenten tijdens stage	2007 t/m 2011	8.0
Presentations		
Symposium plasmacytoid DCs Erasmus MC, Rotterdam	2009	1.0

Publications

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2005 Bouma G, **Coppens JM**, Lam-Tse WK, Luini W, Sintnicolaas K, Levering WH, Sozzani S, Drexhage HA, Versnel MA. An increased MRP8/14 expression and adhesion, but a decreased migration towards proinflammatory chemokines of type 1 diabetes monocytes. Clin Exp Immunol. 2005 Sep;141(3):509-17.

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